University of Thessaly

Department of Biochemistry and Biotechnology

Isolation and study of bacteria able to degrade pesticides contained in the wastewaters produced by the fruit-packaging industry

A thesis submitted by

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Abstract

Fruits are particularly vulnerable to fungal infestations or other physiological disorders during storage thus diminishing their market value. Post-harvest fruit treatments with fungicides like thiabendazole (TBZ), imazalil (IMZ), and *ortho*-phenyl-phenol (OPP), and antioxidants like diphenylamine (DPA) are the most effective means to minimize fruit spoilage in storage. The latter is no more registered for use in the EU, although exemption authorizations for 120 days have been given considering that there are no equally effective alternatives in the market for the control of apple scald.

Pesticide application methods in fruit-packaging plants (dipping, drenching, spraying, or waxing) are water-based generating at the end of the process large volumes of wastewaters containing high concentrations of pesticide (0.2 to 2 g/l depending on the molecule). Direct discharge of these wastewaters into the environment without prior treatment constitutes a serious point-source of contamination. This problem becomes more acute when the high persistence (TBZ, IMZ) and toxicity (all) of the pesticides used is taken in account. Therefore, a wastewater treatment system able to efficiently depurate the wastewaters derived from the post-harvest handling of fruits is required, considering also that authorization for the use of those pesticides TBZ, IMZ, OPP, and DPA was granted under the clause that an effective treatment of the wastewaters produced is operative. However, the only system actually patented (CONTROL TEC-ECO® system) is highly costly precluding its market uptake. Thus, a sustainable, cheap, effective, and environmentally friendly method for the depuration of the fruit packaging wastewater is needed. Microbial degradation of pesticide is considered the most important process for their environmental loss. The development of biological treatment facilities based on the degrading ability of microorganisms seems promising. The implementation of such systems will require inoculation with start-up inocula possessing high degradation capacity against the target compounds. However, very little (OPP, DPA) or nothing (TBZ, IMZ) is known regarding the microbial degradation of those chemicals.

The current doctoral thesis aimed to fulfill this gap and provide novel insights into the microbial degradation of those pesticides with future practical implications for environmental protection. Thus the main research aim of this thesis was to isolate and characterize bacteria able to degrade the major pesticides used in the fruit packaging industry (TBZ, IMZ, OPP, and DPA). Enrichment cultures from soils collected from wastewater disposal sites led to the isolation of TBZ, OPP, and DPA-degrading bacteria, while, despite different attempts, it was not possible to isolate any bacterium able to degrade IMZ.

Three TBZ-degrading consortia able to rapidly degrade the fungicide and use it as a C and N source were obtained. DGGE analysis and cloning showed that all TBZ-degrading

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consortia were composed of two to eight main members of proteobacteria. Different microbiological (antibiotics treatment) and molecular (DNA/RNA-DGGE, cloning, q-PCR) approaches, showed that a *Sphingomonas* sp. strain was responsible for the degradation of TBZ, while the role of other bacterial members was probably supportive regarding nutrition or degradation of metabolites produced (C1-compounds). However, the bacterium was not obtained in pure culture due to its limited capacity to grow on the agar media tested. The TBZ-degrading consortium was able to degrade 750 mg/l TBZ in liquid culture and 500 mg/kg TBZ in artificially contaminated soil. Moreover, it was able to degrade TBZ in a wide range of pH (4.5-7.5) and temperatures (15-37°C) and in the presence of OPP and DPA and their respective degrading bacteria.

An OPP-degrading bacterium identified as *S. haloaromaticamans* was isolated in pure culture. The bacterium was able to rapidly degrade OPP only when supplemented with casamino acids or in the co-presence of at least another bacterial strain (e.g. *Pseudomonas stutzeri*), an observation suggesting the incapacity of the degrading isolate to synthesize some essential amino acids like the ones included in casamino acids. The isolated *S. haloaromaticamans* strain was able to metabolize up to 150 mg/l OPP within 7 days and to use it as an energy source for growth. OPP was metabolized by the isolate in a wide range of pH (4.5-9) and temperatures (4-37°C) and in the presence of different pesticides (TBZ and DPA) used in the fruit packaging industry and of their pesticide-degrading microbes.

Regarding DPA, a *P. monteilii* was isolated which was able to degrade rapidly DPA and use it as a carbon and nitrogen source. Molecular analysis showed that the specific strain had polymorphisms in the different copies of its 16S rRNA gene, a common feature of many Pseudomonads. The isolated bacterium was able to rapidly degrade spillage level concentrations of DPA in liquid media (2 g/l) and in artificially contaminated soil (1 g/kg). Its degrading activity was not impaired in a wide range of pH (4.5-9) and temperatures (4-37°C) or by the co-presence of other pesticides possibly contained in wastewaters (TBZ and OPP) and their respective degrading bacteria.

Overall, the results of this thesis provide novel insights into the largely unknown area of microbial degradation of the pesticides contained in wastewaters from the fruit-packaging industry. The pesticide-degrading bacteria isolated showed promising properties which could make them valuable tools in future biotechnological applications including their use a) as tailor-made inocula in biological wastewater treatment facilities, b) in *in situ* bioaugmentation of pesticide-polluted wastewater disposal sites adjacent to fruit-packaging plants and c) for bioaugmentation of modified biobed systems receiving wastewaters from the fruit packaging industry. Follow up studies will focus on both basic and practical challenges that have arisen

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including a) the elucidation of the metabolic pathway *via* advanced analytical tools and of the genetic mechanisms involved in the degradation of the pesticides studied *via* comparative genomic – proteomic analysis and b) the full-scale application of the isolated bacteria in biological wastewater treatment facilities.

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List of Abbreviations

2-AB	2-aminobenzimidazole
ACN	Acetonitrile
ANOVA	Analysis of Variance
BEM	Biobed Extract Medium
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl sulfoxide
DPA	Diphenylamine
EC	Enrichment Cycle
IMZ	Imazalil
LB	Luria-Bertani medium-Miller
MCP	1-methylcyclopropene
MeOH	Methanol
MRL	Maximum Residue Limit
MSM	Mineral Salt Medium
MSMN	Mineral Salt Medium+Nitrogen
OPP	*Ortho*-phenyl-phenol
PB	Pseudomonas Basal mineral medium
Rt	Retention Time
SEM	Soil Extract Medium
SOPP	Sodium *ortho*-phenylphenol
Ta	Annealing Temperature
TBZ	Thiabendazole
Tm	Melting Temperature
WHC	Water Holding Capacity

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Chapter 1

General Introduction

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1. Pesticides: uses and consumption

As a result of human activities, currently large volumes of wastes with high pollutants load are discharged into the environment. It is estimated that globally more than one billion pounds of toxic chemicals are released into the air and water. Within the same frame, approximately $6x10^6$ chemicals have been produced, 1000 new chemical products are synthetized annually, and between 60000 and 95000 chemicals are commercially used including synthetic pesticides [Shukla *et al*., 2010].

Among those industrial chemicals, pesticides have been identified as significant environmental pollutants [Cerejeira *et al*., 2003; Konstantinou *et al*., 2006; Hildebrandt *et al*., 2008]. As pesticide is defined any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest (insects, mites, nematodes, weeds, rats, etc.) that has a deleterious effect on crops and results in yield losses. Depending on the target-pest, pesticides could be categorized as insecticides, herbicides, fungicides, etc. [FAO, 1989]. Despite minor variations, the core of pesticide definition remains basically the same: any chemical that is poisonous and efficient against target organisms but entails no risk to non-target organisms and the environment [Zhang *et al*., 2011].

The history of pesticide use can be divided into three phases: (1) before 1870s, where natural pesticides were used to control pests; (2) 1870s-1945, the era of inorganic synthetic pesticides; (3) 1945 to now, the era of organic synthetic pesticides [Zhang *et al*., 2011]. The early pesticides production rate (1940s) was estimated at 600000 tons/year and in the '90s their utilization has been vigorously widen, achieving an annual generation of 3 million tn/year (50-fold) [Dich *et al*., 1997]. Over the 1990s the global pesticide sales remained relatively constant, between 27 to 30 billion USD, of which 47.5% were herbicides, 28% insecticides, and 19.6% fungicides/bactericides (Table 1.1).

Table 1.1. Change in pesticide sales globally. Sale: million USD (source Zhang *et al*. [2011]).

Category	1960		1970		1980		1990		2000		2005	
	Sale	%	Sale	%	Sale	%	Sale	%	Sale	%	Sale	%
Insecticides	310	36.5	1002	37.1	4025	34.7	7655	29	7559	27.9	7798	25
Herbicides	170	20	939	34.8	4756	14	11625	44	12885	47.5	14971	48
Fungicides & Bactericides	340	40	599	22.2	2181	18.8	5545	21	5306	19.6	7486	24
Others	30	3.5	159	5.9	638	5.5	1575	6	1354	5	936	3
Total	850	100	2700	100	11600	100	26400	100	27104	100	31191	100

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In recent years the value of the global pesticides market reached to 38 billion US Dollars with herbicides being the most important pesticide group followed by fungicides [Erbach, 2012]. Europe is now the largest pesticide consumer in the world followed by Asia (Figure 1.1).

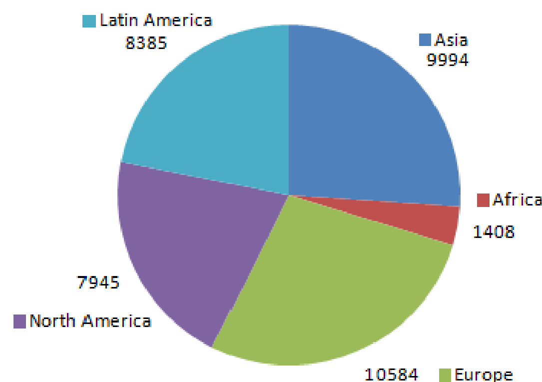

Figure 1.1. Pesticide sales by continent (2010) in million USDs (adapted by Erbach [2012]).

In Europe, the total quantity of pesticides sold (expressed in active ingredient) increased between 2000 and 2005 in Denmark, Estonia, Ireland, Italy, Latvia, Hungary, Poland, Portugal, Finland, and Norway, decreased in France, Slovenia, Sweden and remained relatively stable in Belgium, Germany, the Netherlands, Austria, and the United Kingdom (Table 1.2) [Eurostat, 2012].

Table 1.2. Pesticides consumption in Europe (tons of active ingredients) during the period 2000-2008 (source Eurostat [2012]).

	Total sold quantity									Change
	2000	2001	2002	2003	2004	2005	2006	2007	2008	2000-2005
BE	9 953	8 845	9 204	8 822	9 186	9 776	:	:	:	-2%
DK	2 889	3 127	2 912	2 991	2 941	3 299	3 254	3 354	4 051	14%
DE	30 331	27 885	29 531	30 164	28 753	29 512	31 819	32 683	34 664	-3%
EE	306	329	329	322	357	393	467	459	:	28%
IE	2 133	2 486	2 796	2 913	3 104	2 776	2 874	:	:	30%
EL	11 131	11 111	:	:	:	:	:	:	:	:
ES	34 597	35 700	:	:	:	:	:	:	:	:
FR	94 694	99 635	82 448	74 524	76 099	78 265	71 612	77 255	:	-17%
IT	79 831	76 346	94 711	86 705	84 292	85 073	81 450	:	:	7%
LV	284	369	339	418	597	733	2 239	1 052	:	158%
LU	:	:	:	:	:	:	:	:	:	:
HU	5 473	6 431	8 232	8 726	9 941	9 676	11 523	11 178	12 084	77%
MT	184	217	222	243	:	:	:	:	:	:
NL	9 655	7 987	8 073	7 868	9 071	9 309	9 410	10 740	:	-4%
AT	3 563	3 133	3 080	3 386	3 302	3 404	:	:	:	-4%
PL	8 848	8 855	10 358	7 184	8 726	16 039	17 102	15 303	:	81%
PT	15 469	15 491	17 435	17 046	16 938	16 346	15 703	16 689	17 060	6%
SI	1 469	1 399	1 164	1 361	1 560	1 384	1 281	:	:	-6%
FI	1 146	1 424	1 620	1 667	1 489	1 431	1 645	:	:	25%
SE	1 652	1 738	1 711	2 049	942	1 527	1 707	:	:	-8%
UK	23 601	23 526	23 526	22 564	23 463	23 601	21 151	:	:	0%
NO	378	518	818	658	824	511	690	720	:	35%

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Fungicides and herbicides were the most sold pesticides in 2005 in countries for which data were available. In Germany, France, the Netherlands, and Austria fungicides made up more than one third of the sales of pesticides while in Portugal, Slovenia, and Italy this share was even greater than 60 %. In Belgium, Denmark, Ireland, Latvia, Poland, and Finland herbicides made up more than half of the sales of pesticides, while in Estonia, Sweden, and Norway this share was higher than 80 %. The share of insecticides in total sales of pesticides was negligible in most countries (<5 %), except Belgium (10 %) and Hungary (16 %) (Figure 1.2) [Eurostat, 2012].

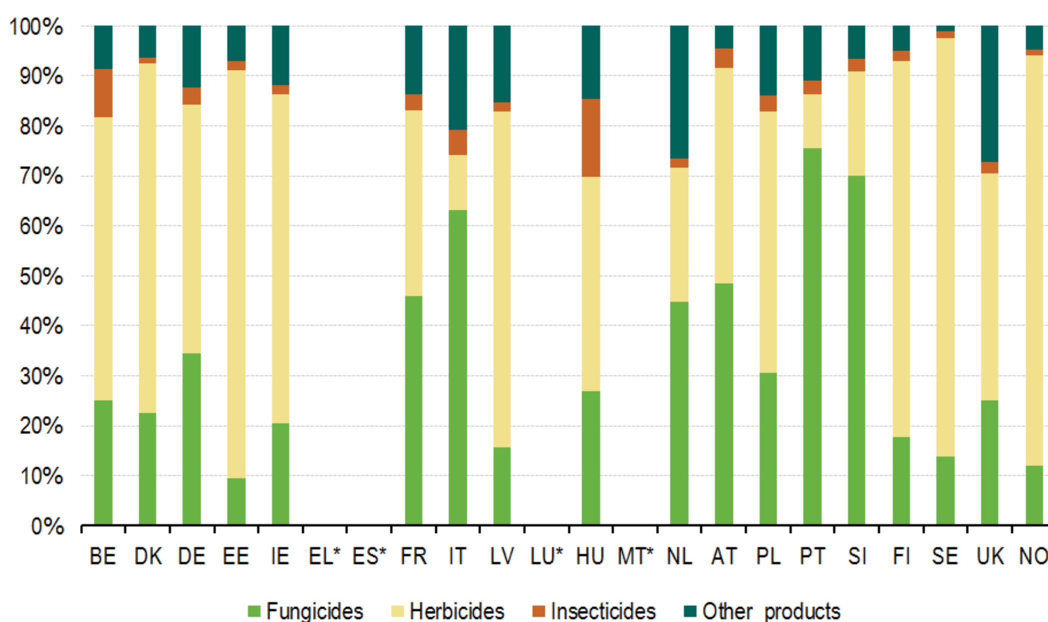

Figure 1.2. The share of the different types of pesticides in total sales of pesticides in selected countries, 2005 (source Eurostat [2012]).

2. Benefits and risks associated with pesticides use

The global agricultural sector is the primary user of pesticides, consuming over 4 million tons annually [Chen *et al.*, 2009]. Chemical pesticides are still an indispensable part of modern agriculture and they are extensively used in most cultivated areas aiming to minimize pest infestations, to protect crop yield losses, and to avoid significant deterioration of product quality [Ortiz-Hernandez *et al.*, 2013]. In fact, worldwide approximately 9000 species of insects and mites, 50000 species of plant pathogens, and 8000 species of weeds threaten crop yields. Estimated yield losses attributed to insect pests, plant pathogens, and weeds are 14, 13 and 13% respectively [Pimentel, 2009a]. Without pesticide applications the loss of fruits, vegetables, and cereals from pest injury would reach 78%, 54%, and 32% respectively [Cai, 2008].

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2.1. Benefits of pesticide utilization

The use of pesticides in modern agriculture improves crop yields by eliminating or reducing competition from weeds and attacks by pests, safeguards the agricultural products quality, and minimizes labour input. Pesticides also play an essential role in ensuring reliable supplies of agricultural products each year, by contributing to prevent fluctuations of annual yields. Moreover, they contribute to ensuring the availability of low-priced fruits and vegetables of good quality, which makes them affordable for all consumers. The use of pesticides reduces demand for land for food production and enables the regional production of a wider variety of food, which in turn can reduce transportation costs and make more land available for other uses, e.g. amenity, natural parks and protection of biodiversity. Conservation tillage and minimum tillage techniques, which reduce the demand for fossil energy in agriculture and decrease erosion and the leaching of nutrients, partly depend on the use of herbicides. Last, the plant protection industry is a significant economic player on the world market and an important employer in Europe with around 26000 employees in the EU-25 [Cooper and Dobson, 2007; Aktar *et al.*, 2009; EC, 2006].

2.2. Risks from pesticides use

On the other hand there are several risks associated with pesticides use. The unregulated and off-label use of pesticides has been shown to induce adverse effects to human health and the environment. The magnitude of those effects depends on the degree of sensitivity of the exposed organisms and the inherent toxicity of the pesticide [Agrawal *et al*., 2010; Piementel, 2009b]. Ideally a pesticide must be lethal to the target pest, but have no effect on non-target organisms including humans. Unfortunately, this is not always the case [Aktar *et al*., 2009]. It has been estimated, for example, that over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species [Miller, 2004].

Risks to humans health by pesticides can occur through direct (workers in agrochemical industries and operators) and indirect (consumers, residents, and bystanders) exposure. Pesticides can enter the human body through inhalation of aerosols, dust, and vapor that contain pesticides, through oral exposure by consuming food and water, and through dermal exposure by direct contact of pesticides with the skin [Damalas and Eleftherohorinos, 2011; Aktar *et al*., 2009; EC, 2006]. There are two types of pesticide intoxication: a) short-term exposure to a single, particularly high dose which can be experienced by workers in agrochemical industries and operators. The symptoms of such intoxication incident could be headaches, vomiting, stomach-aches, and diarrhea, and in extreme high levels of exposure also coma or death; b) long-term

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low-level exposure, which mainly comes from indirect exposure of individuals to residual amounts through consumption of water and food containing low pesticide amounts. Long term exposure could result in tumors, cancer, reproductive and nerve disorders, endocrine disruption, etc. [Damalas and Eleftherohorinos, 2011; Jeyaratnam, 1990].

Apart from human health, the release of pesticide residues in the different environmental compartments including water, air, and soil could result in adverse effects on non-target organisms from all trophic levels including soil microorganisms, beneficial arthropods, plants, aquatics, and birds or mammals [EC, 2006].

Soil microorganisms play a pivotal role in ecosystems functioning by controlling key-steps in the most important geochemical cycles including C, N, P, and S [Falkowski *et al.*, 2008]. Previous studies have shown that exposure of soil to pesticide levels substantially higher that their recommended dose could induce inhibitory effects to the structure and the function of soil microorganisms [Karpouzas *et al.*, 2014]. For example, plants depend on a variety of soil microorganisms that transform atmospheric nitrogen into forms which could be utilized by plants (ammonia and nitrates). Several recent reports have suggested that the application of certain herbicides like acetochlor [Li *et al.*, 2008] and simazine [Hernandez *et al.*, 2011], and fungicides like penconazole [Puglisi *et al.*, 2012] could induce adverse effects on nitrifying microorganisms. Another example are arbuscular mycorrhizal fungi which are obligatory endosymbiotic microorganisms growing in association with the majority of known plants aiding in nutrient uptake. Previous studies have demonstrated their sensitivity towards fungicides [Zocco *et al.*, 2011] and herbicides [Druile *et al.*, 2012; Li *et al.*, 2013].

In addition to killing insect-pests, insecticides are expected to entail an increasing direct risk for non-target beneficial insects including predators and pollinators [Silver and Riley, 2001]. Moreover, herbicides may also have indirect deleterious effects on beneficial insects by destroying the foliage needed for food and shelter [Asteraki *et al.*, 1992].

Herbicides are designed to kill plants, so it is not surprising that they induce phytotoxicity to other plants or to neighboring crops if transferred through volatilization and drift [Silver and Riley 2001]. Such incidents have been reported for several ester-formulations of herbicides that could be transported through volatilization and drift to nearby broadleaf crops inducing severe phytotoxicity [Straathoff, 1986].

Pesticides can induce deleterious effects on birds *via* different exposure means including direct ingestion of granules, baits, treated seeds, and direct exposure to spraying liquid. Indirect bird exposure may result from consumption of treated crops, contaminated water, or feeding on contaminated prey [Fishel, 2005].

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When pesticides residues reach surface water resources, adverse effects on aquatic organisms including fishes could be observed. Previous studies have demonstrated significant intoxication incidents of fishes exposed to insecticides and herbicides [e. g. US EPA, 2000; US EPA, 1996; Cox, 2000; Shafiei and Costa, 1990]. Moreover, since herbicides are designed to kill plants, it is expected their contamination of water resources would have adverse effects on aquatic plants like algae which are considered keystone-organisms in aquatic ecosystems due to their vital role as primary producers [Ambrosi *et al.*, 1978].

3. The environmental fate of pesticides

Following their release into the environment, pesticides could be subjected to different processes regulating their final fate (Figure 1.3). Several factors determine the fate of a pesticide after its release in the environment.

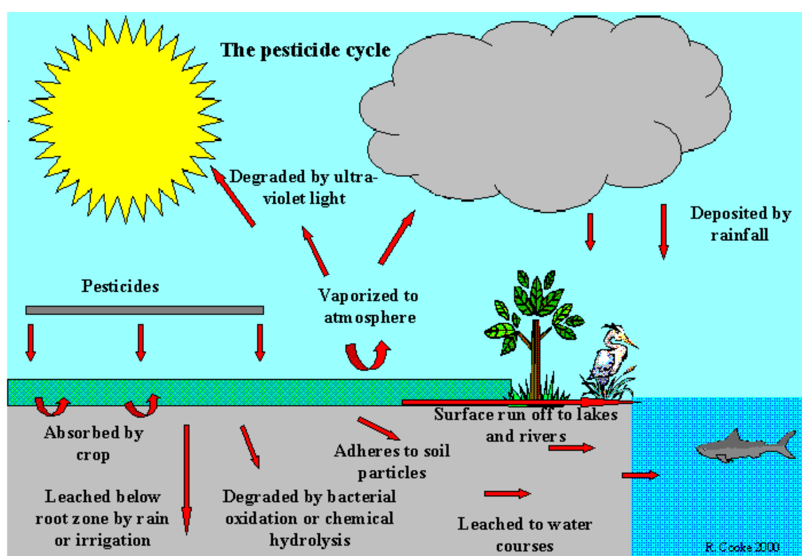

Figure 1.3. Processes involved in the environmental fate of pesticides (source: http://techalive.mtu.edu/meec/module02/Pesticides.htm).

The physicochemical properties of pesticides (vapor pressure, water solubility, and lipophilicity) along with soil characteristics (pH, soil texture, and organic matter content) and edaphoclimatic conditions (soil moisture, temperature) determine to a large extent the persistence of a pesticide in the soil environment (Figure 1.4) [Kellogg *et al.*, 2000]. Pesticides that persist longer in the environment are more likely to move off-site than less persistent pesticides.

Water solubility and lipophilicity are the most important properties which determine the availability of a pesticide in soil for leaching, lateral drainage, adsorption, and biodegradation

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[Kellogg *et al*., 2000; Rathore and Nollet, 2012]. Vapor pressure is an important parameter only for pesticides vulnerable to volatilization.

Soil pH is the most crucial factor controlling the degradation of most pesticides, *via* abiotic mechanisms (e.g. alkaline hydrolysis of organophosphorus compounds) or biotically by favouring the activity of pesticide-degrading microorganisms [Karpouzas and Singh, 2006]. In addition for certain pesticide groups such as sulfonylureas which behave as weak acids, soil pH affects their water solubility and soil availability and thus their potential for biodegradation and leaching [Grey and McCullough, 2012]. Regarding soil texture, the higher the clay content, the greater the number of binding sites for pesticide retention especially for cationic pesticides which strongly bound onto clay particles [Liu *et al.*, 2000]. On the other hand, organic matter content is the most important parameter affecting the adsorption of non-polar pesticides which constitute the majority of the currently available pesticides on the market [reviewed by Gevao *et al.*, 2000].

Soil moisture influences pesticides movement and diffusion, thus influencing the availability for degradation. Rates of pesticides transformation generally increase with the water content. Temperature affects adsorption by altering the solubility and hydrolysis of pesticides, depending on their structure [Pal *et al*., 2006].

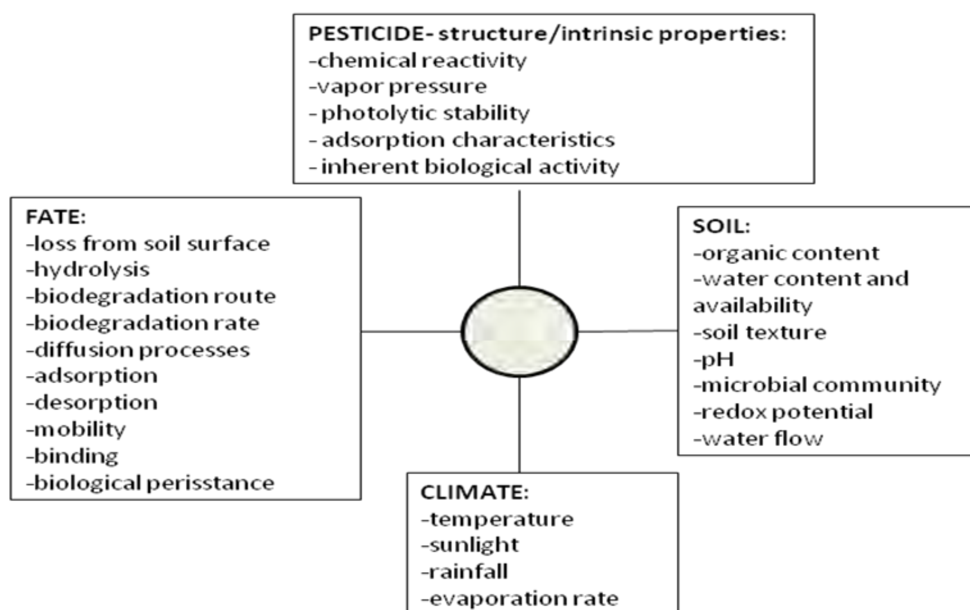

Figure 1.4. Factors governing the fate of pesticides in soil (source Arnold and Briggs [1990]).

The key processes controlling the dissipation of a pesticide in the soil environment are adsorption, transport processes, and biotic and abiotic degradation (Figure 1.5) [Arnold and Briggs, 1990; Ortiz-Hernandez *et al*., 2013].

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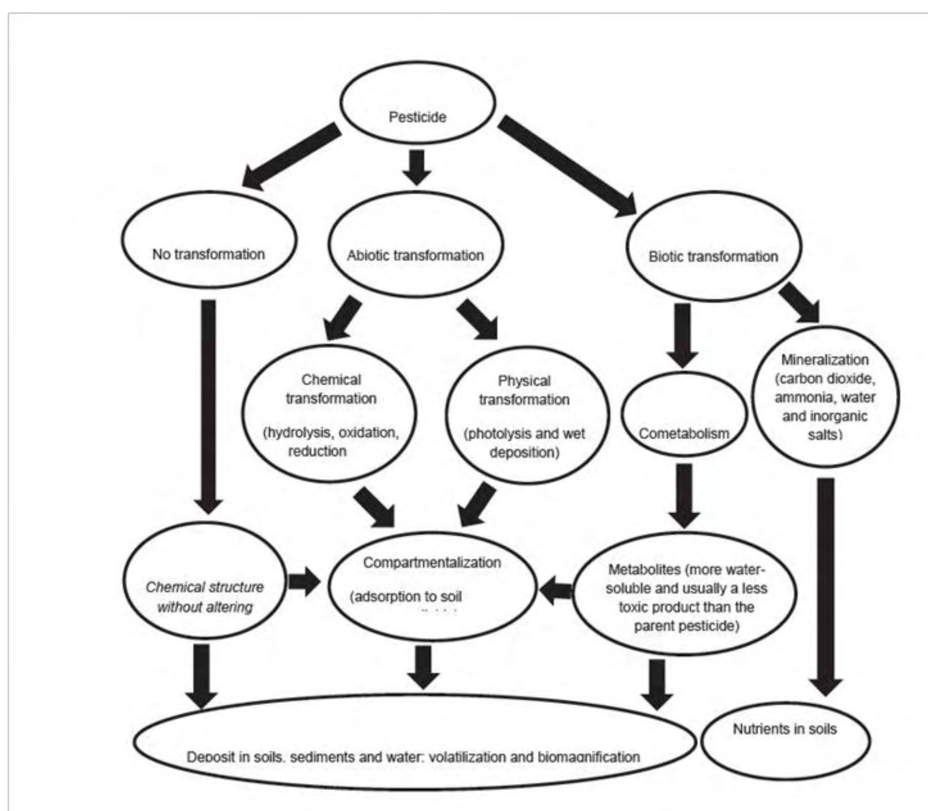

Figure 1.5. Transformation of pesticides in the environment (source Ortiz-Hernandez *et al.* [2013]).

Adsorption of non-polar pesticides is positively correlated with soil organic matter or organic carbon content. Adsorption per unit of organic carbon (K_{OC}) is relatively constant within a two- to three-fold range for a particular compound in surface soils [Arnold and Briggs, 1990], so the higher the organic matter content of a soil, the higher the adsorption of non-polar pesticides. On the other hand, polar pesticides tend to be less adsorbed on the soil organic matter and, depending on their charge, could be either strongly adsorbed by clay particles (cations) or weakly adsorbed by soil colloids (anionic e.g. weak acids like sulfonylureas) and remain available for leaching or biodegradation [Aktar *et al.*, 2009]. Soil adsorption influences not only pesticides transfer but also their availability for biodegradation [Besse-Hoggan *et al.*, 2009].

The main processes controlling pesticide translocation in the soil environment are: volatilization, leaching, and runoff. Volatilization is a major dissipation process for pesticides characterized by high vapor pressure or high values of Henry's law constant which could evaporate from soil and surface water respectively. Previous studies have reported that up to 90% of certain volatile pesticides could volatilize from soil and surface waters within a few days after application [Majewski and Capel, 1995]. Pesticides in the vapor phase could then be transported away from the site of application and be re-deposited on non-target vegetation or soil [Unsworth *et al.*, 1999]. This was demonstrated by analysis of rainwater and airborne dust which

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showed a wide range of pesticides being present in the air and re-deposited over land surfaces remote from the point of application [Coupe *et al*., 2000; Huskes and Levsen, 1997; Charizopoulos and Papadopoulou-Mourkidou, 1999; Vogel *et al*., 2008]. Leaching is the vertical movement of pesticides through soil due to water percolation. It is the process responsible for the contamination of groundwater resources [van der Perk, 2013; Arias-Estevez *et al*., 2007; Tang *et al*., 2012]. Runoff occurs due to excess of rainfall and watering that can cause pesticides to wash off from soil or plant surfaces (or pesticide contaminated soil particles to be carried) into storm drains and nearby waterways. Runoff from treated soil or lawns can contaminate surface water systems [Reichenberger *et al*., 2007; Arias-Estevez *et al*., 2007; Tang *et al*., 2012].

Degradation is a combination of both abiotic and biotic processes [Arias-Estevez *et al*., 2007; Fenner *et al*., 2013]. Abiotic degradation processes could be further distinguished to chemical and photolytic transformations. Chemical transformations could take the form of hydrolysis, oxidation, and reduction. Which of those processes will prevail depends on different factors such as pH (e.g. alkaline hydrolysis), oxygen concentrations, redox potential, moisture content, and the chemical structure of the pesticide itself. Photodegradation is a very important process for pesticides applied on water or soil surface and its rate depends on the intensity and the spectrum of the light, the length of light exposure, and on pesticide properties [Chaplain *et al*., 2011].

Biotic degradation is probably the most important process controlling the dissipation of modern pesticides [Ortiz-Hernandez *et al*., 2013]. The most abundant organisms in soil are bacteria, whereas fungi form the largest biomass. Bacteria dominate the degradation process in alkaline soils and water (pH > 5.5), while fungi dominate the degradation processes in acidic soils [Chaplain *et al*., 2011]. Interestingly, in the soil environment, bacteria and fungi seem to adopt different but complementary metabolic pathways for the degradation of recalcitrant molecules [Ronhede *et al*., 2005]. The ability of soil microorganisms to dissipate high concentrations of pesticides is directly linked to their long-term adaptation to environments where those compounds are deposited [Schroll *et al*., 2004]. Biological degradation of pesticides proceeds *via* different reactions including oxidation, reduction, hydrolysis, and conjugation [Ortiz-Hernandez *et al*., 2011]. Microbial transformation of pesticides could proceed through several sequential steps until complete mineralization of the pesticides occurs. Pesticide mineralization is considered the most desirable process from the environmental clean-up point of view. Both bacteria and fungi utilize a range of intracellular and extracellular enzymes respectively to transform pesticides [Karigar and Rao, 2011]. Microorganisms could actively

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degrade the pesticides and use them as an energy source, a process known as catabolism, or they could transform the pesticides without gaining any energy from this process, a process known as co-metabolism [Ortiz-Hernandez *et al*., 2013; Suthersan, 1999]. Pesticides which resemble in their chemical structure with natural products that soil microorganisms encounter during their life are more likely to be microbially transformed since the enzymatic armory for their metabolism is already present in the microorganisms. Anyway mechanisms to degrade new compounds can gradually or rapidly evolve [Khersonsky and Tawfik, 2010; Wackett, 2009; Russell *et al*., 2011]. Ideally, microbial transformation of pesticides will result in the production of simpler molecules like CO_2, H_2O, or intermediates which could feed the anabolism of microorganisms (pyruvate, acetaldehyde) [Fetzner, 2002]. However this is not always the case and incomplete degradation of pesticides has been also observed and could result in the accumulation of metabolites with similar or even higher toxicity than the parent compound [Navarro *et al*., 2007; Fetzner, 2002]. The metabolic pathway of the same pesticide can follow different pathways depending on the microorganisms and the conditions of the soil microecosystem (aerobic *vs* anaerobic degradation). Aerobic metabolism of pesticides is prevalent in surface soils, while anaerobic processes might be more relevant in water sediments [Suthersan, 1999; Reineke, 2001]. Pesticides that are recalcitrant to biotic and abiotic transformations are likely to accumulate in soils and contaminate groundwater [Fetzner, 2002].

4. Regulation concerning pesticides introduction in the market and use

In view of the global nature of the problems caused by pesticides, significant action has been taken at international level. The Food and Agriculture Organization (FAO) adopted for the first time in 1985 the International Code of Conduct on the Distribution and Use of Pesticides. Parallel to FAO activities, the Organization for Economic Co-operation and Development (OECD) has developed, within the frame of its Environmental Programme, tools for chemical testing and assessment that promote international harmonisation, helps countries to find ways to share the evaluation of different types of chemicals, and provides a forum through which countries exchange information about regulatory approaches and decisions [EC, 2006c]. Also the World Health Organization (WHO) deals with pesticides in its International Programme on Chemical Safety and administers the Pesticides Evaluation Scheme [Erbach, 2012].

In EU existing policies and legislation on pesticides were first introduced in 1979 and have evolved considerably over the years, culminating in the adoption of Directive 91/414/EEC concerning the placing of plant protection products (pesticides) on the market. This was followed by Directive 98/8/EC on maximum residue levels in food and feed of plant and animal origin.

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Directive 91/414/EEC intends to prevent risks at source through a very comprehensive risk assessment procedure for each active substance and the products containing the substance, before they can be authorized for marketing and use. Pesticides that are given EU-wide approval were placed on Annex 1 of the Directive. Regulation (EC) No 396/2005, set maximum residue limits (MRLs) of active substances in agricultural products, thus intending to limit the risk to consumers when pesticide residues enter the food chain. Monitoring pesticide residues is decisive in order to control if recommendations and restrictions in their use have been respected [EC, 2006].

Those Directives have focused on the placing on the market and on the end of the life-cycle of pesticides but did not address the actual use-phase. In order to address this gap and create an overall coherent and consistent policy framework for pesticides, in 2002 the Council and the Parliament adopted, for the period 2002-2012, seven Thematic Strategies (concerning air and soil quality, marine and urban environment, sustainable use of resources and pesticides, waste prevention, and recycling), expressed in the 6th Environmental Action Programme (*6th EAP*) [European Parliament and Council, 2002]. The Thematic Strategy on the sustainable use of pesticides was adopted by the European Commission on 12 July 2006. It was accompanied by a detailed impact assessment whose main objectives were: (i) to minimise the hazards and risks to health and the environment stemming from the use of pesticides; (ii) to improve controls on the use and distribution of pesticides; (iii) to reduce the levels of harmful active substances used, in particular by substituting the most dangerous with safer alternatives; (iv) to encourage low-input or pesticide-free cultivation [EC, 2007].

EU legislation on pesticides was thoroughly reformed in 2009, when new procedures for the authorization of pesticides, a framework for their sustainable use, a new approach to statistics, and new rules on machinery for pesticide applications were approved. The new legislation is based on the precautionary principle, which requires proof of safety before authorization of a product. Regulation 1107/2009 deals with the authorization for placing pesticides on the market and was introduced as a replacement of the 91/414 Directive. The Regulation requires pesticides not to have unacceptable effects on plants or damaging effects on human and animal health, groundwater, or to the environment in general. Active substances are approved by the European Commission (EC) through implementing acts, following a risk assessment by the European Food Safety Authority (EFSA). Pesticides containing approved active substances are then authorized by Member States. The authorization given is valid for ten years and can be renewed. The regulation divides the EU into three geographical zones: North, Centre, and South. Member states must authorize products authorized by other member-states of

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the same zone (with some exceptions). To place a pesticide on the market in another zone, a new authorization must be requested. The EC has completed the review of about a thousand existing active substances in March 2009. A quarter of the substances were authorized, two-thirds were eliminated because dossiers were not submitted, withdrawn or were incomplete, and 7% of the substances failed the review process. The EU pesticide database currently contains 411 approved active substances, 780 non-approved substances, and 75 substances for which approval is pending (March 2012).

Recently a new Directive 2009/128/EC dealing with the sustainable use of pesticides came into force. It aims to introduce solid procedures for pesticide handling and use aiming to minimize environmental disturbance and the risks for human health. Member States must develop national plans that include quantitative objectives, measures, indicators to monitor the use of dangerous pesticides and targets for the reduction of their use. The directive further requires training and certification of professional users and sales personnel. The directive also obliges Member States to protect surface water and drinking water, and to reduce pesticides applications in nature conservation areas.

5. Mitigation strategies to minimize the environmental impact of pesticides

Due to the problems mentioned above, the development of technologies that guarantee pesticides elimination in a safe, efficient, and economical way is important. Different methods have been developed and implemented to remediate contaminated sites and remove pesticide residues and/or obsolete pesticides. Existing technologies could be categorized to those that utilize physical processes, such as adsorption, and those that are based on chemical processes, such as advanced oxidation. The latter involves the generation of powerful transient species, mainly in the form of hydroxyl radicals [Ortiz-Hernandez *et al.*, 2013], UV/ozonation, photo-assisted Fenton reaction, or photocatalysis using TiO_2 [Felsot *et al.*, 2003].

However, these conventional physicochemical approaches are generally expensive and the remediation process is often incomplete due to the conversion of the parent compound to metabolites which are more persistent and equally or more toxic [Singh and Walker, 2006]. Moreover, while large-scale pesticide manufacturers can afford the implementation of sophisticated recovery, treatment, and clean-up techniques, smaller-scale pesticide users face difficulties in the decontamination of polluted soils or handling and detoxification of unused products and wastewaters produced from rinsing of pesticide-exposed equipments. Thus, if pesticide-contaminated waste is not properly disposed of, groundwater and surface water systems could be at risk. For small-scale operators/enterprises which generate small amounts of

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waste, practical technologies for the treatment of those wastes are still too experimental and not easily implemented on an individual farm [Felsot *et al*., 2003].

An alternative for the treatment and detoxification of pesticide-polluted areas or wastes is bioremediation. This technique relies on the ability of microorganisms to convert organic contaminants in simple and non hazardous compounds. Bioremediation overcomes the limitations of traditional methods for the disposal of hazardous compounds, so it has allowed the environmental removal of many organic contaminants at a reduced cost [Ortiz-Hernandez *et al*., 2013]. Thus, bioremediation can offer an efficient and cheap option for the decontamination of polluted ecosystems by pesticides [Singh and Walker, 2006; Vidali, 2001; Singleton, 1994; Blackburn and Hafker, 1993; Dua *et al*., 2002]. For these reasons, bioremediation has emerged as a potential alternative to the conventional techniques and could be the most practical method for small-scale companies generating pesticide-contaminated wastes [Felsot *et al*., 2003].

5.1. Bioremediation

Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities [Mueller *et al*., 1996]. By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade environmental contaminants into less toxic forms. It uses indigenous or exogenous bacteria, fungi and/or plants to degrade or detoxify substances hazardous to human health and/or the environment. Contaminants are transformed by living organisms through reactions that take place as a part of their metabolic processes. Biodegradation of a compound is often the result of the actions of multiple organisms [Vidali, 2001]. Because pesticides are mainly applied in agriculture, soil is the environmental substrate which mostly acts as a sink for those compounds. As a consequence, the vast majority of the microorganisms that have been identified as pesticide-degraders have been isolated from soil sites contaminated with pesticides [Ortiz Hernández *et al*., 2011].

5.1.1. Microbial metabolism of pesticides in soil

Theoretically, the development of the catabolic activity of soil microorganisms against pesticides is an adaptation process and the period required for microorganisms to develop this degradation capacity could reflect: (I) an increase in the degradation activity of a limited number of microorganisms initially present based on increasing expression of specific catabolic genes; (II) an increase in the number of degraders initially present due to microbial growth and/or lateral gene transfer; (III) the evolution of new enzymes necessary for utilization of the chemical as an energy source. It has been shown that bacteria are the dominant microorganisms controlling the

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accelerated biodegradation of pesticides in soil, while fungi have little or no role (Tal *et al.*, 1989; Racke and Coats, 1990). This is in agreement with the fact that in many cases fungal degradation is a co-metabolic reaction attributed to the activity of non-specific lignolytic or cytochrome P450 enzymes [Fernando and Aust, 1994; Kullman and Matsumura, 1996; Yadav *et al*., 2003].

In general, soil microorganisms adapt rapidly to degrade pesticides that are characterized by chemical structures which resemble to naturally occurring substances in the soil environment. In contrast, pesticides with a structure that is different from most naturally occurring molecules are often degraded slowly since the microorganisms do not possess suitable degradation genes or they need time to evolve new catalytic capacities. In the latter case, degradation by non-specific enzymes may still occur at a slow rate [Arbeli and Fuentes, 2007]. Nevertheless in some cases after the introduction of the xenobiotic into the soil environment, degradation rates could accelerate apparently due to the evolution of a new degradation pathway [van der Meer, 1997; Seffernick and Wackett, 2001; Johnson and Spain, 2003]. The Darwinian principles of the origin of species (or genes) by means of natural selection or the preservation of races (or genes) in the struggle for life appears to be valid here [Darwin, 1859].

Although soil may contain up to 10% organic matter, the degradation rate of this carbon, mainly humic and fulvic acids, is slow presumably due to the limited availability of potential degrading agents [Stevenson, 1982]. It is widely assumed that carbon availability is the most common limiting factor for microbial growth in soil [Alden *et al*., 2001; Koch *et al.*, 2001]. Thus the ability of a microorganism to exploit a readily available carbon source in the form of a new molecule would increase its fitness. The concept of the “selfish gene” is especially applicable to degradation genes in bacteria since many of them are mobile located either on plasmids or on transposable elements [van der Meer and Sentchilo, 2003; Top and Springael, 2003]. In this scenario, a gene with higher fitness is expected to proliferate in the environment in different bacterial hosts. These genes can ‘‘select’’ the most suitable host for a given environment and ‘‘as a reward’’ would increase the fitness of their host. Short generation times and high genomic plasticity allow bacteria to evolve new genes at a relatively high rate [Arbeli and Fuentes, 2007].

Among the main microbial groups, bacteria, fungi, and actinobacteria are the main consumers of organic matter and mostly involved in pesticide degradation [Briceño *et al*., 2007]. White rot fungi have been proposed as promising bioremediation agents, especially for compounds not readily degraded by bacteria. This ability arises by the production of extracellular enzymes that act on a broad range of aromatic organic compounds. Some of these extracellular enzymes are involved in lignin degradation, such as lignin peroxidase, manganese

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peroxidase, and laccase. Fungi generally biotransform pesticides and other xenobiotics by introducing minor structural changes to the molecule, rendering it non-toxic. The bio-transformed pesticide is released into the environment, where it is susceptible to further degradation by bacteria [Diez, 2010].

For bioremediation purposes, biotransformation enzymes are mainly categorized in three wider groups based on the reaction catalyzed: oxidoreductases, hydrolases, and transferases [Velázquez-Fernández *et al*., 2012]:

- Oxidoreductases are a broad group of enzymes that catalyze the transfer of electrons from one molecule (electron donor) to another (electron acceptor). Many of those enzymes require additional cofactors, to act as either electron donors, electron acceptors or both. Some enzymes catalyze an oxidation/reduction reaction by utilizing molecular oxygen (O_2) as electron acceptor. In those reactions, oxygen is reduced to water (H_2O) or hydrogen peroxide (H_2O_2) [Scott *et al*., 2008]. Cytochrome P450 constitutes a superfamily of versatile heme monooxygenases which can catalyze reactions of oxidation, reduction, or oxidative breakdown of aromatic or alicyclic compounds. Cytochrome P450 activity on xenobiotics in certain cases might result in an increase in their toxicity [Velázquez-Fernández *et al*., 2012]. Peroxidases and oxidases include some families of enzymes catalyzing redox reactions. Although they are produced by bacteria, fungi, plants, and animals, reports for their involvement in pesticide biodegradation exist only for fungi. It is known that ligninolytic fungi secrete peroxidases and oxidases to decompose lignin [Aust, 1995; Yadav *et al*., 2009].
- Hydrolases are a broad group of enzymes involved in pesticide biodegradation. Hydrolases catalyze the hydrolysis of several major groups of pesticides and generally operate in the absence of redox cofactors, making them ideal candidates for bioremediation strategies [Scott *et al*., 2008]. Among the hydrolases involved in the degradation of pesticides the most well-studied enzymes are phosphotriesterases that specifically hydrolyze phosphoester bonds, such as P–O, P–F, P–NC, and P–S carried by organophosphorous compounds [Ortiz-Hernández *et al*., 2003], and esterases that catalyze the hydrolysis of different ester bonds including carboxylesterases, amidases, phosphatases, etc. [Bansal, 2012].
- Among all known transferases, glutathione-S-transferase (GST) is the one mainly involved in the biodegradation of xenobiotics. Even though they catalyze the transfer of glutathione to electrophillic pesticides, they can also exhibit hydrolytic and peroxidase activities [Toribio *et al*., 1996]. Interestingly, GST can also catalyze the dehalogenation of chloroaromatic xenobiotics [Habig *et al*., 1974].

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5.1.2. Bioremediation strategies

The control and optimization of bioremediation is a complex process affected by a number of factors including: a) the existence of a microbial population capable of degrading the pollutants, b) the bioavailability of pollutants, and c) different environment factors (soil type, temperature, pH, oxygen concentration, electron acceptors, and nutrients) [Vidali, 2001]. The environmental conditions generally required for microbial activity are listed in Table 1.3.

Table 1.3. Optimum environmental conditions for soil bioremediation (source Shannon and Unterman, [1993]).

Environmental Factor	Optimum Conditions
Available soil moisture	25-85% water holding capacity
Oxygen	>0.2 mg/L DO, >10% air-filled pore space for aerobic degradation
Redox potential	Eh > 50 millivolts
Nutrients	C:N:P = 120:10:1 molar ratio
pH	5.5 to 8.5
Temperature	15 - 45°C

Bioremediation technologies could be categorized to *in situ* and *ex situ*. *In situ* techniques are defined as those that are applied to soil and water at the polluted site with minimal disturbance. In contrast, *ex situ* techniques are applied to soil and water once they have been removed from the site *via* excavation (soil) or pumping (water). Regarding the strategies of bioremediation that are followed for the recovery of polluted sites they could be distinguished to biostimulation and bioaugmentation. In biostimulation the removal of the pollutant is performed by the indigenous microbial community whose catalytic activity has been optimized *via* addition of nutrients, optimization of aeration etc. In contrast bioaugmentation relies on the augmentation of soil with exogenous microorganisms which possess high degradation capacities against the target pollutant [Vidali, 2001].

In situ bioremediation

These techniques are generally the most desirable options due to their lower cost and the limited disturbance induced by their use. *In situ* treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60

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cm and greater have been effectively treated in some cases. The most important methods applied in *in situ* bioremediation are [Vidali, 2001]:

- Bioventing: it is the most common *in situ* treatment and involves supplying air and nutrients through wells to contaminated soil aiming to stimulate the indigenous microbial community. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere. It works for simple hydrocarbons and can be used where the contamination is deep under the surface.
- Biosparging: it involves the injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria. The ease and low cost of installing small-diameter air injection points allows considerable flexibility in the design and construction of the system.

Ex situ bioremediation

These techniques involve the removal of the contaminated environmental matrix and its transportation and treatment off-site. The most common *ex situ* bioremediation methods are [Vidali, 2001]:

- Land-farming: it is a simple technique in which contaminated soil is excavated and spread over a prepared bed where it is periodically tilled until pollutants are degraded. The goal is to stimulate the indigenous microbial community for aerobic degradation of pollutants. In practice, this is limited to the treatment of the surface soil (0–35 cm). Since land-farming has the potential to reduce monitoring and maintenance costs it has received much attention as a disposal alternative.
- Composting: it is a technique that involves mixing of contaminated soil with non-hazardous organic amendments such as manure or agricultural organic wastes. The presence of fresh organic matter could stimulate the aerobic decomposition of the organic C of the mixture through the process of composting.
- Biopiles: it is a hybrid of land-farming and composting. Essentially, engineered cells are constructed as aerated composted piles. It is typically used for the treatment of soil surface contamination with petroleum hydrocarbons. Biopiles provide a favorable environment for indigenous aerobic and anaerobic microorganisms.
- Bioreactors or clean-up units: slurry reactors or aqueous reactors are used for *ex situ* treatment of contaminated soil and water respectively. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water in an engineered

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containment system. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil-bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than *in situ* or in solid-phase systems because the conditions in the bioreactor have been optimized. Despite the advantages of reactor systems, there are some disadvantages: the contaminated soil requires pre-treatment (e.g., excavation) or alternatively the contaminant can be stripped from the soil *via* soil washing or physical extraction (e.g., vacuum extraction) before being placed in a bioreactor [Vidali 2001].

5.1.3. Advantages and disadvantages of bioremediation

Bioremediation is a low cost and environmental-friendly process and as such is considered by the public as an acceptable process for the decontamination of polluted environmental matrices. Ideally its application results in the release of products that are harmless to the environment. Moreover bioremediation is less expensive than other non-biological technologies. On the other hand, bioremediation is limited to those compounds that are biodegradable. Not all compounds are susceptible to rapid and complete degradation. There are some concerns that the products of biodegradation may be more persistent or toxic than the parent compound. Biological processes are often highly specific. Important site factors should be optimal for success including the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants. It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations. Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment. Contaminants may be present as solids, liquids, and gases. Bioremediation often takes longer than other treatments such as excavation and removal of soil or incineration. Regulatory uncertainty remains regarding acceptable performance criteria for bioremediation. There is no accepted definition of “clean”, evaluating performance of bioremediation is difficult, and there are no acceptable endpoints for bioremediation treatments [Vidali, 2001].

6. Fruit packaging industry as a source of pesticide-contaminated wastewaters

Fresh fruits and vegetables are considered essential for a high-quality human diet. The competitive global marketing of fresh fruit crops demands the delivery of decay-free fruits. This

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is particularly challenging when long-distance shipping of fruits is involved [Adaskavrg and Forster, 2009]. The fruit-packaging industry is involved in the post-harvest treatment of fruits, aiming at preparing the product for sale and preventing post-harvest losses due to late infestations by pathogens. The extent of postharvest losses varies depending on the type commodities and the country producing the fruits [Amorim *et al*., 2008]. Losses which occur after harvesting (postharvest, processing, and distribution) are most prevalent in developing countries and they can be attributed to the lack of refrigeration facilities and inappropriate post-harvest treatments. In developing countries, 40% of losses occur at post-harvest and processing level while in industrialized countries >40% of yield losses occur at the retail and consumer level (Figure 1.6) [FAO, 2011].

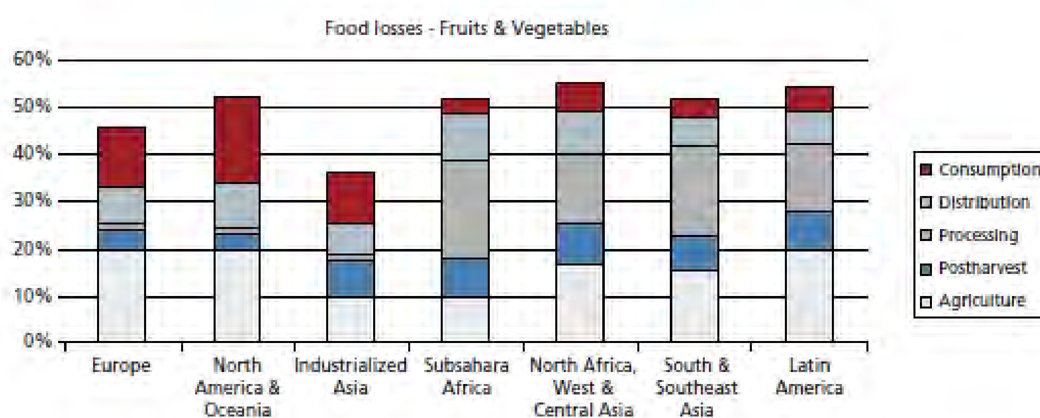

Figure 1.6. Fruits and vegetables losses at the different stages of the fruit production and processing chain. Agriculture: losses due to mechanical damage and/or spoilage during harvest operation (e.g. threshing or fruit picking), crops sorted out post harvest, etc. Postharvest: losses due to spoilage and degradation during handling, storage, and transportation between farm and distribution. Processing: losses due to spoilage and degradation during industrial or domestic processing. Losses may occur when crops are sorted out if not suitable to process or during washing, peeling, slicing and boiling or during process interruptions and accidental spillage. Distribution: losses and waste in the market system, at e.g. wholesale markets, supermarkets, retailers. Consumption: losses and waste during consumption at the household level (source FAO [2011]).

6.1. Postharvest processes and handling of fruits

The fruit packing industry is responsible for preparing, storing, and packing any fruits which are not immediately processed. Fresh fruits must be either immediately processed or stored (cold storage) for later shipment to the markets. During storage, fruits are susceptible to several post-harvest diseases and physiological disorders. In order to reduce their incidence, fresh fruits are subjected to various chemical or non-chemical treatments (Figure 1.7). A more detailed description of those processes is given below.

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Fresh fruits are placed on the drencher where they are allowed to float in a stream of water treated with fungicides. At this stage dirt and fungal spores residing on the surface of fruits are eliminated [Blanco *et al*, 2005]. There are two drenching methods currently in use: truck-drenching or bin-drenching. In the former**,** typical for processing more than 50000 bins per year, the drench solution is applied onto the fruits while they are still in bins on the truck. A typical truck-drencher has one 1500- to 3000-gallon storage tank with side and overhead coarse-spray nozzles. Post-applied drenching solution could be collected in storage tanks and re-circulated onto fresh bins of apples until a decision is made to drain out the solution and make up a new batch. In bin-drenching, typical for processing less than 50000 bins per year, the drench solution is applied to the individual bins of fruits, which have been removed from the truck, by spraying them while on a conveyor [Washington State Department of Ecology, 2004].

After drenching, a decision has to be made whether the fruit is going to be processed or placed in cold storage. If processed, the fruit is transferred at the start of the packing line which consists of float tanks that are used to remove the fruit from the bins. The number of float tanks per packing house usually ranges from one to four, with each ranging in size from 500 to several thousand gallons. Those tanks are typically discharged on a weekly or biweekly basis depending on the number of bins treated and the turbidity of the solution. The float tanks contain water which could be warmed up, chlorinated or acidified. Fungicides, if applied, are usually applied on the line. The float solution disinfects the fruit prior to packing which could be done following either the non-presize or the presize scheme [Washington State Department of Ecology, 2004].

(I) Non-presize schemes are applicable to all fruits. The fruit are conveyed out of the float tank solution by means of a continuous large-mesh chain screen. This accomplishes both the drainage of the excess of the float solution and the culling of under-sized (unmarketable) fruits. The marketable fruits which remain on the screen are placed onto a conveyance system of horizontal cylindrical rollers, laying perpendicular to the process pathway. Next, fruits are washed with a detergent solution for the removal of soil particles and other debris. Fruits are then rinsed with freshwater to flush off excess chemicals/detergents. The fruits are then rolled over a series of sponge-covered rollers which aim to absorb any water that has remained on fruit surface. Sometimes, additional devices (i.e. fans, heat, dehumidifiers) are used to expedite the drying of fruits. Once dried, fruits are waxed with or without a fungicide and after a short drying session are sorted either mechanically or manually. At the end of the packing line, the fruit is given a final visual quality control check and placed into a variety of packaging containers including boxes, bulk bags, totes, etc. These are then put into regular cold storage until time for shipment [Washington State Department of Ecology, 2004].

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(II) Presize schemes are used mainly with apples and can be followed either before or after storage. Presize schemes are more extensive and tend to use greater quantities of water than the non-presize schemes. This is because fruit conveyance is done by water "flumes" rather than by mechanical devices which are used in the non-presize schemes. A typical presize fruit packer utilizes a number of flumes at any one time, from 6 to 18. The most important factor is that all sorting is completed separately of the packing line, which itself is nearly identical to that of the non-presize scheme.

When presizing occurs before storage, harvested fruits are first drenched with a fungicide/antioxidant solution and then floated, sorted, and packed or re-binned. The filled bins are then placed into storage. When market orders arrive, the bins of properly sized apples are subjected to the non-presize scheme (as described above), with the exception of sorting which has been already completed. When presizing occurs after storage, binned fruits are floated, washed, rinsed, and sorted. Once the sorting has been accomplished, the apples are re-binned and placed into regular cold storage. When market orders arrive, the bins of properly sized apples are retrieved from storage and sent through the non-presize scheme (as described above), with exception of sorting, since that has been already completed [Washington State Department of Ecology, 2004].

Figure 1.7. A foreshortening of fruit packaging industry phases (sources: http://www.redseal-quality.com/articles/view/packing-house#/catalogs/get/36/gallery; S. Horvitz, INTA E.E.A. Balcarce; Adam Blasberg)

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6.2. Postharvest diseases and disorders of fruits

Microbial decay is one of the main factors that compromises the quality of the fresh product. Fruits, due to their low pH, higher moisture content, and nutrient composition are particularly prone to fungal infestations, which in addition to causing rots may also make them unfit for consumption due to the production of mycotoxins [Phillips, 1984; Moss, 2002]. Eckert and Ratnayake [1983] estimated that more than 100 fungal species are the causal agents of postharvest diseases. On the contrary, the low pH of most fruit acts as a barrier for bacterial infestations which are generally of less importance [Coates and Johnson, 1997].

Postharvest diseases of fruits can be the result of latent infections that occur in the field during the growing season and infections from wounding during harvest and handling operations [Eckert and Sommer, 1967; Michailides and Elmer, 2000]. Latent infection of plants by pathogenic fungi at the field involves an asymptomatic parasitic phase that eventually gives rise to visible symptoms when conditions become favorable for disease development [Verhoeff, 1974; Sinclair and Cerkauskas, 1996]. The physiological changes which occur during fruit ripening are often the trigger for reactivation of quiescent infections. On the other hand, major postharvest diseases arise from infections initiated during and after harvest. Many common postharvest pathogens are unable to directly penetrate the fruit cuticle. Such pathogens therefore infect through surface injuries or natural openings such as stomata and lenticels. Injuries can vary in size from microscopic to clearly visible and may arise in a number of ways. Mechanical injuries such as cuts, abrasions, pressure, and impact damage commonly occur during harvesting and handling. Insect injuries may occur before harvest yet remain undetected at the time of grading, providing ideal entry points for many postharvest pathogens. The natural resistance of fruits to disease declines with storage duration and ripeness. Weak pathogens which normally require a wound to infect can become a serious problem in products that have been stored for long periods [Coates and Johnson, 1997].

Many of the fungi which cause postharvest disease belong to the phylum Ascomycota and the associated Fungi Anamorphici (Fungi Imperfecti). In the case of the Ascomycota, the asexual stage of fungus (the anamorph) is usually encountered more frequently in postharvest diseases than the sexual stage of the fungus (the teleomorph) [Coates and Johnson, 1997]. The main fungal pathogens that cause important postharvest losses on fruits in Europe are: *Penicillium expansum* and *Phlyctema vagabunda* on pome fruits, *Monilinia* sp. on stone fruits, *Penicillium digitatum* and *Penicillium italicum* on citrus fruits, and *Botrytis cinerea* on table grape, strawberries, and kiwifruits [Mari *et al.*, 2009].

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6.2.1. Blue mould of pome fruits (P. expansum Link)

It is one of the main postharvest diseases in pome fruits [Jones and Aldwinckle, 1990]. In Europe, the pathogen causes extensive decay [Amiri *et al*., 2008], particularly in pears [Spotts *et al*., 1998]. The pathogen infests fruit tissues *via* wounds such as punctures, bruises, and limb rubs which are formed during fruit processing. The infested area appears light tan to dark brown. The decayed tissue is soft and watery and the lesion has a very sharp margin between diseased and healthy tissues. Blue or blue-green spore masses may appear on the decayed area (Figure 1.8). This disease has raised concerns also for the fruit-processing industry due to the capacity of some strains of *P. expansum* to produce a mycotoxin called patulin which affects the quality of juices [Rosenberger, 1990]. In the orchard, *Penicillium* spp. survives in organic debris on the orchard floor, in the soil, and perhaps on dead bark on the trees. Conidia are also present in the air and on the surface of fruit. In the packing-house, antioxidant- or fungicide-drench solutions, flume water and dump-tank water are common sources of *Penicillium* spores for fruit infection during the handling and packing processes. Spores of *P. expansum* are also commonly present in the air and on the walls of storage rooms.

Orchard sanitation to remove decayed fruits and organic debris helps to reduce inoculum levels of *Penicillium* spp. Good harvest and handling management to minimize punctures and bruises on the fruits help to prevent fruit infestations by *P. expansum* and other *Penicillium* species [Tree Fruit Research & Extension Center, Washington State University, 2005]. In the packing-house, the control of blue mould is based on the use of thiabendazole (TBZ) and imazalil (IMZ). The development of resistance to those fungicides has reduced the effectiveness of such treatments [Baraldi *et al*., 2003]. Alternatively, two relatively new fungicides, fludioxonil and pyrimethanil have been used for the control of blue mould with promising results [Li and Xiao, 2008; Tree Fruit Research & Extension Center, Washington State University, 2005]. Sanitizing dump-tank and flume water is an essential practice to reduce infection of fruits by *Penicillium* spp. during the packing process. Killing spores in dump tanks, bins, and in flume water with chlorine or sodium *ortho*-phenylphenol (SOPP) has been effective in reducing the spore load and the levels of fungal infestation [Janisiewicz, 1999].

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Figure 1.8. Blue mould infestations in a Red Delicious apple and a d'Anjou pear. White mycelium and blue spore masses at the decayed area are visible (source: http://decay.tfrec.wsu.edu/display Page.php?id=pathlab&pn=20).

6.2.2. Lenticel rot [Neofabrea alba (EJ Gutrie) Verkley, anamorph P. vagabunda Desm., syn. Gloeosporium album Ostew]

It is one of the most frequent and damaging diseases occurring in stored apples (more rarely in pears) in Italy, France, and other European apple-producing countries [Pratella, 2000; Amiri *et al*., 2008]. Fruit infection occurs in the orchard, but disease symptoms appear only several months after harvest. Several late maturing varieties of apples are particularly susceptible to the disease, with an incidence level of 15–30% after 120 days of cold storage [Bompeix and Cholodowski-Faivre, 1998; Mari *et al*., 2002; Maxin *et al*., 2005; Weibel *et al*., 2005]. The disease produces conidiomata on the surface of decayed areas of the fruit after prolonged storage. Early symptoms appear as small dimples. As firmness decreases, pits grow in size and depth (Figure 1.9).

Current measures to control *N. alba* infections include pre- and postharvest treatments with synthetic fungicides. In Italy, the postharvest use of TBZ is allowed only for apples and pears stored longer than 31 December of each year. Among non-chemical control means dipping in hot water (45°C for 10 min) leads to a consistent reduction of infection, without causing any damage to the fruit [Maxin *et al*., 2005].

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Figure 1.9. Lenticel rot infection in apple (source: L. Lindner).

6.2.3. Green and blue moulds of citrus fruits (P. digitatum Pers.: Fr. Sacc. and P. italicum Wehmer respectively)

They are the most important post-harvest diseases of citrus fruits [Eckert and Eaks, 1989; Palou *et al*., 2001b]. Both pathogens grow optimally at 24°C and although green mould predominates at room temperature, blue mould is more important for cold-stored citrus fruits, since *P. italicum* grows faster than *P. digitatum* at temperatures below 10°C [Brown and Eckert, 2000]. Those fungi are wound parasites and can infect fruits in the grove, in the packing-house, and during distribution and marketing. Fungal spores are practically always present on the surface of the fruit throughout the growing season and after harvest they can build up to high infestation levels unless appropriate sanitization measures are adopted at the fruit packaging plant level [Kanetis *et al*., 2007]. A white mold is first seen growing on the peel. When the infection is caused by *P. digitatum* the mold later turns green, whereas the infection turns blue if *P. italicum* is the causal agent (Figure 1.10).

Application of synthetic fungicides is generally the main method to control green and blue moulds where the frequency of fungicide-resistant isolates is low. However, the intense use of fungicides such as sodium *o*-phenylphenol (SOPP), TBZ, and IMZ has led to the proliferation of fungicide-resistant isolates [D'Aquino *et al*., 2006]. Heat treatment [Plaza *et al*., 2003], ultraviolet light exposure (UV-C) [D'hallewin *et al*., 1999], and storage in an ozoned atmosphere [Palou *et al*., 2001] have been found to control *P. digitatum* and *P. italicum*; nevertheless, each of these methods have certain limitations. Three biological fungicides called Aspire™ (*Candida oleophila*, limited to USA and Israel), BioSave™ (*Pseudomonas syringae*, limited to USA) and Shemer™ (*Metschnicowia fructicola*, limited to Israel) have been utilized for the postharvest control of green and blue moulds. However, the commercial use of those products remains limited and accounts for only a very small fraction of the market. Preventive measures such as

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careful picking and handling of fruit and sanitization of packing and storage facilities could substantially reduce the level of infestations by those fungal pathogens [Mari *et al*., 2009].

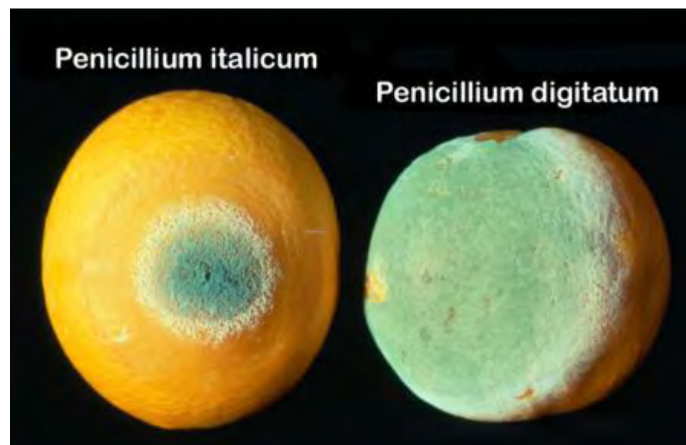

Figure 1.10. Blue mold (left) and green mold (right) causing soft decay of citrus fruit (source: Edwards Don, University of California, Davis).

6.2.3. Grey mould (B. cinerea Pers. Fr.)

Grey mould is an important postharvest disease because environmental conditions prevailing in storage facilities favor its development. *B. cinerea* is a pathogen of many crops but it has become a main problem in cold-stored apples, pears, grapes, strawberries, and kiwifruits. The control of the disease is particularly important in storage because it develops at low temperatures (-0.5°C) and spreads quickly among stored fruits (Figure 1.11) [Mari *et al*., 2009]. *Botrytis* rot first appears as a light brown to grey spot without any distinct margin around the infected area. After a few days, if conditions are favorable, a brown to grey velvet growth will appear on the surface of the infected fruit. The fungus grows well on senescent, dead tissues. Spores are blown by the wind or splashed by the rain onto flower and fruit tissues. Because *Botrytis* is an aggressive colonizer of plant wounds, direct infection can also occur if the fruit is injured. Mature fruits are especially susceptible to infestations because of their high sugar content and sensitive tissues [Bolda and Koike, 2012]. Secondary infection of fruit through fruit-to-fruit contact during storage is commonly seen after a long period of storage and can cause significant losses. Mycelial growth is enhanced by high relative humidity. Fruit infected by grey mold due to secondary infection in storage bins may not have visible symptoms or lesions are very small at the time of packing, and thus infected fruit may be packed but symptoms develop during storage or transportation (Figure 1.11) [Lennox *et al*., 2003].

Preventive fungicide applications during bloom are essential to control this disease. Orchard sanitation to remove decayed fruit and organic debris from the orchard floor helps to

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reduce inoculum levels of *B. cinerea*. Good harvest management to minimize punctures and bruises on the fruit skin helps to avoid decay from wound infections. A postharvest drench treatment with TBZ applied prior to storage is effective to control grey mold, particularly for those infections that originate from wounds [Barkai-Golan, 2001].

Figure 1.11. Extensive *Botrytis* sporulation on grey mold on strawberries (left) (source: Koike S., UCCE) and nesting of grey mold due to fruit-to-fruit spread during storage of pears (right) (source: http://decay.tfrec.wsu.edu/printPage.php).

6.2.4. Scald

It is a term loosely applied to a group of skin disorders of apples and pears. They appear as brown or grey irregularly shaped discolorations on the surface of the fruit during or following storage (Figure 1.12). This spotting could easily be mistaken for lenticel scald, but the main difference is that the scald is a physiological disorder and it is not caused by pathogens. It has been shown that early in storage fruits accumulate a chemical called alpha-farnesene, a volatile compound. Under prolonged storage alpha-farnesene is oxidized to form conjugated trienes which do not evaporate and continue to accumulate in the fruit during storage resulting into cell death appearing as a brown or black discoloration on the fruit. The severity of scald appearance depends on a variety of factors including fruit cultivars, weather conditions and fruit maturity. Generally, hot and dry weather increases scald susceptibility while mature fruits are less susceptible to scald. Finally, green apples are generally more susceptible to scald [Bramlage 1988; Rupasinghe *et al*., 1998; Rowan *et al*., 2001].

Numerous approaches to control scald have been developed. Storage of fruits under low oxygen conditions impedes oxidation of alpha-farnesene thus minimizing the appearance of scald. However, the most reliable scald-control measure is still the use of antioxidant chemicals like diphenylamine (DPA) and ethoxyquin. These pesticides interfere with the oxidation of alpha-farnesene. However their use is not without problems. The materials must be used with

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care, since excessive dosage can cause severe fruit injury. Even use at recommended dosage often leads to injury due to entrapment of solution in cavities, between fruit, or in wooden containers. There is also concern about the risks to consumers from residual antioxidants. However, since these compounds are volatile, little or no residue should persist at the end of storage if their application is done properly [Bramlage 1988; Ju and Curry, 2000]. At the moment neither DPA nor ethoxyquin are authorized for use at EU level for different reasons which will be described later in the introduction. An alternative chemical used against apple scald is the fumigant MCP (1-methylcyclopropene) which is registered for use in most fruit-producing countries. MCP is an ethylene action inhibitor that delays ripening of many fruits, including pears [Sisler and Blankenship, 1996; Bai *et al.*, 2006; Calvo and Sozzi, 2009]. Although it appears as a good alternative to existing antioxidants its application should be done timely in order to avoid total halting of fruit ripening [Villalobos-Acuna *et al.*, 2011].

Figure 1.12. Scald on apple (source: http://postharvest.ucdavis.edu/produce_information/Fruit_Physiological_Disorders/Apple_StorageScald/).

6.3. Strategies for postharvest disease control

An integrated approach for controlling the development of post-harvest decays and disorders could include a) preventive measures including injury prevention, sanitization, and maintenance of high hygiene level in the fruit-packaging plants, b) non chemical measures like heat treatment or irradiation, and c) chemical measures like the application of fungicides and antioxidants [Adaskaveg and Forster, 2009]. A more detailed description of the different measures follows.

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6.3.1. Prevention of injury

As many postharvest pathogens gain entry through wounds or infect physiologically-damaged tissues, prevention of injury at all stages during production, harvest, and postharvest handling is critical. Injuries can be mechanical (e.g. cuts, bruises, and abrasions), chemical (e.g. burns), biological (e.g. insect, bird, and rodent damage), or physiological (e.g. chilling injury, heat injury). Injuries can be minimized by careful harvesting and handling of fruits, appropriate packaging, and storage at the recommended temperature. Where injuries are present, the process of wound healing can be accelerated in some instances through manipulation of the postharvest environment (e.g. temperature and humidity) or by application of certain chemicals [Coates and Johnson, 1997].

6.3.2. Heat treatments and ionizing irradiation

Heat treatment of harvested fruits has been used as a non-chemical method to minimize fungal infestations at storage. Postharvest heat treatments include hot water treatment, short hot water rinsing and brushing, and treatment with hot air. Such treatments appear to reduce rot development, enhance fruit resistance to chilling injury in cold-sensitive cultivars, and retain fruit quality during cold storage and shelf life. Despite the beneficial effects of heat treatments, the complete control of fruit decays is rarely accomplished by heat therapy alone and combination with other treatments is essential to establish acceptable control levels [Schirra *et al*., 2011]. Moreover, fruits vary greatly in their physiological tolerance to heat treatments. For example, most temperate fruit types are quite susceptible to heat injury, particularly at the temperatures required to achieve disease control [Coates and Johnson, 1997].

Ionizing irradiation is another physical treatment that can be used after harvest to reduce the incidence of fungal infestations in some commodities. However during ionization free radicals are produced. These free radicals react with various food constituents and may cause injury to the cells. Since fresh fruits contain 80-95% water and their intracellular spaces (about 20% of total volume) contain oxygen, the most common free radicals are those of water and oxygen. Consequently, treating fresh fruits with ionizing energy in nitrogen atmosphere can reduce the amount of free radicals and possible injuries to the plant tissue. However, this will also reduce the treatment effectiveness. Like in the case of the heat, fruits must be able to tolerate the doses of ionizing radiation required to achieve disease control [Kader, 1986; Hallman, 2011]. Combination treatments, such as heat+irradiation, may be synergistic. In that case, lower levels of irradiation could be used which are expected to be less detrimental to fruits quality [Kader, 1986; Pan *et al.*, 2004].

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6.3.3. Hygiene practices

Maintenance of high hygiene levels during postharvest treatment of fruits is critical for minimizing the possibility of development of postharvest diseases. To most effectively reduce inoculum, a good knowledge of the life cycle of the pathogen is essential. Inoculum for infections occurring at post-harvest level commonly originates from the packing shed and storage environment. Water used for washing or cooling fruits can become contaminated with pathogen propagules if not changed on a regular basis or if it is not properly disinfected. Fruits of low market value which have been rejected from further processing could act as an ideal substrate for postharvest pathogens proliferation and should be removed from the fruit packaging plant [Coates and Johnson, 1997]. Fruit debris that are released during fruit processing should be removed from the floor and the machinery of the packaging plant on a daily basis to reduce inoculum sources. Packing and grading equipment, particularly brushes and rollers, should be cleaned and disinfected on a regular basis [Coates and Johnson, 1997]. A sanitizing agent (e.g., chlorine, peroxyacetic acid, etc.) should be used to treat surfaces coming in contact with fruits after the equipment is cleaned at the end of each day [Ritenour *et al.*, 2012].

6.3.4. Postharvest storage conditions

Temperature is perhaps the single most important factor influencing disease development after harvest. Temperature not only directly influences the rate of pathogen growth, but also the rate of fruit ripening. Because the development of many postharvest diseases is closely associated with fruit ripeness, treatments which delay ripening tend also to delay disease development. Low temperature storage of fruits is used extensively to delay ripening and the development of diseases. However, those low storage temperatures are not lethal for fungal pathogens. Thus, cold-stored fruits which are transferred to the market at ambient temperature may rapidly breakdown due to the late development of postharvest decays [Coates and Johnson, 1997; Kulkarni, 2012].

Modifying the storage atmosphere is a measure commonly used to delay fruit ripening. The rate of fruit respiration can be reduced by increasing CO_2 and decreasing O_2 levels in the storage environment. Storage atmosphere can also have a direct effect on pathogen growth, although levels of CO_2, or O_2 required to achieve this are often damaging for fruits if applied for extended periods. The relative humidity of the storage environment can have a major impact on the development of postharvest diseases. High humidity is often used to minimize water loss of produce. This however can increase disease levels so the humidity chosen for storing fruits is

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frequently a trade-off between minimizing water loss and disease [Coates and Johnson, 1997; Kulkarni, 2012].

6.3.5. Chemical control with synthetic pesticides

The postharvest fruit treatment with pesticides is the most effective mean to minimize losses due to fungal decays or physiological disorders [Eckert and Ogawa, 1988; Adaskaveg *et al*., 2002]. Ideally, fungicides protect fruits from fungal infections occurring either at pre- and postharvest level and prevent the appearance of decays until the fruit reaches the market [Adaskaveg and Forster, 2009]. Systemic fungicides are generally used for this purpose. How successful fungicides are in achieving high level of control depends largely on the extent to which infection has developed at the time of fungicide application and how effectively the fungicide penetrates the host tissue. In general, fungicides for the control of wound-invading pathogens should be applied immediately after harvest. If infection is advanced at the time of postharvest treatment, control will be difficult to achieve. For postharvest pathogens which infect products before harvest, field application of fungicides is also often necessary although this is out of the scope of this thesis.

Postharvest pesticides used in fruit-packaging plants can be applied as dips, sprays, fumigants, treated wraps, and box liners or in waxes and coatings. The most common mode of application is dipping or spraying which is done usually in aqueous solutions, suspensions, or emulsions [Coates and Johnson, 1997]. For wax-applied fungicides higher concentrations are used compared to aqueous applications, since the fungicide which is impregnated in wax is not readily available to penetrate into the wounds or cracks of the surface of the fruit. The combination of heat treatment with fungicides could provide a more effective control compared to fungicides applied at ambient temperature [Schirra *et al*., 2011]. This has been attributed to the more efficient diffusion and penetration of pesticides into fruits at increasing temperature. The combined application of heat and pesticides could provide successful control of fruit pathogens at lower pesticide dose rates [Baur and Schonherr, 1995]. However, undesirable effects of high temperatures on fruits have been observed in those combinatory applications and have been attributed to the prolonged exposure of fruits to high temperatures or to localized accumulation of high pesticide residues on fruits surface [Schirra *et al*., 2011]. Various factors affect the deposition of fungicides and their dissipation rates in fruit, including fungicide concentration, treatment mode (spray, drench, or dip), type of mixture (aqueous- or wax-based mixtures), type of fruit, cultivar, fruit age, treatment duration, temperature, and pH of the fungicide mixture [Dezman *et al*., 1986; Papadopoulou-Mourkidou, 1991].

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Despite the key role of pesticides in the control of postharvest decays and disorders in fruit packaging plants, their use leads to the establishment of various problems which should be managed accordingly. A first major concern is the production of large volumes of wastewaters containing fungicides/antioxidants by the fruit packaging plants. These wastewaters constitute a serious point source for the contamination of natural water resources. Previous monitoring studies reported the frequent detection of TBZ and IMZ in surface water systems of Costa Rica which was attributed to the presence of fruit packaging plants adjacent to the river systems monitored [Castillo *et al*., 2000; Campo *et al*. 2013; Masia *et al.* 2013]. The risk for point source contamination by the postharvest activities of the citrus production industry has been identified by the EC which has given authorization to these fungicides under the clause that *appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place* [EC, 2001; EC, 2010]. Despite that, the only depuration system currently available is based on pesticide adsorption onto granular activated carbon [Garcia Portillo *et al*., 2004]. Although this system achieved 7000 times reduction in TBZ concentrations its cost is prohibitive [EC, 2000]. Thus there is an urgent need for the development and implementation of a cost- and depuration-effective system for the detoxification of those wastewaters.

Another problem concerning the use of fungicides in fruit-packaging plants is the evolution of fungicide-resistant fungal strains [Reimann and Deising, 2000; Dianz *et al.*, 2002]. Resistant pathogens develop primarily by selection from a small number of naturally occurring, less sensitive individuals within the population upon prolonged and repeated exposure to the same fungicides or fungicides with similar mode of action [Brent and Hollomon, 1998]. The continued use of the same fungicide will eliminate all sensitive strains while allowing the survival of resistant strains which in the absence of competition will multiply. Once the size of a less sensitive, competitive sub-population reaches a threshold, treatments with the fungicide will no longer be effective [Adaskaveg and Forster, 2009]. The extent of potential fungicide resistance development within a population is influenced by the intrinsic properties of the fungicide and the pathogen in a given environment. In addition, a range of packing-house practices that affect fungicide efficacy also affects the risk of resistance development. For postharvest fungicides that have a single-site mode of action, the selection of resistant individuals is more likely to occur. Among postharvest fungal pathogens, a more rapid development of resistance is expected for those pathogens that have a high reproductive potential and short generation time [Adaskaveg and Forster, 2009]. Packing-house practices that increase the likelihood of resistance development include all methods that lead to sub-optimal fungicide

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coverage (e.g. uneven distribution over the fruit surface) and residue concentrations at infection sites (e.g. mostly fruit injuries). Appropriate sanitation practices are another key aspect of resistance management. The goal is to keep postharvest pathogen populations that are exposed to fungicide selection pressure at a minimum [Adaskaveg and Forster, 2009].

The most common method to minimize the possibility of cross resistance between fungicides is to rotate chemicals with different mode of actions. In theory, any fruit lot should only be treated once with a fungicide of the same class or mode of action. Ideally, rotations of mixtures should be used for fruit crops that are being treated more than once, such as some citrus and pome fruits. New and planned postharvest fungicide pre-mixture registrations accommodate this strategy. In mixture applications, the resistance potential is much reduced as compared to applications with single active ingredients because of a lower resistance frequency [Adaskaveg and Forster, 2009]. Among other pivotal components in resistance management the routine monitoring for fungicide sensitivity in the pathogen population is of outmost importance [Adaskaveg *et al.*, 2004]. The goal is to detect any shifts in sensitivity as compared to baseline values at an early stage before practical resistance and lack of fungicide efficacy occurs.

6.4. Emerging technologies for postharvest disease control

The problems associated with the use of synthetic pesticides in the fruit-packaging industry have stimulated interest in alternative approaches [Cross and Berrie, 2008]. In accordance with this, a number of strategies including biological control agents, natural products with fungicide activity or elicitors for plant defense mechanisms have been tested and used in the fruit-packaging industry [Wilson and Wismewski, 1989].

6.4.1. Biological control agents (BCAs)

BCAs have been reported to act against a number of postharvest pathogens [Wisniewski *et al.*, 2001]. So several BCAs have been commercialized for this purpose including Aspire™ (Ecogen Inc., Langhore, PA) which is based on the yeast *Candida oleophila*, BioSave™ (JET Harvest Solution, Longwood, FL) which is based on a *Pseudomonas syringae* strain, YeldPlus™ (Anchor Yeast, Cape Town) which is based on the yeast *Cryptococcus albidus* and Shemer™ (AgroGreen, Asgdod) which is based on *Metschnikovia fructicola*. These BCAs are currently registered for postharvest use in the USA, South Africa, and Israel but not in Europe. CANDIFRUIT™ (SIPCAM INAGRA, S.A. Valencia) based on a *Candida sake* strain is commercially available in Spain since 2008 [Mari *et al*., 2009]. BCAs show diverse mode of actions which could include direct inhibition due to secretion of antibiotics, outcompeting

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pathogens for nutrients and/or space, induction of resistance responses by the host or direct parasitism of the pathogen [Wilson *et al.*, 1991]. Despite their obvious advantages, BCAs suffer from specific problems which limit their wider market uptake. Such problems include limited spectrum of pathogens that they could control, short shelf-life, and generally high cost compared to their chemical counterparts [Coates and Johnson, 1997; Mari *et al.*, 2009].

6.4.2. Natural products with fungicidal activity

Many compounds produced by plants through their secondary metabolism exhibit fungicidal properties [Rosenthal and Jansen, 1979; Goyal *et al.*, 2012]. Such compounds could be extracted and used as pesticides to control fungal pathogens.

Several plant volatiles like acetaldehyde, benzaldehyde, cinnamaldehyde, ethanol, benzyl alcohol, nerolidol, 2-nonanone have been found to exhibit antifungal activity against different fruit pathogens during *in vitro* trials [Utama *et al.*, 2002]. The general antifungal activity of essential oils is also well documented [Tripathi and Dubey, 2004]. The advantage of essential oils is their bioactivity in the vapor phase, a characteristic that makes them attractive as possible fumigants for stored product protection. Most of the essential oils have been reported to inhibit postharvest fungi *in vitro* [Bishop and Reagan, 1998; Singh and Tripathi, 1999; Bellerbeck *et al.*, 2001; Hidalgo *et al.,* 2002]. However, the *in vivo* efficacy and practical activity of only a few essential oils has been studied [Dubey and Kishore, 1988; Tiwari *et al.*, 1988; Smid *et al.*, 1994.; Dixit *et al.*, 1995]. Glucosinolates, a large class of approximately 100 compounds produced by brassicas, exhibit high biocidal activity which has been mostly attributed to their hydrolysis products, isothiocyanate, thiocyanate, and nitrile. The antifungal activity of six glucosinolates has been tested on several postharvest pathogens, both *in vitro* [Mari *et al.*, 1993] and *in vivo* [Mari *et al.*, 1996] with encouraging results. Acetic acid is a metabolic intermediate that is formed naturally in many fruits and its application for surface-sterilization of a wide range of fruits has been shown to be an effective method for commercial use on apricots and plums [Liu *et al.*, 2002], grapes [Sholberg *et al.* 1996] and sweet cherries [Sholberg, 1998; Chu *et al.*, 1999; Chu *et al.*, 2001]. It is a natural compound which poses little or no residual hazard at the low levels required to kill fungal spores. In addition, it is inexpensive, compared to other fumigants such as acetaldehyde, and can be used in relatively low concentrations to treat fruits in air-tight storage rooms or containers [Tripathi and Dubey, 2004].

Biological compounds, because of their natural origin, are certainly more biodegradable and thus less persistent in the environment than their synthetic counterparts. Although these compounds may be more desirable than synthetic chemicals from a consumer viewpoint, their

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potential toxicity to humans needs to be evaluated before commercialized. Proper organoleptic tests are also necessary before any recommendation. The lowest suitable dose of the chemicals for practical application should also be determined. Encouraging results on the use of natural products to control postharvest fungal rotting indicate that it could be possible to develop natural fungicides that would be as effective as synthetic fungicides, and presumably safer for man and the environment [Tripathi and Dubey, 2004].

6.4.3. Induction of systematic resistance of fruits

Relatively little is known about host defense responses in harvested commodities. Resistance responses have been demonstrated in harvested citrus fruits [Brown *et al*., 1978; Brown and Barmore, 1983; Bandoin and Eckert, 1985] and apples [Lakshminarayana *et al.*, 1987; Sommer and Fartlage, 1988]. Compartmentalization of the pathogen within the plant tissue appears to be an important resistance mechanism. Baudoin and Eckert [Bandoin and Eckert, 1985] showed that lignin-like substances were deposited in the outer peel of fruits upon wounding. Chitosan, which is a natural compound present in the cell wall of many fungi could act as an elicitor of host defense responses. Chitosan can stimulate a number of processes including production of chitinase, accumulation of phytoalexins and increase in lignification. A wide range of other compounds (e.g. salicylic acid, methyl jasmonate, and phosphonates) and treatments (e.g. heat) can induce host defences in harvested commodities. Identification and manipulation of elicitors in harvested fruits may provide an effective novel way to control post-harvest diseases [Coates and Johnson, 1997].

7. Main fungicides and antioxidants used in postharvest treatments of fruits

In Europe the main fungicides used to prevent postharvest diseases of fruits are thiabendazole, imazalil, and *ortho*-phenylphenol (Table 1.4). Regarding scald the only currently registered pesticide for its control is the fumigant MCP [EC, 2006b], although exemption authorizations for 120 days have been given for diphenylamine by certain member-states in Europe considering that there are no equally effective alternatives in the market for the control of apple scald.

7.1. Thiabendazole (TBZ)

Thiabendazole (*2-(4-thiazolyl)benzimidazole*; CAS number: 148-79-8; trade names: Mertec®, Arbotect®, Vitavax®, Agrosol®, Metasol®, Sealbrite®) is a benzimidazole fungicide used to control fruit and vegetable diseases. It is mainly used in postharvest treatments of fruits (bananas, citrus and pome fruits) and potatoes (seed and ware potatoes). TBZ is also used as a

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broad spectrum anthelminthic compound in various animals and in humans [Coulet *et al*., 1998]. TBZ chemical structure is presented in Figure 1.13.

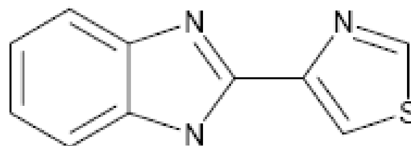

Figure 1.13. The chemical structure of thiabendazole.

Physicochemical properties: Technical TBZ (Molecular Mass: 201.26 g/mol) is a colourless crystalline solid with a melting point of 304-305^oC. It has moderate to low solubility in water (28-30 mg/l at 25^oC and pH 7) and is soluble in several organic solvents. Its relatively low vapour pressure (5.3 x 10^{-7} Pa at 25 °C) and Henry's law constant (3.7 x 10^{-6} Pa m^3 mol^{-1}) suggest limited volatility. It has a $logP_{ow}$ of 2.39 (at pH 7 and 20°C) [EC, 2001].

Toxicity-ecotoxicity: TBZ has low acute oral and dermal toxicity (Category III). It is not an eye or dermal irritant nor a dermal sensitizer. US EPA has classified TBZ as "*likely carcinogen at doses high enough to cause a disturbance of the thyroid hormone balance. It is not likely to be carcinogenic at doses lower than those which could cause a disturbance of this hormonal balance*" [US EPA, 2002]. TBZ is not mutagenic, teratogenic, neurotoxic, genotoxic, and it does not induce reproductive toxicity. It shows low acute and short-term dietary toxicity to rats and birds (LC_{50} rats = 3100 mg/kg bw; LC_{50} birds acute >2250 mg/kg bw; LC_{50} short-term birds >5620 mg/kg diet). However, it is highly toxic to non-target aquatic organisms as it is indicated by its low toxicity endpoint values for indicator organisms (EC_{50} *Daphnia magna* = 0.81 mg/l, NOEC 21 days *D. magna* = 0.084 mg/l; LC_{50} rainbow trout = 0.55 mg/l; EC_{50} algae (96h) 9.0 mg/l) [EC, 2001].

Environmental fate: TBZ appears to be extremely persistent in the soil environment, particularly in soils with low organic carbon content [EC, 2013b]. Extrapolated $t_{1/2}$ values ranged from 833-1100 days in cropped plots and from 1093-1444 days in fallow plots [US EPA, 2002]. Similar regulatory studies at EU level also noted that the $t_{1/2}$ values of TBZ were > 1 year in a laboratory soil dissipation study conducted at 20°C under both aerobic and anaerobic conditions [EC, 2001]. Similarly, its $t_{1/2}$ in the field was longer than 2 years suggesting accumulation in the soil environment. In a similar lab study Kesavan *et al*. [1976] reported a $t_{1/2}$ for TBZ of more than 17 weeks compared with longer $t_{1/2}$ values in sterilized soils suggesting the involvement of soil

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microflora in the slow dissipation of TBZ. More recent studies by Omirou *et al.* [2012] reported shorter $t_{1/2}$ =77.8 days, although it was still the most persistent chemical from the ones used by the fruit-packaging industry. According to biodegradability tests, TBZ is classified as 'not readily biodegradable'. It is adsorbed strongly on soil colloids as it is indicated by its high K_{oc} values (1104-22467 ml/g) which suggest a limited mobility in the soil environment [EC, 2001] verified by different studies [Solel *et al*., 1979; Omirou *et al*., 2012].

In water/sediment test systems TBZ showed a limited persistence in water ($t_{1/2}$ = 1.6-2.3 days) compared to its extreme persistence in the sediment compartment ($t_{1/2}$ = 375 – 4332 days) [EC, 2001]. It is not prone to hydrolytic degradation in a wide range of pH values (4-9). In contrast TBZ is vulnerable to photolysis in water where, in aqueous pH 5 buffer solution exposed to xenon lamp for 96 hours at 25°C, underwent rapid photolytic degradation, with a $t_{1/2}$ of approximately 29 hours [US EPA, 2002]. Similar photolysis studies in the presence and absence of humic and fulvic acids suggested a more rapid transformation of TBZ ($t_{1/2}$ = 1.1 h) with the main products being benzimidazole-2-carboxamide and benzimidazole [Murthy *et al*., 1996].

Mode of action: TBZ inhibits fungal microtubular function. In particular it binds to β-tubulin and prevents cytoskeleton-dependent cellular transport processes, including chromosome transport and cell division [Watanabe-Akanuma *et al*., 2005; Hollomon *et al*., 1998].

Agricultural uses: TBZ is registered for use as a pre-planting dust treatment to potato seed-pieces, soybean, and wheat seeds. It is also registered for use on mushrooms. However, its main use in Europe and elsewhere is as spray and dip application during waxing for the protection of citrus fruits, apples, pears, bananas, mangos, papaya, plantain, carrots, avocados, peas, and potatoes from post-harvest fungal infestations. The target fungi include *Verticillium fungicola, Mycogone perniciosa, Aspergillus* spp.*, Penicillium sp.*, [US EPA, 2002; EC, 2013b]. TBZ is usually applied by drenching on pome fruits, at a dose of 1.2 g/l, while citrus fruits can be treated by spraying, drenching, or more rarely by dipping at concentrations of up to 2 g/l [EC, 2013c].

TBZ has been included in the Annex I of the Directive 91/414/EEC and its authorization was renewed until 31 December 2015 under the clause that *member-states should pay particular intention to the protection of aquatic- and sediment- dwelling organisms and must ensure that the conditions of authorisation include, where appropriate, risk mitigation measures. Suitable risk mitigation measures (e.g. depuration with diatom earth or activated carbon) have to be implemented to protect surface waters from unacceptable levels of contamination via wastewater*

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[EC, 2010b]. This is a follow up of the initial evaluation by the Scientific Committee on Plants (2000) who concluded that "*a discharge of thiabendazole waste water (from dips and/or drenches) poses a potential risk for the functioning of the sewage treatment plant and for the receiving surface water, but will not pose an unacceptable risk to the aquatic organisms when adequate mitigation measures have been carried out*" [EC, 2000]. It is thus necessary that the wastewaters produced by the use of TBZ in the fruit-packaging plants to be treated in appropriate systems before released in the environment or in the sewage treatment plants.

7.2. Imazalil (IMZ)

Imazalil (*1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1H-imidazol*; CAS number: 58594-72-2; trade names: Bromazil®, Deccozil®, Fungaflor®, Freshgard®, Fungazil®, Imazacure®) is a systemic fungicide used for the control of a wide range of fungal diseases on fruits, vegetables, and ornamentals [Tomlin, 1994] and also as a fungicidal veterinary drug [Lanthier and Chalifoux, 1991]. The chemical structures of IMZ and of its major identified metabolite (imazalil ethanol) are shown in Figure 1.14.

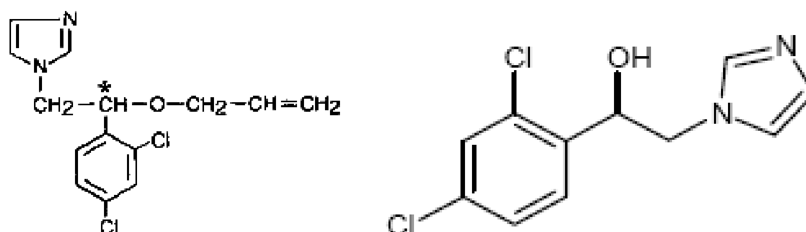

Figure 1.14. Chemical structure of imazalil (left) and of its main metabolite, imazalil ethanol (right). The asterisk indicates the chiral C in the molecule of imazalil.

Physicochemical properties: Pure IMZ (Molecular Mass: 297.2 g/mol) is a yellow or brown crystalline solid with a melting point of 50°C. It is a racemic mixture of two enantiomers with the R enantiomer being more potent than the S enantiomer. IMZ is moderately soluble in water (184 mg/l at 20°C, pH 7.6) and it is very soluble in methanol, ethanol, 2-propanol, dimethylbenzene, acetonitrile, N,N-dimethylformamide, tetrahydrofuran, 1-methyl-2-pyrrolidinone, 1,2-ethanediol, 1,2-propanediol, and glacial acetic acid (>500 g/l at 25°C) [EFSA, 2010]. Its low vapour pressure (1.58 x 10^{-4} Pa at 25 °C) and Henry's law constant (0.000108 Pa m^3/mol) indicate low volatility [EC, 2009].

Toxicity-ecotoxicity: IMZ is classified as "a *likely carcinogen to humans*" according to EPA's July 1999 Draft Guidelines for Carcinogen Assessment. Carcinogenicity studies in rodents

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indicate that IMZ is carcinogenic to male Swiss albino mice and Wistar rats. In addition, IMZ is placed in Category II, II, and IV for oral, dermal, and inhalation toxicity respectively. It is highly irritating to the eyes (Category I), but it is not a skin irritant (Category IV) or a dermal sensitizer [US EPA, 2003].

IMZ is considered moderately toxic to birds (LD_{50} acute 510 mg/kg, LD_{50} short term dietary > 5620 mg/kg feed) and moderately to highly toxic to mammals (LD_{50} acute rats = 227 mg/kg and NOEL short term dietary = 2.5 mg/kg diet). It is generally considered toxic for aquatic organisms as it is indicated by the low toxicological endpoint values for different indicator species of fishes (LC_{50} acute *Onchorynchus mykiss* = 1.48 mg/l; NOEC chronic = 0.043 mg/l), invertebrates (EC_{50} *D. magna* 3.5 mg/l), and algae (*Pseudokirch subcapitata* E_bC_{50} = 0.87 mg/l and E_rC_{50} = 1.20 mg/l). The main metabolic product of IMZ, imazalil-ethanol, was generally less toxic to aquatics (LC_{50} acute fish = 21.26 mg/l; LC_{50} *D. magna* = 13.6 mg/l) with the only exception of algae where slightly higher toxicity was exhibited compared to the parent compound (*Desmodesmus subcapitata* E_bC_{50} = 0.68 mg/l and E_rC_{50} = 1.04 mg/l) [EC, 2009].

Environmental fate**:** IMZ is chemically stable at room temperature in the absence of light. It is stable to abiotic hydrolysis at a wide pH range. Decomposition occurs at elevated temperatures (80°C) and under the influence of light (it photodegrades relatively rapidly with a $t_{1/2}$ of 36 hours in water) [US EPA, 2003]. In aqueous solutions the product is stable up to 40°C at pH ranging from 2.4 to 7.0 for at least 8 weeks [Anonymous, 1975].

IMZ degrades very slowly in soil under aerobic conditions with $t_{1/2}$ values ranging from 44 to 128 days [US EPA, 2003]. The long persistence of IMZ was also verified by similar regulatory studies at EU level with $t_{1/2}$ mean values of 137 days (aerobic soil laboratory study at 20°C) [EC, 2009]. Recent studies by Omirou *et al*. [2012] showed a shorter persistence of IMZ with $t_{1/2}$ values in soil of 29 days. In the field the dissipation of IMZ proceeds faster with $t_{1/2}$ ranging from 5.7 to 7.1 days [EC, 2009]. A recent study by Kreuzig *et al*. [2010] also verified the long persistence of IMZ in soil ($t_{1/2}$ = 83 days), although amendment of soil with manure accelerated its dissipation ($t_{1/2}$ = 29-48 days). In soil IMZ is metabolized to imazalil ethanol (Figure 1.14) which is not particularly persistent ($t_{1/2}$ = 5.1 -10.4 days) [EC, 2009].

Regarding its mobility in soil, IMZ is strongly adsorbed on soil colloids with mean K_{foc} value of 4357 ml/g [EFSA, 2010]. Similar values of K_{oc} = 4059 ml/g were reported by Kreuzig *et al.* [2010]. Similarly, imazalil ethanol, the main soil metabolite of IMZ, showed high soil adsorption affinity (K_{foc} 757-1663 ml/g). All those data suggest that IMZ and its main metabolite

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are not particularly mobile in soil and they are not expected to contaminate surface water and groundwater resources.

Mode of action: IMZ is a systemic fungicide of the group of N-substituted imidazoles. Members of this class act on the permeability of fungal membranes. In particular, IMZ inhibits CYP51 (lanosterol 14 alpha-demethylase), a key enzyme for sterol biosynthesis in fungi. Inhibition of this enzyme leads to a depletion of ergosterol, a vital ingredient of the fungal cell wall, resulting in growth inhibition and death [Heusinkveld *et al*., 2013; Becher and Wirsel, 2012].

Agricultural uses: IMZ is used at post-harvest level for the control of fungal infestations in fruits (citrus and pome fruits) and potatoes, as a pre-harvest foliar application to tomatoes, cucumbers, melon and roses (against powdery mildew), and as a seed-dressing on cereal grains, cotton seed, and seed potatoes [EC, 2009]. However, its main use is in post-harvest treatment of fruits where it controls infestations by *Botrytis cineria*, *Alternaria* spp., *Pencillium* spp., *Diaporthe* spp., *Diplodia* spp. [EC, 2009]. It can be applied *via* dipping, drenching, spraying, or waxing and depending on the way it is applied the dose rates vary. Thus when applied *via* dipping or drenching lower dose rates are used (1 g/l), while higher concentrations are utilized when IMZ is applied *via* spraying or waxing (1.5 g/l and 2 g/l respectively) [EC, 2009].

IMZ was included in Annex I of Directive 91/414/EEC and its registration was renewed in 2011 under the new Regulation 1107/2009 until 31 December 2021. However, the use of IMZ was authorized only under the clause that *appropriate decontamination system for the treatment of wastewaters produced by its use would be implemented on site* [EC, 2009]. In the U.S.A., IMZ was first registered in 1983 for postharvest use on citrus fruits, and its registration was extended for seed treatment in 1984 [US EPA, 2003].

7.3. 2-phenyl-phenol (or *ortho*-phenyl-phenol, OPP)

2-phenyl-phenol (*biphenyl-2-ol*; CAS number: 90-43-7; trade names: Dowcide1®, Preventol O Extra1®, Torsite®) has been used broadly for over 40 years in many countries for its general antimicrobial properties. This compound has been used as a preservative for citrus fruits and vegetables [FAO, 1999], in households, industry, and hospitals as surfaces disinfectant, as a preservative in the cosmetics, plastics, leather, textile, and paper industries and in mushroom units [Coelhan *et al*., 2009]. Its chemical structure is presented in Figure 1.15.

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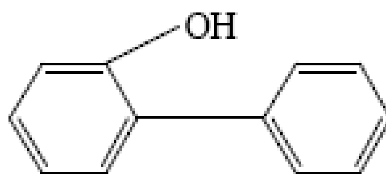

Figure 1.15. The chemical structure of 2-phenyl-phenol.

Physicochemical properties: OPP (Molecular Mass: 170.2 g/mol) appears like white to light buff crystals, with a melting point of 56-58^oC and a water solubility of 700 mg/l at 25^oC It is also soluble in different organic solvents and stable to photolysis and hydrolysis [US EPA, 2006]. Based on its vapour pressure (0.474 Pa) and its Henry's law constant (0.14 Pa×m^3×mol^{-1} at pH 7 at 20 °C) it is considered as moderately volatile. Its logK_{ow} is 3.18 which suggests that it is rather fat soluble [EFSA, 2008].

Toxicity-ecotoxicity: OPP shows moderate acute toxicity *via* the oral route of exposure (Toxicity Category III). It is considered as severe dermal irritant (Toxicity Category I), but not dermal sensitizer. It is not mutagenic [US EPA, 2006] and has been classified as "likely to be carcinogenic to humans", based on the presence of urinary bladder tumors in rats and the presence of liver tumors in mice at doses above 200 mg/kg/day [Zheng *et al*., 2011].

Regarding its toxicity to non-target organisms, OPP is non-toxic to mammals and birds as it is indicated by the high values of the relevant endpoints (LD_{50} rats >2000 mg a.i./kg bw/day; LD_{50} birds > 2250 mg a.i./kg bw/day). However it is highly toxic to aquatic organisms including fishes (LC_{50} *Onchorynchus mykiss* = 4 mg/l; NOEC 21d = 0.036 mg/l), invertebrates (*Daphnia magna* EC_{50} = 2.7 mg/l) and algae (*Pseudokirch subcapitatus* EC_{50} biomass and EC_{50} growth rate 1.35 and 3.57 mg/l respectively) [EFSA, 2008].

Environmental fate**:** OPP is stable and persistent in abiotic aqueous medium at pH 5, 7, and 9. When exposed to sunlight in neutral aqueous medium, it degrades with a $t_{1/2}$ of 14 days. Exposure to UV light (at 253.7 nm) results in its transformation to phenyl benzoquinone, phenylhydroquinone, and 2-hydroxy benzofuran. Its $t_{1/2}$ in air is 14 hours. Adsorption of OPP to soil is moderate with measured K_{oc} values of 894-1793 ml/g [Zheng *et al*., 2011] indicating that OPP could be moderately mobile in soil. Similar regulatory adsorption studies reported average K_{oc} values of 347 ml/g although no equilibrium had been reached during the duration of the study [EFSA 2008]. Studies by Omirou *et al.* [2012] in columns and in on-farm biopurification systems showed that OPP is the most mobile chemical among the fungicides used by the fruit-

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packaging industry; however its limited persistence prevents its extensive leaching. Indeed, aerobic soil dissipation studies showed a $t_{1/2}$ <1 day with most of OPP becoming non-extractable rapidly [EFSA, 2008]. However in more recent studies, Omirou *et al.* [2012] reported longer $t_{1/2}$ values for OPP in soil ($t_{1/2}$ = 43.3 days). Based on biodegradability assays, OPP could be characterized as readily biodegradable [EFSA, 2008]. In a sediment/water aerobic system OPP was not persistent with $t_{1/2}$ values in the water column of 5.5 to 19 days.

Monitoring studies have regularly detected residues of OPP in groundwater and drinking water [IACR, 1983], marine and freshwater systems [Jonkers *et al*,. 2010], and sewage treatment plants (STP) effluents [EFSA, 2008; Jonkers *et al*., 2010]. Studies in municipal STP in Germany revealed a 98-99% removal of the OPP residues with maximum concentrations in the effluents reaching 2.6 μg/l [EFSA, 2008]. Moreover, relatively recent studies revealed the common presence of OPP residues at max levels of 16.9 and 40 μg/l in canned soft drinks and canned beer respectively [Coelhan *et al*., 2006; Coelhan *et al*., 2009].

Mode of action**:** The fungicidal mode of action of OPP is not yet clearly defined. One theory is that active oxygen species generated in the presence of the fungicide lead to the peroxidation of unsaturated fatty acids thereby disturbing membrane functions. Several NADPH-dependent flavin enzymes were shown to be inhibited *in vitro* and this inhibition was considered to be responsible for the origin of active oxygen [Dekker, 1999].

Agricultural uses**:** OPP is used in postharvest treatments of fruits for the control of infestations during storage caused by moulds and rots (*Penicillium* sp. and *Rhizopus* sp.) [US EPA, 2006]. The mainly application of OPP at EU level is *via* drenching: the fruits are drenched in a closed drencher cabinet for 30 sec. with an aqueous solution of OPP (max recommended concentration: 600 mg/l) [EC, 2008]. At the EU level OPP was listed in Annex I of the 91/414/EC Directive and its authorization was recently renewed until 2019 under the new Regulation. Based on the proposed uses of OPP at postharvest level the EC stated that all member-states *should pay particular attention to put in place appropriate waste management practices to handle the waste solution remaining after application, including the cleaning water of the drenching system. Member States permitting the release of those wastewaters into the sewage system shall ensure that a local risk assessment is carried out* [EC, 2010c].

In the U.S.A. there are 120 products containing OPP and its salt as an active ingredient. US EPA after completing its assessment on the dietary, occupational, drinking water, and ecological risks associated with the use of pesticide products containing the active ingredient

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OPP concluded that risk mitigation measures should be adopted in order to minimize the chance for water contamination by the use of OPP-containing agrochemicals [US EPA, 2006].

7.4. Diphenylamine (DPA)

Diphenylamine (*N-phenylbenzenamine*; CAS number: 122-39-4; trade names: DPA, Shield DPA, Big Dipper, Decoscald 282, No Scald DPA 283) is a highly reactive chemical and it has a very broad range of applications. It is predominantly used as stabilizer for single- or multi-base propellants and nitrocellulose-containing explosives [Drzyzga, 2003]. Another significant application of DPA is in the fruit-packaging industry where it prevents the establishment of apple and pear scald during cold storage [Kim-Kang *et al.*, 1998; Mir and Beaudry, 1999]. Further applications include its use as a) an antioxidant for various polymers and elastomers and as condensates for the insulation of rubber, b) a precursor in the production of azo dyes [Lye and Freeman, 1999]; c) a stabilizer in perfumery products [Bazin *et al.*, 1986]; d) a precursor of non-steroidal anti-inflammatory drugs [Masubuchi *et al.*, 1999]. The DPA chemical structure is showed in Figure 1.16.

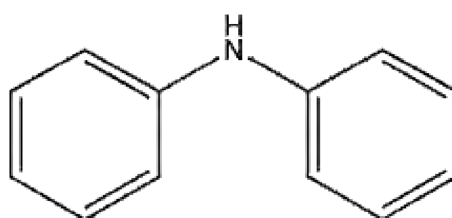

Figure 1.16. The chemical structure of DPA.

Physicochemical properties: Pure DPA (Molecular Mass: 169.23 g/mol) is a colourless solid (at room temperature and normal pressure) with a floral odor. DPA is a very reactive compound which is attributed to its imine hydrogen atom [Drzyzga, 2003]. DPA has moderate to low water solubility (40 mg/l at 25°C) and it is moderately volatile from water with a Henry's law constant value of 0.321 Pa.m^{3}.mol^{-1} [EU, 2008]. It has a pKa of 1.99 which means that it is a weak base and its logK_{ow} of 3.82 (at 20ºC) suggests that it is fat soluble [EFSA, 2008b].

Toxicity-ecotoxicity: DPA can cause severe eye irritation but showed no genotoxic potential or carcinogenicity [EFSA, 2012]. However, diphenylnitrosamine (an impurity of technical grade DPA), is classified as a "probable human carcinogen" based on increased incidence of bladder tumors in male and female rats, reticulum cell sarcomas in mice, and on structural relationship to carcinogenic nitrosamines [US EPA, 1998].

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Regarding its toxicity to non-target organisms, DPA exhibits low acute (LD_{50} > 2250 mg/kg bw/day) and short-term dietary toxicity to birds (LD_{50} > 2293 mg/kg bw/day) and it is not acutely toxic to mammals (*Oryctolagus cuniculus* LD_{50} > 15000 mg/kg bw/day) [EFSA, 2012]. In contrast, it is very toxic to aquatic organisms as it is demonstrated by the very low toxicity endpoint values for indicator species of fishes (EC_{50} *Oncorhynchus mykiss* 2.2 mg/l), algae (*Selenastrum capricornutum* biomass and growth rate EC_{50} 0.18 and 0.30 mg/l respectively), and invertebrates (*Daphnia magna* EC_{50} 1.2 mg/l) [Drzyzga, 2003; EFSA, 2012].

Environmental fate: DPA is stable to hydrolysis at pH 5, 7, and 9 with $t_{1/2}$ ranging from 315 to 358 days [US EPA, 1998]. Photolysis appear to be a major route of degradation of DPA with $t_{1/2}$ values ranging from 4.39 h under buffered sterile conditions to 1.31 h in distilled water [EFSA, 2008b]. DPA shows relatively high adsorption onto soils (K_{oc} = 1212-6593 ml/g) which suggests a limited mobility and low leaching potential [US EPA, 1998]. Little is known about its persistence and metabolism in the soil environment. However regulatory studies conducted in USA suggested that the molecule is not persistent in soil with $t_{1/2}$ of <1 day. A ready biodegradability study according to OECD guidelines suggested that DPA should be classified as "not readily biodegradable" [EC, 2007b].

Agricultural uses: the main agricultural use of DPA is for the control of apple scald in fruit packaging plants. It is applied *via* dipping, drenching, and spraying or fogging of fruits [Cagoulia *et al*., 2012] at concentrations ranging between 0.4 and 2 g/l [EFSA, 2012]. Up to 95% of DPA at EU level is applied *via* drenching while only 5% is applied *via* dipping. The excess of spraying liquid is collected in storage tanks and after filtering is recycled several times until replaced by fresh solution [EU, 2007b].

In 2008 and following a re-evaluation at 2012, the EC decided against the authorization of DPA and all products containing this active ingredient [EFSA, 2008; EFSA, 2012]. The decision of non-inclusion was based on lack of sufficient data on the levels and toxicity of unidentified metabolites of the substance, the possible formation of nitrosamines during storage of the active substance and during processing of treated apples, and the lack of data on the potential breakdown or reaction product of DPA residues in processed commodities [EFSA, 2012]. Recent decisions at EU level substantially reduced the MRLs of DPA on apples to 0.1 mg/kg compared to the previous value of 5 mg/kg [EC, 2013]. Despite the non-inclusion of DPA in Annex I, main fruit-producing countries like Spain, Portugal, and Greece have provided exemption authorization for 120 days for its use in 2012. Their decision was based on the absence in the market of equally effective alternatives. Meanwhile, DPA is still used for the

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postharvest treatment of apples in most other areas of the world including USA, Canada, Australia, Chile, Argentina, Uruguay, South Africa, and countries adopting Codex standards.

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Table 1.4. The registration status and authorizations for use at EU level of pesticides utilized by the fruit-packaging industry.

Pesticides	Approval	Clause for authorization	References
Thiabendazole	**Approved in** Cyprus, France, Greece, Spain, Italy, Portugal **until 31/12/2015**	Particular conditions to be taken into account on short term basis by Member-States in relation to the granting of authorisations of plant protection products containing thiabendazole: *Suitable risk management measures (e.g. depuration with diatom earth or activated carbon) have to be implemented to protect surface waters from unacceptable levels of contamination via wastewater from post-harvest and potato seed treatments.*	Review report for the active substance thiabendazole Finalised in the Standing Committee on Plant Health at its meeting on 12 December 2000 in view of the inclusion of thiabendazole in Annex I of Directive 91/414/EEC. http://ec.europa.eu/food/plant/protection/evaluation/existactive/list1-26_en.pdf
Imazalil	**Approved in** Cyprus, France, Greece, Spain, Italy, Portugal **until 31/7/2021**	Member States should pay particular attention to: *ensure that appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place. Prevention of any accidental spillage of treatment solution. Member States permitting the release of waste water into the sewage system shall ensure that a local risk assessment is carried out.*	Commission Directive 2010/57/EU of 26 August 2010 amending Annex I to Council Directive 91/414/EEC to renew the inclusion of imazalil as active substance. Official Journal of the European Union L225/5. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:225:0005:0009:EN:PDF
2-phenyl-phenol	**Approved in** Cyprus, Greece, Spain **until 31/12/2019**	In this overall assessment Member States must pay particular attention*: to put in place appropriate waste management practices to handle the waste solution remaining after application, including the cleaning water of the drenching and other application systems. Member States permitting the release of wastewater into the sewage system shall ensure that a local risk assessment is carried out.*	Final Review report for the active substance 2-phenylphenol. Finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 27 November 2009 in view of the inclusion of 2-phenylphenol in Annex I of Directive 91/414/EEC http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_2_phenylphenol_en.pdf
Diphenylamine	**NOT approved in the EE** possibility to grant emergency uses for a period of 120 days	Particular conditions proposed to be taken into account to manage the risk(s) identified: *Therefore management measures tailored to local practice and legislation need to be put in place to control the waste disposal of spent application solution and prevent accidental spillage entering sewers or surface water drains. Member States indicated that they may wish to have additional environmental data to support and inform the management measures that they have to put in place. For example the proposal made in the DAR (section B.8.4.4) that holding the solution in lagoons to allow photolysis to degrade diphenylamine before being applied to soil, may be ill advised in the absence of any soil degradation or mobility data, or data on effects on soil dwelling organisms of the known aqueous photodegradation products).*	EFSA Scientific Report (2012), 10(1), 2486 Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine http://www.efsa.europa.eu/en/efsajournal/doc/2486.pdf

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8. Wastewater production and treatment facilities

The postharvest treatment of fruits with fungicides or antioxidants *via* drenching, dipping, spraying, or waxing requires the use of copious amounts of water. At the end of the treatment process large volumes of wastewater characterized by high concentrations of suspended solids, organic sugars, starches, and, more importantly, particularly toxic and persistent pesticides are produced. The direct disposal of these wastewaters in the environment will constitute a serious point source for the contamination of natural water resources [Castillo *et al*., 2000]. Furthermore, the discharge of those wastewaters in municipal sewage treatment plants could have devastating effects for the function of those treatment facilities. Thus those wastewaters should be treated and detoxified prior to their release in the environment [Rushing *et al*., 1995]. This need was also identified by the EC which, as mentioned in the previous section, has provided authorization to the pesticides used in the fruit packaging industry only under the clause *that appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place* (Table 1.4). Thus there is an urgent need for the development and implementation of an effective system for the depuration of those pesticide-contaminated wastewaters.

Currently, disposal of pesticide wastewater is carried out by several methods including land cultivation or land-spreading, dumping in soil pits, ditches, artificial lagoons and evaporation ponds. However in several cases totally unsafe disposal means are followed including discharge in municipal sewer systems or neighboring surface water systems like creeks, streams, or rivers.

In contrast to direct disposal methods, there are treatment methods which could be employed to treat pesticide-contaminated wastewaters such as incineration and chemical and physical treatments. These treatment methods either require land or are expensive and suffer from erratic efficiency [Kowal *et al.,* 1982]. Thus, the development of safe, economic and cost-effective technique for the depuration of pesticide-contaminated wastewaters is of paramount importance [Al Hattab and Ghaly, 2012]. The development of biological treatment facilities based on the degrading ability of microorganisms seems promising and should be explored.

8.1. Wastewater disposal methods

The methods for the disposal of pesticide-containing wastewaters include land spreading, disposal pits, and evaporation ponds.

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Land spreading: in this method, wastewaters are spread over surface soil which is then tilled in order to ensure high aeration. This method allows natural chemical and biological processes to transform and degrade the pesticides. Soil generally contains microorganisms with versatile catabolic properties against organic xenobiotics including pesticides [Felsot *et al*., 2003; Krueger and Severn, 1984]. Land spreading is only effective for compounds that can be biotransformed or biomineralized by soil microorganisms [Felsot, 1998].

Disposal pits: there are three types of disposal pits: soil, plastic and concrete pits. A primary method for disposing of pesticide-contaminated wastewater is by dumping in an unlined soil pit [Winterlin *et al*., 1989]. Factors such as chemical structure and concentration of pesticides play a major role in the degradation of pesticides in soil pits [Schoen and Winterlin, 1987]. Plastic lined pits have been also used for the disposal of pesticide-contaminated wastewaters. They usually have a depth of 0.5 - 1 m. Their bottom is insulated with a plastic liner and a layer of soil is laid on top of the liner. The pit is open to the atmosphere to allow water evaporation. Similar to the plastic lined pit, concrete pits have usually a depth of 0.5 to 1 m, a length of 8 - 10 m and a width of 3.5 m and they are reinforced with 0.20 m thick concrete walls. The pit consists of a 4-cm top and bottom layer of gravel. The two gravel layers are interrupted by a middle topsoil layer. The pit should be cover to prevent flooding by rain or snow but remain open to the atmosphere to allow for water evaporation [Al Hattab and Ghaly, 2012]. Johnson and Hartman [1980] tested the microbiological activity in a concrete pit and concluded that the degradation process in the pit was effective and no accumulation of pesticide residues occurred. Junk and Richard [1984] tested the depuration efficacy of a 30000 l concrete disposal pit receiving over 50 kg of 40 different types of pesticides for 8 years and concluded that this method was effective for disposal of pesticide waste with negligible release to air and water surroundings.

Evaporation beds: lined soil evaporation beds (Figure 1.17) have been also used for the disposal of pesticide-contaminated wastewaters. Tubes underneath the soil surface supply the beds with wastewaters which saturates the soil matrix slowly. Pesticide residues are then dissipated *via* photochemical, chemical, and biological means while volatilization also occurs for volatile pesticides [Al Hattab and Ghaly, 2012].

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Figure 1.17. Evaporation ponds used for the disposal of pesticide-contaminated wastewaters (source: LANL, "Aerial tour of Los Alamos National Laboratory," Los Alamos Study Group, Albuquerque, 2004. http://www.lasg.org).

8.2. Wastewater treatment methods

Wastewater treatment technologies are used to dissipate pesticides and other pollutants from wastewaters. The treated effluent could be recycled in industries with high water requirements, discharged to municipal sewage treatment plants, or released in the environment if the load of pollutants has decreased to negligible levels and certainly below the maximum allowed values set by environmental legislation [Gogate and Pandit, 2004].

Wastewater pre-treatment is often a necessary step to facilitate the removal of pollutants from wastewaters. In fact, many pesticide products are formulated by mixing pesticide active ingredients with inert materials (surfactants, emulsifiers, petroleum hydrocarbons) to achieve specific application characteristics. When those co-formulants are mixed with water they produce emulsions which might reduce the performance of many wastewater treatment operations, such as chemical oxidation and activated carbon adsorption. So in many situations, emulsion breaking is a necessary pretreatment step. Facilities can break these emulsions through several methods with temperature control and acidification being the most popular, simple and inexpensive ones. In addition to emulsion breaking, a variety of other technologies could effectively pre-treat the wastewater, including membrane filtration (ultrafiltration), chemically-assisted clarification and settling [US EPA, 1998b].

Methods used for the treatment of pesticide-contaminated wastewaters could be categorized into thermal, chemical, and physical treatments. Thermal treatments include incineration and open burning. Chemical treatments include oxidation, hydrolysis, and precipitation. Physical treatments are based on absorption using activated carbon, inorganic, and organic materials [Al Hattab and Ghaly, 2012].

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Thermal methods: pesticide incineration is a high temperature oxidation process where the pesticide is converted into inorganic gases (CO_2, volatile acids, particles, and metal oxides) and ash [Felsot *et al.*, 2003; Alloway and Ayres, 1997]. Incineration of pesticide should be operated at temperatures higher than 1000°C so that the pesticide can be treated within the first 2 seconds because lower temperatures tend to produce toxic intermediate products [Oberacker, 1988]. Ferguson and Wilkinson [1984] reported that incineration has 99.99% removal efficiency. The advantages of incineration include: I) effectiveness in degrading chlorinated organics, II) destruction efficiency of 99.99%, and III) setup at locations next to plants generating the waste. The disadvantages of incineration technology include: I) needs for sophisticated equipment, II) production of cyanide in the off gas during the incineration of organonitrogen pesticides, III) too costly and complex, IV) it is intended for centralized large scale disposal and V) it is not recommended for inorganic pesticides [Felsot *et* al., 2003].

Chemical methods: a) Oxidation is employed for the treatment of pesticide-contaminated wastewaters. It works through the addition of an oxidizing agent in the wastewater resulting in the formation of less toxic forms of the parent compound. During chemical oxidation, one or more electrons are transferred from the oxidant to the target pollutant, causing its transformation [EPA, 1998b]. Because non-selective oxidation occurs, this treatment allows the parallel transformation of multiple pesticides in a single step [Foo and Hameed, 2010]. One common method of chemical oxidation is the alkaline chlorination, which uses chlorine under alkaline conditions to transform pollutants. However, chemical oxidation reaction may generate toxic chlorinated organic compounds as by-products. Chemical oxidation can also be performed with other oxidants (e.g., hydrogen peroxide, ozone, and potassium permanganate) or with the use of ultraviolet light. Although these other methods of chemical oxidation can effectively treat wastewaters, they typically entail higher capital and/or operating and maintenance costs, greater operator expertise, and/or more extensive wastewater pretreatment than alkaline chlorination [EPA, 1998b]

b) Hydrolysis is a chemical reaction in which organic constituents react with water and break into smaller (and less toxic) compounds. This method is rather common for cleaving the ester bonds commonly found in several pesticide compounds, including pyrethroids, carbamates, organophosphates and acetaniledes [Al Hattab and Ghaly, 2012]. The rate of the hydrolysis reactions can be pH-dependent and could be accelerated at acidic or alkaline conditions depending on the pesticide pollutant. Hydrolysis rates increase as temperature increases [EPA, 1998b].

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c) Chemical precipitation involves the addition of chemicals (e.g. sulfides, hydroxides, and carbonates) in the wastewaters which react with organic and inorganic pollutants forming insoluble precipitates that are then filtrated, settled, or decanted. This treatment tends to be effective on a narrow range of contaminants [EPA, 1998b].

Physical methods: Adsorption has been shown to be the most promising option for the removal of non-biodegradable pesticides from wastewaters. Activated carbon has been the most common adsorbent for this process due to its effectiveness and versatility [Aksu, 2005]. Activated carbon is a material which is characterized by extremely high adsorption capacity due to a large surface area and high number of internal pores per unit mass. In general, organic pesticides with aromatic functional groups, high molecular weights, and low water solubility are amenable to activated carbon adsorption. Wastewater flows through a bed of activated carbon and pesticides are adsorbed on the pores of the stationary carbon material resulting in maximum removal of pesticides from the wastewaters. Carbon adsorption capacity depends on the characteristics of the adsorbed compounds, the types of compounds competing for adsorption, and characteristics of the carbon itself. If several constituents that are amenable to activated carbon adsorption are present in the wastewater, they may compete with each other for carbon adsorption capacity. This competition may result in low adsorption or even desorption of some constituents.

Activated carbon is regenerated by removing the adsorbed organic compounds through steam, thermal, or physical/chemical methods with thermal and steam regeneration being the most common methods. However, activated carbon is quite expensive and the higher the quality the greater the cost. Both chemical and thermal regeneration of spent carbon is also expensive, impractical on a large scale, produces additional effluent and results in considerable loss of the adsorbent [Aksu, 2005].

Biological methods: All the treatments mentioned above are generally cost-prohibitive for small-scale wastewater-generating enterprises [Felsot *et al*., 2003]. Biological treatments could be a cheaper, more environmental friendly, and equally effective method for the treatment of pesticide-contaminated wastewaters. Biological treatment of pesticide-wastewaters could take the form of bioaugmentation and phytoremediation. On-farm biodepuration systems have been used extensively for the treatment of pesticide-wastewaters and their utilization will be also discussed.

a) Bioaugmentation: it refers to the augmentation of wastewater treatment facilities with acclimated microorganisms with specialized catabolic capacities or their catabolic enzymes in

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order to enhance the removal efficiency of the wastewater treatment facility against the target pesticides [Balest *et al.*, 2008]. Operatively, the implementation of bioaugmentation in conventional wastewater treatment facilities requires proper inoculation with well-acclimated microorganism strains during startup operations or during plant malfunctioning that requires system rehabilitation. The practice of utilizing selected microorganisms to carry out biochemical transformations has long been applied in the fields of pesticides and pharmaceuticals, where the wastewater composition is well-characterized, but it is still not a common practice in municipal wastewater plants. A deeper insight into the degradation efficiency and the identification of the intermediate metabolites (degradation byproducts) is required, together with the elucidation of the mineralization mechanisms [Basile *et al.,* 2011].

b) Phytoremediation: in this method plants are used to contain and remove harmfull environmental contaminants from wastewaters [Cunningham *et al.,* 1996]. Plants like *Kochi* sp. [Coats and Anderson, 1997], *Lemna minor*, *Cabomba aquatica,* and *Elodea canadensis* [Olette *et al*., 2008] were capable of removing different pesticides present in wastewater.

c) Biobeds: they were first introduced in Sweden in 1993 as a response to the need for simple and effective methods to minimize environmental contamination from pesticide use, especially when filling spraying equipment, a typical point source of contamination [Castillo *et al.*, 2008]. Biobeds are simple and cheap constructions intended to collect and degrade spills of pesticides on farms [Torstensson, 2000; Torstensson and Castillo, 1997]. In its simplest form it consists of a pit on the ground which could be between 0.5-1 m deep. This pit is generally lined at the bottom and filled in with an organic substrate called biomix. The depuration efficiency of biobeds is based on the high biodegradation and adsorption capacity of the biomix used. The original Swedish biomixture consists of straw, peat, and soil in the proportions 50:25:25 vol. %. Each component of the biomixture plays an important role in the efficiency of retention and degradation of the pesticides. The straw is the main substrate for pesticide degradation and microbial activity, especially from lignin-degrading fungi (such as white rot fungi), which produce phenoloxidases. The soil provides sorption capacity and is also an important source of pesticide-degrading microorganisms, especially bacteria with the ability to metabolically degrade such chemicals [Castillo *et al*., 2008]. The peat in the biomixture contributes to sorption capacity, moisture control, and also abiotic degradation of pesticides. It also decreases the pH of the biomixture as shown by the significant negative correlation between the pH and the volume of peat in the biomixture [Castillo and Torstensson, 2007]. However, the use of peat has been criticized due to its relatively high cost and limited availability, especially in southern Europe [Coppola *et al*., 2007]. In addition, its extensive use is not considered plausible with sustainable

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agricultural practices [Fogg *et al*., 2003]. A series of studies showed that both straw and peat could be effectively replaced by other lignocellulosic (grape stalks) and agricultural composted materials (e.g. olive leaves compost, grape marc, and grape stalks compost) respectively resulting in increasing biodegradation efficiency especially compared to peat-based biomixtures [Karanasios *et al*., 2010; Karanasios *et al*., 2010b; Karanasios *et al*., 2012; Karanasios *et al*., 2012b; Omirou *et al*., 2012].

Since its first introduction in Sweden, the original biobed system was modified in order to accommodate local needs, agricultural practices and conditions. The results of this were the development of a wide range of modified systems called Phytobac (France), Biofilters (Belgium, [Pussemier *et al*., 2004]), and Biomassbed (Italy, [Vischetti *et al*., 2004]). However all those systems could be categorized into two main categories: I) Drive over or Direct System which includes the original Swedish biobed and II) Offset or Indirect System which includes UK biobeds, Phytobac, and Biofilters (Figure 1.18). The latter indirect systems have the advantage that could handle larger amounts of wastewater compared to the drive over system which was originally constructed to receive only spills occurring during spraying preparations [De Wilde *et al*., 2007].

Several efficacy studies in Europe with full scale systems showed that the depuration capacity of biobeds ranges between 97-99.9% depending on the biomixture composition and the wastewater management of the biobed system [De Wilde *et al*., 2007]. The implementation of biobeds in Sweden has resulted in a substantial decrease in the levels of pesticide residues in surface waters and groundwater systems [Kreuger and Nilsson, 2001].

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Figure 1.18. Photos of the main types of biobed systems used in Europe: (a) original driver over biobed system; (b) biofilter system used in Belgium; (c) Offset type Phytobac system originated from France, and (d) UK offset type biobed system in Cyprus.

9. Methods of treating wastewaters from the fruit-packaging industry

Despite the urgent need of treatment systems for wastewaters generated by the fruit-packaging industries, at the moment there are no well-accepted methods to decontaminate those wastewaters. The only depuration system currently available is based on pesticide adsorption onto granular activated carbon [Garcia Portillo *et al*., 2004], but, although its efficiency in depuration, it is cost-prohibitive [EC, 2000]. Biobeds might be a possible solution for the treatment of wastewaters from the fruit packaging industry after certain modifications in their construction and water management schemes. Flaim and Toller [1989] proposed the use of a filter system composed of a mixture of peat moss, manure, clay and dolomite sand which was efficient in removing >98% of TBZ, benomyl, and DPA. However this system was not able to treat large wastewater volumes, while the use of materials like peat moss and clay increased the cost. The two systems actually available are:

a) Biobeds: A recent study by Omirou *et al*. [2012] demonstrated that biobeds could be used also for the depuration of pesticide contaminated wastewaters produced during citrus production (containing high concentrations of the fungicides TBZ, IMZ, and SOPP) at both pre- and at post-harvest level, using low cost materials. However, there are certain limitations for their use in the treatment of wastewaters from the fruit-packaging industry including mostly water management limitations. Indeed, depending on their size, biobeds could receive a certain

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volume of wastewaters per season without compromising their depuration efficiency. Thus it is anticipated that biobeds could be an effective solution for the depuration of those wastewaters produced by small to medium size fruit-packaging plants producing not more than 50 m^3 of wastewater per season. Such volumes could be stored and gradually disposed of in biobed systems maintain optimum moisture conditions and avoiding oversaturation and the establishment of anaerobic conditions which will compromise the efficacy of the system. More details regarding the implementation of biobeds for the treatment of those wastewaters will be presented in the PhD of Mr. Panagiotis Karas which was performed in the laboratory of the Group of Plant and Environmental Biotechnology in parallel.

b) CONTROL TEC-ECO® system: It has been developed by the Spanish company Technidex for the depuration of wastewaters from the fruit-packaging industry (Figure 1.19), it is a physical treatment system which acts by passing the wastewaters through an activated carbon filter that adsorbs the pesticides contained in the wastewaters. Tests with this system for the depuration of wastewaters containing TBZ resulted in the reduction of its concentration by 7000 times in the effluent suggesting high depuration efficiency [EC, 2000]. However its high cost of implementation prohibited so far its uptake in the fruit producing regions of Europe.

Figure 1.19. The CONTROL-TEC ECO® system used for the treatment of wastewaters from the fruit packaging industry.

10. The aim of the present thesis

Postharvest fungal decays still constitute the most acute problem for the fruit-packaging industry [Kanetis *et al.*, 2007]. Fungicides are still the major mean of controlling postharvest fungal infestations [Schirra *et al.,* 2011]. However, postharvest pesticide treatments lead to the production of large volumes of wastewaters containing high concentrations of the pesticides used [Omirou *et al.*, 2012]. These wastewaters constitute a serious point source for the contamination

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of natural water resources, as has been identified also by the EC which has given authorization to all these chemicals under the clause that "*appropriate decontamination system is available or a risk assessment has demonstrated to the authorizing Member State that the discharge of the treatment solution does not have an unacceptable risk to the environment and in particular to aquatic organisms*" [European Council, 1991]. However, so far there is no sustainable and efficient means of treating these wastewaters. The existing physicochemical methods are not applicable to the fruit packaging industry due their high land requirements, high costs, and erratic efficacy. So, inappropriate handling and disposal of those wastewaters are currently employed raising concerns about the integrity of adjacent water and terrestrial ecosystems. Thus, the development of a safe, effective, and economical method for the treatment of those pesticide-contaminated wastewaters is urgently needed. Within this frame, biological treatment methods exploiting the versatile catabolic capacities of microorganisms could be a possible solution.

Based on these facts and established problems, a study was initiated in the laboratory of the Group of Plant and Environment Biotechnology of the Department of Biochemistry and Biotechnology, University of Thessaly, in order to investigate the possibility of developing a biological system for the decontamination of the wastewaters produced by the fruit-packaging plants. Biobeds offer a possible solution for treating those wastewaters under the clause that their volume will not exceed 50 m^3 per season, thus targeting mostly small to medium enterprises. A more mechanised alternative will be therefore required for the treatment of the large volumes of wastewaters produced by large fruit-packaging plants (producing > 50 m^3 of wastewater per season) during a relatively short period of time. Considering the limited number of pesticides that should be removed from the wastewaters and their recalcitrance to biodegradation, specialized microbial inocula will be needed for the augmentation of such biological treatment systems ensuring high efficacy of the depuration process. Such microbial inocula could include fungi or bacteria depending on their capacity to degrade the target pesticides. Preliminary studies with the white rot fungi *Trametes versicolor* and *Pleurotus ostreatus* showed that they were able to rapidly degrade high concentrations of some of the pesticides contained in these wastewaters (OPP, DPA) when cultivated in straw extract substrate which resembles their natural growth substrate [Karas *et al*., 2011]. However fungi failed to degrade TBZ and partially degraded IMZ at a slow rate. This led us to further explore the potential of bacteria for the degradation of those pesticides.

So the main aim of this thesis was to isolate bacteria able to degrade TBZ, IMZ, OPP, and DPA and to characterize their degrading ability. The kinetic of the degradation reaction, the formation of metabolites and the capacity of the isolates to degrade other molecules of similar

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chemical structure or potential metabolites was explored in order to elucidate the metabolic pathway. Further studies evaluated the impact of temperature, pH, and pesticides concentration on the degradation capacity of the isolates. In order to assess their bioremediation ability in a more realistic situation, degradation was also tested in artificial contaminated soil samples and in liquid biobed extraction medium.

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Chapter 2

General Materials and Methods

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1. Pesticides

Analytical standards of pesticides were used in all experiments and for analytical purposes unless stated otherwise. A description of the analytical standards purity and supplier is given in the following experimental chapters. For every pesticide a stock solution of 1000 mg/l in methanol was prepared. A series of 10-fold dilutions in methanol were subsequently prepared to obtain a range of pesticide solutions with concentrations of 100, 10, 5, 2, 1, 0.5, and 0.1 mg/l. Methanol standard solutions with pesticide concentrations ranging from 0.1 to 10 mg/l were used for the preparation of the standard curves for the quantification of pesticide residues. All pesticide stock solutions were maintained at -20°C.

An aqueous stock solution of 100 mg/l was also prepared for all pesticides studied using the analytical standard. Upon preparation the aqueous solutions were placed in an ultrasonic water bath until completely dissolved. Subsequently, solutions were filter sterilized through a syringe filter (0.22 μm, sterile MS®PES Syringe Filter, Membrane Solution) and used for the preparation of the pesticide-containing growth media. The concentration of all pesticides in the growth media was adjusted to 20 mg/l unless stated otherwise. All stock solutions were kept at 4°C for not longer than 1 month.

In cases were aqueous stock solution with concentrations exceeding the 100 mg/l had to be prepared, commercial formulations of the studied pesticides were utilized to overcome solubility problems of the analytical standards. The solutions obtained were filter sterilized using a syringe filter (0.22 μm, sterile MS®PES Syringe Filter, Membrane Solution) and were kept at 4°C for subsequent use.

2. Preparation of growth media

2.1. Selective media

The selective media used for the isolation of pesticide-degrading bacteria were a Minimal Salt Medium supplemented (MSMN) or not supplemented (MSM) with nitrogen. In those media the pesticide constituted the only carbon or the only carbon and nitrogen source, respectively. The selection of the medium utilized was dependent on the pesticide chemical structure.

For the preparation of MSMN/MSM three stock solutions were prepared with the following composition:

- Stock solution 1 (g/l): 22.7 KH_2PO_4; 59.7 $Na_2HPO_4 \cdot 12H_2O$; 10 NH_4Cl. For MSM NH_4Cl was substituted with equal amounts of NaCl to eliminate any extra N sources.
- Stock solution 2 (g/l): 5 $MgSO_4 \cdot 7H_2O$; 0.1 $CaCl \cdot 2H_2O$; 0.15 $MnSO_4 \cdot H_2O$.
- Stock solution 3 (g/l): 0.46 $FeSO_4 \cdot 7H_2O$.

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Regarding preparation of the final media, Stock solutions 1 and 2 were sterilized by autoclaving for 25 min at 120ºC, while Stock solution 3 was filter sterilized (0.22 μm, sterile MS®PES Syringe Filter, Membrane Solution). All stock solutions were maintained at 4^oC. For the preparation of 1 liter of medium, 100 ml of Stock solution 1 were added to 780 ml of deionised water. After sterilization by autoclaving and cooling, 100 ml of Stock solution 2 and 20 ml of Stock solution 3 were added and the medium was kept at 4^oC.

For the preparation of MSM/MSMN containing the studied pesticides, appropriate volumes of the aqueous stock solution of the pesticides (100 mg/l) was added to Stock solution 1 + water after autoclaving and cooling. In order to avoid altering the concentration of minerals in the media, the amount of water added to the medium as pesticide solution was deducted from the 760 ml of deionised water mixed with Stock solution 1 prior to autoclaving.

2.2. Non-selective media

Non-selective media routinely used for the cultivation of clones or pesticide-degrading microorganisms were as follows:

- Soil Extract Medium (SEM): for its preparation, 500 g of soil (collected from a field of the National Agricultural Research Foundation of Greece, Larissa) were mixed with 1 liter of deionized water and autoclaved for 25 minutes at 120ºC. The mix was centrifuged for 10 min at 10000 rpm and the clear supernatant was collected and sterilized again *via* autoclaving for 25 minutes at 120ºC.
- Luria-Bertani (LB) medium-Miller: it is a rich medium composed of 10 g/l NaCl, 10 g/l bacteriological peptone, 5 g/l yeast extract. The medium was autoclaved for 25 minutes at 120ºC.
- Biobed Extract Medium (BEM): it is a rich medium prepared in order to simulate the nutritional condition of biobed substrates (volumetric content: 50% spent mushrooms substrate, 25% soil, and 25% straw). For its preparation 100 g of the biomix were mixed with 1 liter of deionised water and, after a first heat-sterilization and centrifugation, the supernatant was autoclaved again and kept at 4^oC.

To prepare SEM, LB, or BEM + pesticides, appropriate amounts of aqueous stock solutions of each pesticide (100 mg/l) were added to the media once cooled. The medium was kept at 4ºC until used.

Solidified agar media with or without pesticides (20 mg/l) were prepared in a similar way to the liquid media, except that Bacto Agar (15 g/l) was added before autoclaving.

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All isolation and cultivation procedures were performed at standard temperature of 26°C and under agitation at 160-180 rpm in a shaking incubator.

3. Antibiotics

LB medium was supplemented with antibiotics when used in standard cloning procedures and also in further studies which will be described in more detail in the relevant chapters. All the antibiotics were purchased from Sigma-Aldrich. The antibiotics used in the present work were: amikacin, ciprofloxacin, colistin, doxycycline, lincomycin, nalidixic acid, polymyxin B, trimethoprim (10 g/l), ampicillin, penicillin, piperacillin, streptomycin, vancomycin (100 g/l). Antibiotics stock solutions were prepared in sterilized deionized water at concentration of 10 or 100 g/l stocks. After filter sterilization (0.22 μm, sterile MS®PES Syringe Filter, Membrane Solution) the solutions were kept at 4°C. In the case of ciprofloxacin and doxycycline the water was acidified with HCl to facilitate their dissolution. Working concentrations depended on the experiment and are described in the specific chapters. The antibiotics were added in the media when necessary, after sterilization and cooling of the medium.

4. Pesticides residue analysis

4.1. Pesticides extraction

Pesticides were extracted from liquid media *via* methanol. Briefly, 0.5 ml of culture was mixed with 1 ml methanol (HPLC Grade, Merck) in glass vials and vortexed for 30 sec. at maximum speed. Samples were then centrifuged for 3 min at maximum speed and the clear supernatant was carefully removed, collected in new bottles, and stored at -20°C until analyzed. Recovery tests with MSMN/MSM containing each pesticide at three concentration levels (20, 2 and 0.2 mg/l) were performed and in all cases recoveries were higher than 80%. Extraction of pesticides from soil was performed with different protocols for each pesticide and the procedures are described in the relevant chapters.

4.2. Instrumentation for HPLC analysis

Pesticide residues were analyzed in an HPLC system (Marathon III, RigasLabs, Greece) composed of a Marathon III HPLC gradient pump system and a Fasma 500 UV/Vis detector. Two reverse phase columns were used throughout the thesis: a) a Grace Smart RP C18 150 mm x 4.6 mm and b) a CNW Athena RP C18 150 mm x 4.6 mm. The HPLC system was directly linked with a personal computer with the Clarity™ software which was used for HPLC operation and chromatographic analysis (LA2903 Data Apex Clarity HPLC Integration Software). A

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variety of mobile phases and UV wavelengths were used for the different pesticides and they are described in the relevant chapters.

5. Enrichment cultures for the isolation of pesticide-degrading bacteria

MSMN and MSM amended with the relevant pesticide (20 mg/l) were used as selective media for the isolation of pesticide-degrading bacteria able to use the pesticides as sole carbon or as sole carbon and nitrogen source respectively.

Soils were collected from sites used for the disposal of wastewaters from fruit-packaging industries located in Agia (Larissa) and in Lemesos (Cyprus) and used as sources for the isolation of pesticide-degrading bacteria. In order to stimulate the pesticide-degrading microflora in the soil, the samples were treated two or three times, on biweekly intervals, with 20 μg of pesticide *per* g of soil (on a dry weight basis). The pesticide was applied from a 1000 mg/l methanol stock solution. After methanol evaporation, soils were well mixed by hand, placed in a plastic bag, and incubated under aerobic condition in the dark at 25°C.

Subsequently, for each liquid media, 0.5 g of the treated soil were added in flasks (100 ml) containing 20 ml of medium (triplicates) + 20 mg/l of pesticide (1st-Enrichment Cycle (EC)). Two non-inoculated flasks were always co-incubated to serve as abiotic controls. The flasks were placed in an orbital shaker and incubated in the dark at 26°C and 180 rpm. Pesticide degradation in the enrichment culture was determined by regularly collecting aliquots of the liquid culture (0.5 ml) and analysing for pesticide levels as described elsewhere. When more than 50% degradation was observed in the 1st-EC, an aliquot (1 ml) was transferred in fresh medium and degradation was followed on the same way. In total four more EC were followed. When more than 50% degradation was observed at the 5th-EC, all replicates were mixed and 1 ml of culture was used for the preparation of 10-fold dilution series in water. Aliquots from the 10^{-3} up to 10^{-6} dilutions were spread on agar plates of the corresponding medium + pesticide (20 mg/l). All plates were placed in an incubator at 26°C in the dark and allowed to grow for 4-5 days. Morphological distinct and well separated bacterial colonies were selected with sterilized toothpicks and tested in corresponding liquid media for their degrading ability. Degradation of pesticides was determined at different times after inoculation and colonies showing >50% depletion of the pesticide initial concentration compared to the abiotic controls were considered as positive.

Positive cultures were streaked on LB agar plates to verify purity. If not pure, morphologically different colonies were selected again, put in liquid medium, and their degrading ability was checked. When finally purified, 20% glycerol stocks were prepared and

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stored at -80ºC. The isolated degrading bacteria were then sub-cultured in fresh liquid medium and DNA was extracted for identification and downstream assays as described below.

6. DNA extraction and PCR amplification

6.1. Soil DNA extraction

DNA extraction from soil was performed with the commercial kit Power Soil DNA isolation kit according to manufacturers' protocol (MOBIO, USA).

6.2. DNA extraction from bacteria

DNA extraction from bacteria was performed with two different commercial kits following always the manufacturers' instructions: a) Tissue Kit (Macherey-Nagel) and b) Purelink Genomic DNA Minikit (Invitrogen). Briefly, bacterial pellet for DNA extraction was collected either from fresh liquid cultures or from agar plates. In the former case, bacterial biomass was harvested by centrifugation at maximum speed for 4 min., while in the latter case growing bacteria were scraped from the agar plate with 1 ml of sterilized water and then centrifuged at maximum speed for 4 min. After centrifugation the supernatant was removed and the pellet was processed following kit instructions. The quality and the size of the DNA extracted was checked *via* electrophoresis on 0.7% agarose gel, stained with ethidium bromide, and visualized under a UV light. For electrophoresis 0.3 μl of bacterial DNA extract were mixed with 1 μl of 6x loading buffer (50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.2% (v/v) TAE 50x). In all gels a molecular size ladder (Quick-Load® 2-Log DNA Ladder, New England BioLabs Inc.) was also loaded (fragment size range 0.1 to 10 kbp).

6.3. PCR amplification

The 16S rRNA gene is by far the most common genetic marker used to study bacterial phylogeny and taxonomy [Janda and Abbott, 2007]. The 16S rRNA gene is a component of the 30S small subunit of prokaryotic ribosomes. Its full size sequence is about 1542 bp and it is composed of both variable and conserved regions (Figure 2.1). Universal primers are usually chosen as complementary to the conserved regions and the sequence of the variable region in between is used for attaining taxonomy information [Chen *et al*,. 1989; Relman, 1999].

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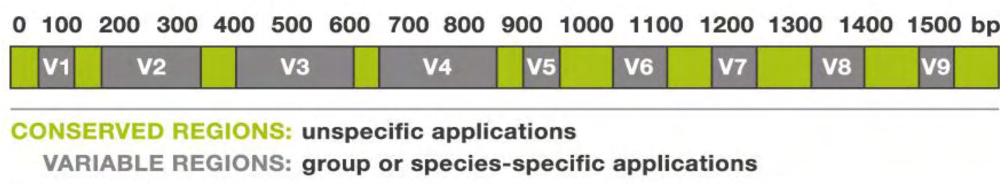

Figure 2.1. A schematic representation of the 16S rRNA gene illustrating the conserved (green) and variable (grey) regions (source: http://www.alimetrics.net/en/index.php/dna-sequence-analysis).

When studying the composition of the bacterial community or verifying the purity of isolated bacteria, three different pairs of universal primers for amplification of the 16S rRNA gene were used (Table 2.1).

Table 2.1. Set of primers used to amplify the bacterial 16S rRNA gene.

Primers	5'- sequence - 3'	Size (bp)	Reference
8f	CACGGATCCAGACTTTGATYMTGGCTCA	1500	Felske *et al.*, 1997
1512r	GTGAAGCTTACGGYTAGCTTGTTACGAC		
63f	AGGCCTAACACATGCAAGTC	1000	Marchesi *et al.*, 1998
1087r	CTCGTTGCGGGACTTAACCC		Hauben *et al.*, 1997
357f *	CCTACGGGAGGCAGCAG	200	Muyzer *et al.*, 1993
534r	ATTACCGCGGCTGCTGG		

* A 40 bp GC clamp at the 5 end of primer 357f was used. This GC clamp stabilizes the melting behavior of the DNA fragment, making it suitable for analysis by denaturing gradient gel electrophoresis (DGGE) [Sheffield *et al.*, 1987].

The PCR products obtained after amplification with primers 8f-1512r or 63f-1087r were used as template for a nested PCR using the 357f+GC/534r set of primers to obtain a product giving a good resolution of the bacterial community in DGGE (Figure 2.2).

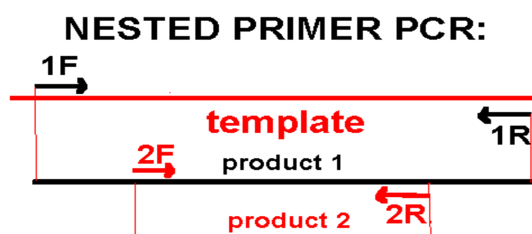

Figure 2.2. Schematic representation of a nested PCR (source Rybicki [2001]).

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Depending on samples and purpose of amplification, two different polymerases were used in all PCR efforts: a) DyNAzyme™ EXT (Finnzymes), having a proof-reading activity, was generally used to amplify the total 16S rRNA gene from isolated bacterial to be processed for sequencing and bacterial identification, and b) KapaTaq (KapaBiosystems) was utilized in all other routine PCR. The composition of the respective reaction mixtures are given in Tables 2.2 and 2.3. In cases where soil DNA was used as template the reaction was amended with 400 ng/μl of Bovine Serum Albumin (BSA, 10 μg/μl) to minimize inhibition of polymerase by humic and fulvic acids that are usually co-extracted from soil. Thermocycling conditions for the different primer sets are given in the Tables 2.4, 2.5, and 2.6.

Table 2.2. Concentrations of the different reagents used for PCR amplifications with the DyNAzyme™ EXT polymerase. The usual PCR reaction volume was 25 μl.

Component	**Final concentration**
10X Buffer	1X
$MgCl_2$ (50 mM)	1.5 mM
dNTPs (10mM)	200 μM
Forward Primer (20μM)	0.2 μM
Reverse Primer (20μM)	0.2 μM
Template DNA	Variable
Polymerase EXT (1U/μl)	1U/25 μl
PCR grade water	Up to final volume

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Table 2.3. Concentrations of the different reagents used for PCR amplifications with the KapaTaq polymerase. The usual PCR reaction volume was 25 µl.

Component	Final concentration
10X Buffer with Mg^{2+}	1X
dNTPs (10mM)	200 µM
Forward Primer (20µM)	0.4 µM
Reverse Primer (20µM)	0.4 µM
Template DNA	Variable
KapaTaq (5U/µl)	1U/50 µl
PCR grade water	Up to final volume

Table 2.4. Thermocycling conditions for amplification of the 16S rRNA gene with primers 8f-1512r.

Steps	Temperature (^oC)	Time (min.)	Cycles
Initial denaturation	95	5	1
Denaturation	95	1	
Annealing	55	1	25
Extension	72	2	
Final extension	72	10	1

Table 2.5. Thermocycling conditions for amplification of the 16S rRNA gene with primers 63f-1087r.

Steps	Temperature (^oC)	Time	Cycles
Initial denaturation	95	5 min.	1
Denaturation	95	30 sec.	
Annealing	55	30 sec.	25
Extension	72	75 sec.	
Final extension	72	10 min.	1

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Table 2.6. Thermocycling conditions for amplification of the 16S rRNA gene with primers 357f-534r.

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 min.	1
Denaturation	95	30 sec.	
Annealing	55	30 sec.	20
Extension	72	20 sec.	
Final extension	72	40 min.*	1

* The final extension step was increased to 40 minutes to improve DGGE gel quality preventing the formations of artifactual double bands [Janse *et al*., 2004].

The PCR products obtained after amplification with the 8f-1512r and 63f-1087r set of primers were visualized on a 1% agarose gel electrophoresis, while for the visualization of shorter PCR products obtained after amplification with primers 357f-534r a 1.5% agarose gel was used. PCR products purifications were done with the NucleoSpin® Extract II kit (Macherey-Nagel, GmbH, Germany) according to manufacturers' instructions.

7. RNA extraction and cDNA synthesis

RNA was extracted from bacterial liquid cultures using the Nucleospin RNA II kit (Macherey-Nagel). Bacterial pellet was collected from liquid cultures after centrifugation at maximum speed for 4 min. and processed following the instructions of the manufacturers'. The purity and integrity of RNA was checked on a 1.5% agarose gel where 3 μl of each sample mixed with 1 μl of loading buffer were loaded. RNA extracts were kept on ice at all steps and stored at -80°C.

The contamination of RNA with DNA was checked in subsequent PCR amplifications where no template was added. In most cases a DNAse treatment step was essential to remove DNA residues from extracted RNA. In those cases extracted RNA was treated with DNAse (DNAse I, Amplification Grade, Invitrogen) and incubated at 37°C for 1 hour in the presence of RNAseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen). The enzyme was inactivated with the addition of 1 μl of 25 mM EDTA solution. PCR amplification with primers 63f/1087r was routinely used to check for DNA contamination. DNA-free RNA was then reverse transcribed (kit Superscript II, Invitrogen) using random hexamers (Takara) following the manufacturers' instruction and cDNA was obtained.

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8. DGGE analysis

Denaturing gradient gel electrophoresis (DGGE) is a commonly used molecular technique for fingerprinting the microbial community in environmental samples [Green *et al*., 2010]. DGGE analyses are employed for the separation of double-stranded DNA fragments that are identical in length, but differ in sequence. The technique exploits the difference in stability of G-C pairing (3 hydrogen bonds per pairing) as opposed to A-T pairing (2 hydrogen bonds). A mixture of DNA fragments of different sequence are electrophoresed in an acrylamide gel containing an increasing gradient of DNA denaturating substances. In general, DNA fragments richer in GC will be more stable and remain double-stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate better in the acrylamide gel, while denatured DNA molecules become effectively larger and slow down or stop in the gel. In this manner, DNA fragments of differing sequence can be separated in an acrylamide gel [Green, 2005].

DGGE analyses were carried out on an INGENYphorU-2x2system (Ingeny International BV, The Netherlands) (Figure 2.3). In all cases 8% polyacrylamide gels in 1X TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM disodium EDTA, pH 8.3) were used. Polyacrylamide gels were prepared with a denaturating gradient of 45-65% (50-70% was used only in a few cases) by mixing low (0%) and high (100%) concentration denaturant solutions (Table 2.7) at appropriate ratios (Table 2.8). A peristaltic pump was used to form the denaturant gradient in the acrylamide gel. Aliquots of fresh solutions of ammonium persulfate (APS, 0.1 g/ml of nanopure water) and TEMED (N,N,N',N'-tetramethylenediamine) were added to the denaturant solutions to induce polymerization of acrylamide.

Figure 2.3. The DGGE system used (source: INGENYphorU-2, Instruction manual).

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Table 2.7. Composition of the 0 and 100% denaturant solutions.

Components	0%	100%
Acrylamide 40%	20%	20%
TAE 50X	2%	2%
Deionized formamide	---	40%
Urea	---	7M
Deionized water	Up to 100 ml	Up to 100 ml

Table 2.8. The ratios of denaturant solutions 0 and 100% mixed to obtain 25 ml of denaturant solutions with concentration of denaturants ranging from 45 to 70%.

Concentration of denaturants (%)	0% (ml)	100% (ml)
45	13.75	11.25
50	12.50	12.50
65	8.75	16.25
70	7.50	17.50

In all DGGE analysis of bacteria the PCR product analysed was obtained after nesting with primers 357+GC/534r. Samples were prepared for gel loading by mixing 1 μl of PCR product with 5 μl of loading buffer. In order to be able to compare the composition of samples run on different gels, a bacterial DGGE marker was created by mixing PCR products obtained after amplification of the 16S rRNA gene of known bacteria (Figure 2.4). In all cases electrophoresis was run for 16 hours at 60°C and 75 V and gels were silver stained as previously described by McCaig *et al*. [2001]. The image was captured using a digital camera and further processed for identification of clones or bands as described elsewhere.

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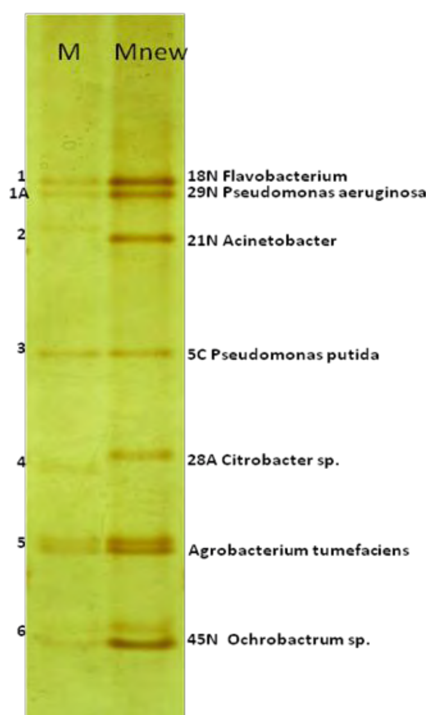

Figure 2.4. The two bacterial DGGE markers used in the present work (Denaturating Gradient 50-70%).

9. Development and screening of clone libraries

To assess microbial composition and identify the different members of the bacterial communities or consortia analyzed clone libraries were constructed based on the 16S rRNA gene, using the fragments generated by the first PCR step (1500 or 1000 bp fragments).

9.1. Clean up, ligation, and transformation

PCR products were purified using the NucleoSpin II PCR clean-up kit (Macherey-Nagel GmbH, Germany) and ligated to the pGEM®-T plasmid vector (Promega, Madison, USA) with T4 DNA ligase (Invitrogen) in an overnight incubation at 4°C. PCR product concentrations were estimated by comparison with the ladder bands after gel agarose run. A 3:1 (vector:insert) molar ratio was used and the ng of insert necessary to achieve optimum ligation were calculated by the following equation:

(ng of vector) x (kb size of insert) /(Kb size of vector) x (insert:vector molar ratio) = ng of insert.

The ligation product was then used to transform competent *E. coli* DH5a cells following standard procedures [Sambrook *et al.*, 1989]. Transformed cells were spread-plated onto LB+100 μg/ml ampicillin plates in the presence of 50 μl 2% X-gal and 10 μl 0.1M IPTG for easy identification of transformants by white/blue color screening (Figure 2.5). Colonies that have been transformed

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become ampicillin-resistant because of the corresponding resistance gene carried by the vector. Moreover, successful cloning of an insert into the pGEM®-T vector interrupts the coding sequence of β-galactosidase, so clones containing PCR products produce white colonies, while clones without insert metabolize X-gal in the presence of the gene inductor IPTG becoming blue.

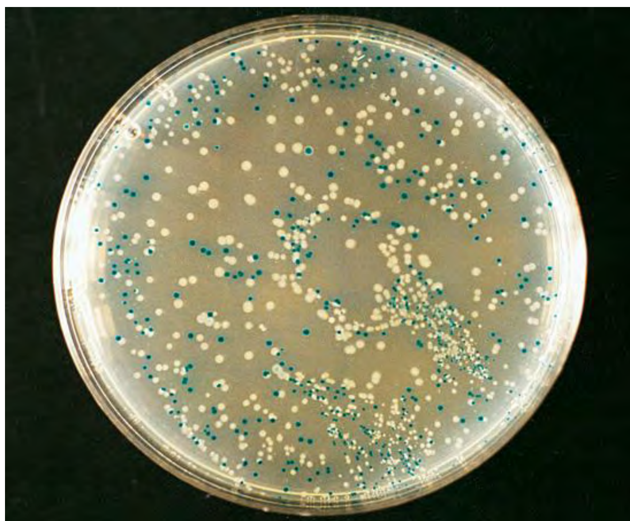

Figure 2.5. White/blue transformed *E. coli* DH5a colonies on LB+ampicillin plate in the presence of X-gal and IPTG.

9.2. Colony PCR and DGGE

After overnight incubation at 37°C, white colonies were selected for colony PCR (cPCR) and subsequent DGGE screening of clones. The number of colonies selected usually depended on the complexity of the sample which was fingerprinted *via* DGGE. Thus more clones were screened in rather complex communities to cover more effectively the diversity encrypted. For every selected colony the following procedure was followed:

(a) The insert of the vector (16S rRNA gene) was amplified *via* colony PCR with the 357f+GC/534r set of primers. The composition of the reaction mix for cPCR is presented in Table 2.9. The cPCR buffer was composed of 0.5M KCl, 0.1M Tris-HCl (pH 9), and 1% (v/v) Triton-X. The following thermal cycling conditions were used: 5 min at 95°C of initial denaturation, then 25 cycles of (I) denaturation (30 sec. at 95°C), (II) annealing (30 sec. at 55°C), (III) extension (1 min at 72°C), with a final extension step of 10 min at 72°C. The 200 bp PCR product obtained was run in a 1.5% agarose gel electrophoresis and visualized under UV light and then a DGGE was run to check the mobility of the clones against the band pattern of the original environmental sample.

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(b) At the same time, every selected colony screened *via* cPCR-DGGE analysis was also inoculated into a well of a 96-well plate containing 150 µl LB + ampicillin (100µg/ml) and it was grown overnight at 37°C.

(c) Representative clones for each band type matching the DGGE migration pattern of bands in the original environmental or consortium samples were chosen for sequencing. Thus, 1 µl from the corresponding LB-grown colony were used to inoculate fresh cultures of 5 ml of LB+100µg/ml ampicillin. The bottles were grown overnight at 37°C/180 rpm and the bacterial pellet obtained was used for plasmid extraction and sequencing as described below.

Table 2.9. The reagents used for colony PCR.

Component	Final concentration
10X cPCR buffer	1X
$MgCl_2$ (25 mM)	1.5 mM
dNTPs (10mM)	0.2 mM
Primer 357f+GC (20µM)	0.4 µM
Primer 534r (20µM)	0.4 µM
KapaTaq polymerase (5U/µl)	1U/50 µl
PCR grade water	Up to 15 µl

9.3. Plasmid extraction and sequencing

Bacterial pellet was collected in eppendorf tubes by centrifugation (max speed/3 min.) of 1.5 ml of fresh culture. Plasmid DNA was extracted and purified using the NucleoSpin Plasmid kit (Macherey-Nagel GmbH, Germany) according to the manufacturer's instructions. The extracted plasmids were checked by 1% agarose gel electrophoresis (1µl of sample mixed with 1µl of loading buffer) and then sent for sequencing of the insert. The pGEM®-T vector in which the inserts were cloned contains T7 and SP6 RNA polymerase promoters that are used for sequencing reactions (Table 2.10). The 1500 bp fragment was sequenced in both directions to obtain the full length sequence of the 16S rRNA gene (Sanger sequencing provided maximum readable sequence of approximately 800 bp). The obtained sequences were edited manually and analysed for best match with the on-line tool BLAST® (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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Table 2.10. The sequence of the T7 and SP6 primers used for sequencing the inserts ligated in the pGEM®-T vector.

Primers	Sequences
T7	5'-TAATACGACTCACTATAGGG-3'
SP6	5'-ATTTAGGTGACACTATAG-3'

10. Construction of phylogenetic trees

Phylogenetic trees were prepared based on the whole 16S rRNA gene sequence of the bacterium of interest unless otherwise stated. The first step was to identify a large number (about 40-50) of sequences belonging to the same genus of the studied bacterium *plus* a sequence of a different genus to utilize as an out-group for rooting the resulting phylogenetic tree. For this purpose, different BLAST® searches were run and the best hits were selected.

Subsequently all the 16S rRNA sequences selected were aligned using the T-Coffee software (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi) at the "expresso" mode. This software uses a consistency method, meaning that it combines several algorithms for building an alignment and joins their results. The most important fact is that it searches several databases to consider secondary structure. The only problem is that also global alignment is performed so usually the edges of the alignment are not good and have to be discarded or manually curate. So, the misalignments and parts of the alignment (blocks) that cannot be aligned due to very low conservation were rejected using the GBlocks software (http://molevol.cmima.csic.es/castresana/Gblocks_server.html).

The Topali software (http://www.topali.org/) was then employed to look for the best evolutionary model that describes the alignment. Finally the best model had to be applied to the aligned blocks in order to obtain the phylogenetic tree. The problem with Topali is that the number of bootstrap replicates is limited to 100. For this last step is so preferable to use the PhyML or RaXML software that apply the chosen model with 1000 bootstrap replicates, obtaining a better statistical support of the branching of the best tree. Branch bootstrap values of above 0.7 were considered acceptable.

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11. Experiments for the characterization of the bacterial degrading ability

11.1. Assessing the impact of pH on the degrading capacity of bacterial isolates

The degrading ability of the pure bacterial isolates and bacterial consortia capable of degrading pesticides was tested at a range of pH (4.5, 5.5, 6.5, 7.5, and 9). The medium used was MSMN or MSM amended with the corresponding pesticide (20 mg/l). The pH of those media is 6.5 and it was adjusted to higher and lower levels by addition of appropriate amounts of NaOH or HCl respectively. The pH was adjusted prior to autoclaving of the medium when Stock solutions 1 and 2 and deionised water were added. For every pesticide and pH level, two replicates were inoculated (0.6 ml of degrading culture in 12 ml of medium corresponding to 10^6 cells/ml measured by spread plating in LB plates). In addition, two not-inoculated samples were also included as abiotic controls. All samples were put in a shaking incubator at 26°C/160 rpm and degradation was daily measured by HPLC as described above until degradation was completed.

11.2. Assessing the impact of temperature on the degrading capacity of bacterial isolates

The effect of temperature on the degrading ability of the isolated bacteria or bacterial cultures was determined in a range of temperatures (4, 15, 26 and 37°C). The experiment was carried out in MSMN or MSM + 20 mg/l of the corresponding pesticide. For every pesticide - temperature combinations two inoculated (0.6 ml of degrading culture in 12 ml of medium corresponding to 10^6 cells/ml measured by spread plating in LB plates). All samples were put in shaking incubators of the corresponding temperature at 160 rpm and degradation was measured by HPLC at the day of inoculation and 1, 2, 3, 4, 8, 11, and 30 days later.

11.3. Combinations of pesticide-degrading bacteria and their effect on the degrading ability

In this experiment all possible combinations of the pesticide-degrading bacteria isolated were tested to evaluate if their co-presence would alter their overall degrading capacity. This is essential since in future applications and development of tailor-made inocula for biofiltration systems it is expected that depending on the composition of the wastewaters combinations of bacteria would be needed as starting inocula. The same molarity (1.7 mM) for all pesticides studied was used in the culture medium (MSMN). For each pesticide, all bacteria combinations had two inoculated and two non-inoculated controls. The degrading capacity of the pesticide-degrading bacteria was tested both individually and also in dual and triple mixtures. The density of the inoculum was measured by spread plating and was determined to be 2x10^6 cfu/ml. Upon inoculation all samples were incubated in a shaking incubator at 26°C/160 rpm. The degradation of the different pesticides was determined at 1, 2, 3, 4, 5, and 8 days post inoculation *via* HPLC.

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11.4. Assessing the bioaugmentation potential of pesticide-degrading isolates in soil

In the soil experiments the bioaugmentation potential of the isolated degrading bacteria was tested on soil artificially contaminated with the specific pesticides. A soil collected from a field of the National Agricultural Research Foundation of Greece in Larissa with no recent history of pesticides application was used. After sieving, the moisture content and the water holding capacity (WHC) of the soil were determined in order to adjust soil moisture to around 40% of the water holding capacity. The water content was determined by measuring water loss from 10 g of soil after overnight drying in an oven at 100°C. The WHC of the soil was measured gravimetrically following saturation of the soil (20 g) with distilled water in a filter funnel with Whatman N° 1 filter paper and allowing to drain for 24h. The following formula was applied to calculate WHC:

$$\mathbf{WHC=} \frac{(\text{T gr of soil} - X) + (W2 - W1)}{X} \cdot 100$$

Where: W1= soil+funnel+filter paper prior to the addition of water

W2= soil+funnel+filter paper after the addition of water and overnight drainage

X= the amount of dry soil in 20 g of fresh soil

Soil pH was determined in a 1:2.5 air-dried soil:water ratio (10 g : 25 ml). The mixture was shaken for 20 minutes and pH was determined to be 8.3.

The bioaugmentation potential of the isolated bacteria was tested against three pesticide concentration levels. Appropriate aqueous solutions of the given pesticides were prepared as it will be described in more detail in the following chapters and aliquots of those solutions were added in soil to achieve the intended concentrations. After pesticide application, soils were left to equilibrate at 10°C for 30 days in plastic bags in order to allow aging of pesticide residues in soils simulating an *in situ* pollution situation. At the end of the 30-day period, soils were removed from the incubator and were inoculated with appropriate amounts of degrading bacterial inoculum resulting in an inoculation level in soil of $2x10^{6}$ cfu/g of soil. The inoculum density was measured by spread plating as described above. After inoculation, soils were left to equilibrate for a day at 25°C and then they were divided into subsamples of 20 g which were placed individually into aerated plastic bags and incubated in the dark at 25°C. The moisture content of the soil was adjusted on a weekly basis by regular additions of distilled water when needed. The degradation of the pesticides tested was measured at different times after

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inoculation *via* HPLC. In all cases, for every pesticide and concentration level three replicates of inoculated and non inoculated controls were analysed at each sampling time.

11.5. Assessing the degrading ability of pesticide-degrading bacteria in BEM and their survival upon maintenance at composting temperatures

The degrading ability of the isolated bacteria was tested in liquid BEM that resembled as much as possible the nutritional status of biomixtures used for the packing of on-farm biobed systems [Castillo *et al.*, 2008]. BEM was produced as described above and pesticides (25 mg/l) were added as aqueous filter-sterilized solutions after medium sterilization. For comparative purposes the biodegradation of all pesticides was concurrently tested in MSMN or MSM + pesticide. Three replicates were inoculated for every medium and the inoculum level was determined by spread plating in LB as $2x10^6$ cfu/ml. Two non-inoculated controls for every medium were included. All samples were incubated in a shaking incubator at 26^oC/160 rpm. Degradation was measured with HPLC at the beginning of the experiment (T0) and at 2 and 5 days post-inoculation.

A second experiment was conducted in both MSMN and BEM media to assess the survival and activity of the pesticide-degrading bacteria during the composting process of a spent biomixture substrate. The bacterial cultures were incubated in the growth media following a daily temperature profile similar to the temperature profile occurring during composting of the biobed material (Figure 2.6). During the 28 days of the experimental duration, degradation was measured on a weekly basis. After the final sampling day, the survival and activity of the bacteria was checked, by transferring the inoculated cultures into fresh media. In parallel new controls were utilized. All samples were placed again in a shaking incubator at 26^oC/160 rpm and degradation was measured on a weekly basis for the next 28 days.

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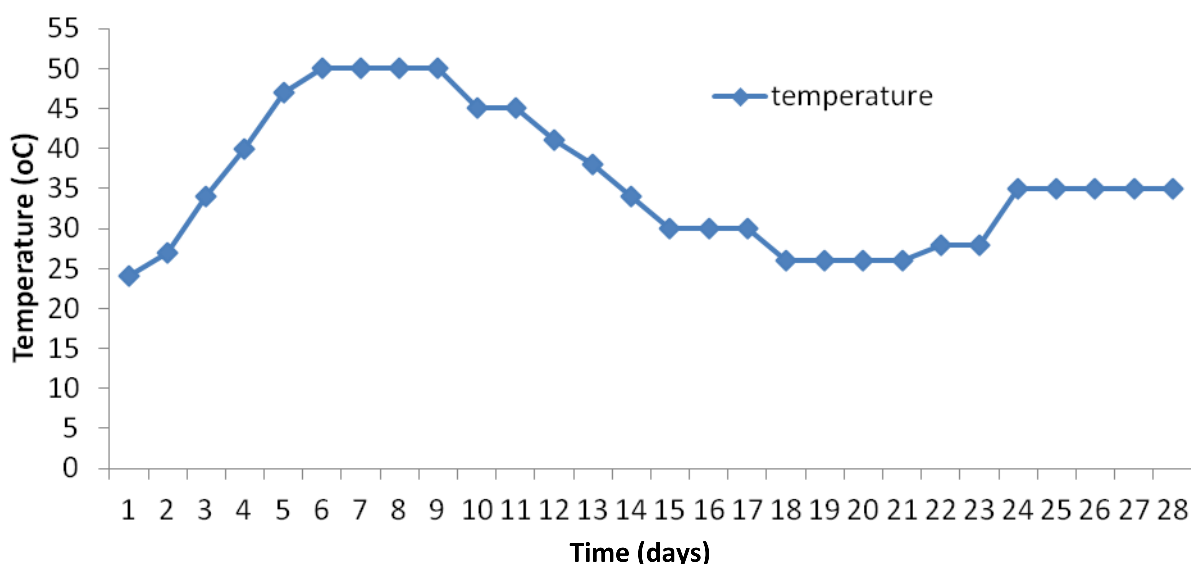

Figure 2.6. The composting temperature profile followed during the incubation of the pesticide-degrading cultures.

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Chapter 3

Isolation and characterization of bacteria able to degrade thiabendazole

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1. Introduction

Thiabendazole (*2-(4-thiazolyl) benzimidazole*) is widely used as a postharvest fungicide on various agricultural products including fruits (bananas, avocado, mango, citrus and pome fruits), potatoes, and mushrooms [Roberts and Hutson, 1999; EC, 2013]. It controls molds and rots caused by a range of fungal pathogens including *Penicillium* spp., *Fusarium* spp., *Colletotrichum* spp., *Botrytis* spp., *Gloesporium* spp., and *Phoma* spp. [US EPA, 2002; EC, 2013].

TBZ can be applied by dipping, drenching, and spraying, or during waxing. Depending on the type of agricultural product treated, different dose rates and methods of applications are utilized. The highest application rates are used for the treatment of potato seeds (up to 30 g/l), while less concentrated solutions are utilized for the treatment of citrus fruits (2 g/l), pome fruits (1.2 g/l), banana, avocado, mango, papaya (0.25-0.45 g/l) [EC, 2013b]. Pome fruits are mostly treated with TBZ *via* drenching while both dipping and drenching are used for the treatment of citrus fruits.

All modes of TBZ applications (drenching, dipping, spraying) are generally water-based and large amounts of water are used resulting in the production of large wastewater volumes containing high TBZ loads. The direct environmental release of those wastewaters could have deleterious effects on the ecological and chemical quality of natural resources [Washington DC, 2004]. This becomes more hazardous when considering the particularly high acute and long-term toxicity of TBZ to aquatics [US EPA, 2002; EC, 2013]. A monitoring study in Costa Rica demonstrated that fruit packaging plants were associated with the frequent detection of postharvest fungicides like TBZ in receiving surface water systems [Castillo *et al*., 2000]. Similarly, different studies have revealed long term contamination of soil and water resources with TBZ in the region of the Alto Valle de Río Negro-Neuquén (Northern Patagonia, Argentina), where the production and packaging of fruits is the main economic activity [Loewy *et al*., 1999.; Lombardi *et al*., 2003b; Roca *et al*., 2013].

To avoid such environmental contamination, the use of TBZ is authorized by the EC under the clause that "*appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste, are put in place* [EC, 2001]. Despite that, there are a limited number of methods for the treatment of those effluents. The only depuration system currently available (patented and on the market with the commercial name CONTROL TEC ECO®) is based on pesticide adsorption onto granular activated carbon after a pre-treatment of wastewater with filtration and flocculation to eliminate solids [Garcia Portillo *et al*., 2004]. This system showed high efficiency for the dissipation of TBZ and was able to treat high wastewater volumes (2500 l/h) [EC, 2000], however its high cost has hampered its wider uptake by small-

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medium enterprises. Alternative treatment systems based on physicochemical processes were tested by Roca *et al.* [2013] and Bemabeu *et al.* [2011]. The former suggested the use of aluminum pillared clays as adsorbents for TBZ removal from wastewaters. The latter showed that solar TiO_2 photocatalysis could be utilized as a tertiary treatment for TBZ-contaminated wastewater. In all those cases, dissipation efficiency was showed at laboratory scale and pilot scale studies are required to establish their full market potential [Santiago *et al*., 2011].

Considering the high cost of the existing physicochemical treatment systems and the lack of alternative ready-to-be-implemented methods, direct disposal into creeks, sewage treatment plants, and evaporation ponds, or land spreading on adjacent field sites, are currently employed to dispose off these agroindustrial effluents. This is particularly problematic since TBZ appears to be extremely persistent in soil with extrapolated field dissipation $t_{1/2}$ of 833-1100 days in cropped plots and of 1093-1444 days in fallow plots [US EPA, 2002]. However more recent dissipation studies showed that TBZ was not that persistent in soil with $t_{1/2}$ values ranging from 45.2 to 153 days [Omirou *et al.* 2012; EC, 2013]. In water/sediment systems TBZ appears to partition gradually in the sediment phase where it persists for long [EC, 2013].

Biological treatment methods might be a good alternative for the treatment of TBZ-containing wastewaters. In a recent study Omirou *et al*. [2012] showed that on-farm biopurification systems like biobeds dissipated 85% of the TBZ amount disposed off in those systems. However, biobeds could not handle the large wastewater volumes produced daily by citrus fruit-packaging plants, where TBZ is mostly utilized. Thus more mechanized biological systems able to handle large wastewater volumes are needed for citrus fruit-packaging plants in particular. In such systems, tailored made microbial inocula will facilitate their implementation. So far, little is known regarding the capacity of microorganisms to degrade TBZ. In the only study available, Karas *et al*. [2011] showed that selected lignolytic fungi like *Trametes versicolor* had limited degradation capacity against TBZ.

Considering the versatility of bacteria in the degradation of organic pollutants and the limited capacity of white rot fungi, the most versatile fungal degraders, to degrade TBZ, we aimed a) to isolate and identify bacteria able to rapidly degrade TBZ and b) to characterize their degrading ability. This will be a key step towards the future implementation of biofilter systems for the depuration of wastewaters from the fruit-packaging industries.

2. Materials and methods

2.1. Pesticides and other chemicals

High purity standards of TBZ (Pestanal® 99%), fuberidazole (Pestanal®, 99.4%), carbendazim (Pestanal®, 99.2%), 2-aminobenzimidazole (Pestanal® 99.9%), and imazalil (Pestanal® 99.8%)

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were purchased from Fluka, Sigma-Aldrich, and were used for analyses and other experimental purposes. Methanol stock solutions and water solutions of TBZ were prepared as described in Chapter 2. A 30 g/l stock solution of TBZ in dimethyl sulfoxide (DMSO, 99.9%, ChemLab) was also prepared, filter sterilized (0.22μm, PTFE Syringe Filter, sterilized) and stored at 4°C.

Other chemicals including methylamine hydrochloride (purum, >98% purity), benzimidazole (98% purity) and 1*H*-benzimidazole-2-carboxylic acid (97% purity) identified as potential metabolites of TBZ were purchased from Sigma-Aldrich. The latter was utilized for the synthesis of 1*H*-benzimidazole-2-carboxamide which has been also described as a potential metabolite of TBZ in aqueous photolysis studies [EC, 2013]. Synthesis and verification of its identity were performed in the Laboratory of Pesticide Chemistry, Aristotle University of Thessaloniki by Prof. U. Menkissoglu-Spiroudi. For all chemicals mentioned above, methanol and aqueous solutions (100 mg/l) were prepared as described for TBZ. The only exception was 1*H*-benzimidazole-2-carboxylic acid for which a 1000 mg/l solution in DMSO was prepared and used for the preparation of a series of dilutions in DMSO:methanol (50:50 by volume) which were used in analyses.

2.2. Growth media

The minimal media MSM and MSMN plus the non-selective SEM amended with TBZ (20 mg/l unless stated otherwise) were used for isolation of TBZ-degrading bacteria. LB was routinely used in liquid or solid form. Other media were also utilized in order to either characterize the degradation capacity of the TBZ-degrading bacteria (Biobed extract medium, BEM) or to purify members of the TBZ-degrading consortia (MSM/MSMN + Casamino Acids; Mandelbaum Medium; L9). The protocols for their preparation have been given in Chapter 2 (BEM) or are described below:

a) **MSM/MSMN + casamino acids** (Fluka, Sigma-Aldrich): 0.15 g/l of casamino acids were added before autoclaving. After sterilization and cooling of Stock solution 1 + water, appropriate amounts of TBZ stock solution were added together with Stock solutions 2 and 3 in order to get a TBZ concentration in the final medium of 20 mg/l;

b) **Mandelbaum medium** [Mandelbaum *et al*., 1993]: the components of this medium are listed in Table 3.1. The vitamin and the salts solutions were filter sterilized and added to the medium after autoclaving and cooling together with the TBZ stock solution (100 mg/l). The pH of the medium was adjusted to 7.3.

c) **Modified L9 minimal salt medium**, selective for *Sphingomonas* spp. [Yim *et al.*, 2010]: the components of the medium are presented in Table 3.2. After autoclaving and cooling, the medium was amended with the filter sterilized trace elements solution, the filter

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sterilized solutions of the antibiotics streptomycin and piperacillin (100 g/l), and the filter sterilized stock solution of TBZ (100 mg/l). The pH of the medium was adjusted to 7.

All the above liquid media were also prepared in solid form as described before (Chapter 2).

Table 3.1. Composition of the Mandelbaum medium.

Components	**Concentration (g/l)**
K_2HPO_4	1.6
KH_2PO_4	1.4
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
$CaCl_2$	0.02
Salt Stock Solution	20 ml
Vitamin stock solution	20 ml

Salt stock solution:	
Components	**Concentration (g/l)**
EDTA	2.5
$ZnSO_4$	11.1
$FeSO_4$	5
$MnSO_4 \cdot H_2O$	1.54
$CuSO_4 \cdot 5H_2O$	0.4
$Co(NO_3)_2 \cdot 6H_2O$	0.25
$Na_2B_4O_7 \cdot 10H_2O$	0.18
H_2SO_4	5

Vitamin stock solution:	
Component	**Concentration (mg/l)**
Thiamine-HCl	0.005
Biotin	0.002
Folic acid	0.002
Nicotinamide	0.010
Pyridoxine-HCl	0.010

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Table 3.2. Composition of the modified L9 minimal salt medium.

Component	Concentration (g/l)
$Na_2HPO_4 \cdot 2H_2O$	8.8
KH_2PO_4	3
NH_4Cl	1
NaCl	0.5
$MgSO_4$	0.2
Trace element solution	2.5 ml
Streptomycin	100 μg/ml
Piperacillin	50 μg/ml

Trace element solution:

Component	Concentration (mg/l)
$MnCl_2 \cdot 2H_2O$	23
$MnCl_4 \cdot H_2O$	30
H_3BO_3	31
$CoCl_2 \cdot 6H_2O$	36
$CuCl_2 \cdot 2H_2O$	10
$NiCl_2 \cdot 6H_2O$	20
$Na_2MoO_4 \cdot 2H_2O$	30
$ZnCl_2$	50

2.3. Antibiotics

Antibiotics stock solutions were prepared as described in Chapter 2. The working concentration of the different antibiotics in the subsequent experiments is shown in Table 3.3. Streptomycin nominal concentration in the medium was 200 μg/ml, but when combined with piperacillin its concentration was adjusted to 100 μg/ml as suggested by Yim *et al*. [2010]. Agar plates amended with antibiotics were prepared by adding the appropriate amount of filter sterilized antibiotic stock solution after autoclaving and cooling of the medium.

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Table 3.3. The concentrations of antibiotics used in the different media.

Antibiotics	Concentration (mg/l)
Amikacin	16
Ampicillin	100
Ciprofloxacin	2
Colistin	2
Doxycycline	25
Nalidixic acid	23
Penicillin	300
Piperacillin	50
Streptomycin	100/200
Trimethoprim	20
Vancomycin	200

2.4. Pesticide extraction and HPLC analysis

Pesticide extraction from liquid medium was performed as described in Chapter 2. Regarding extraction of TBZ from soil, a two-step extraction process was followed. In the first step, 10 ml of methanol (HPLC Gradient Grade) were mixed with 5 g of soil in a conical flask. The mixture was shaken for an hour in an orbital shaker (200 rpm) at 26°C. Subsequently, the mixture was centrifuged for 5 min at 11000 rpm and the clear supernatant was recovered in a glass bottle. The remaining soil was re-extracted with further 10 ml of methanol as described before. After centrifugation the clear supernatant from the second extraction cycle was combined with the first extract resulting in a final extraction volume of *ca*. 20 ml. The extract was passed through a 0.45μm syringe filter (PTFE Syringe Filter) and kept at -20°C until analysed.

TBZ residues were determined in an HPLC-UV system (described in Chapter 2) at a wavelength of 254 nm using a mobile phase of 39/60.5/0.5 acetonitrile (ACN)/water/25% NH_3 solution (by volume). Under these conditions, the retention time of TBZ ranged from 3.3 to 4.3 minutes depending on the column used. Regarding the other compounds analysed, the HPLC conditions used are shown in Table 3.4.

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Table 3.4. The HPLC conditions used for the analysis of the different compounds studied.

Compound	Wavelength (nm)	Mobile Phase (by volume)	Retention time (min.)
Fuberidazole	250	60:40 Water:ACN	3.2
Carbendazim	270	50:50 MeOH:Water + 0.6% NH_3 solution	3.6
2-aminobenzimidazole			3.0
Imazalil	204	80:20 MeOH:25% NH_3 solution	3.3
1*H*-benzimidazole-2-carboxylic acid	205	80:20 Water:ACN	5.2
1*H*-benzimidazole-2-carboxamide	211		5.4
benzimidazole	243		5.3

2.5. Enrichment culture for the isolation of TBZ-degrading bacteria

The enrichment culture technique, as described in Chapter 2, was used for the isolation of TBZ-degrading bacteria. A soil collected from a site used for the disposal of wastewaters from a citrus-fruits packaging plant in Lemesos, Cyprus was used as a source of TBZ-degrading bacteria. Upon its collection and transfer in our laboratory the soil was stored at 4°C until further processing. The soil was sieved through a 2 mm sieve and received a single fresh application of TBZ in order to stimulate the TBZ-degrading bacterial community. TBZ was applied into the soil as a methanol solution (1000 mg/l) resulting in a soil concentration of 20 mg/kg. Further details about the soil treatment are given in Chapter 2. After two weeks of incubation at 25°C the soil (0.5 g per flask) was used to inoculate MSM, MSMN, and SEM. The degradation of TBZ was recorded at 0, 2, 5, 7, 9, 12, and 14 days depending on the enrichment culture and cycle. After 5 enrichment cycles (4 in SEM), an aliquot of the degrading culture was used for the preparation of 10-fold serial dilutions which were spread on the corresponding agar medium + 20 mg/l TBZ. Single colonies growing on the plate were picked up and tested for their degrading ability in the corresponding liquid media.

The enrichment cultures of MSM and MSMN from the 5th-EC were maintained under continuous subculturing for the following 4 years aiming to gradually reduce the diversity of the bacteria constituting the TBZ-degrading consortia and to finally obtain stabilized consortia (regarding their composition) or ideally the TBZ-degrading bacterium in pure culture. Thus, on a weekly basis, 0.5 ml of the degrading cultures were transferred into 10 ml of the corresponding medium plus 20 mg/l of TBZ and left in a shaking incubator at 26°C/180 rpm. The degrading ability and the bacterial composition of the subcultured TBZ-degrading cultures were regularly checked.

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2.6. Molecular analysis of pesticide-degrading bacteria

Details on the methods used for DNA and RNA extraction from bacterial cultures, cDNA synthesis, DGGE fingerprinting of bacteria and pesticide-degrading consortia, and the construction and screening of clone libraries were done as described in Chapter 2.

2.7. Experiments aiming to identify the TBZ-degrading bacteria

2.7.1. DNA/RNA-DGGE analysis of the TBZ-degrading consortia

The composition of the TBZ-degrading consortia obtained by enrichment cultures and upon further purification attempts was compared at different intervals during the 4-year sub-culturing *via* comparative DGGE analysis of their DNA and RNA profiles. It is anticipated that DNA profiles give information on which bacteria are present in a community, while RNA profiles indicates only the members of the bacterial community actively growing on the given substrate (in this case TBZ). The latter is based on the observation that actively growing bacteria show high ribosomal transcription activity. In each case, MSMN and MSM + TBZ (25 mg/l) were prepared and inoculated with the corresponding TBZ-degrading culture as described in Chapter 2. Triplicate inoculated and duplicate non-inoculated samples were prepared for each medium. All samples were incubated in an orbital shaker at 26°C/180 rpm. Aliquots of the different cultures were daily removed and used for the determination of TBZ residues by HPLC and for the collection of the bacterial pellet which was used for the extraction of DNA and RNA. The latter was used for cDNA synthesis. Subsequently DNA and cDNA from all samples were subjected to PCR-DGGE analysis. The identity of selected bands showing differences in their intensity or in their presence in the DNA *vs* RNA fingerprints was determined *via* the development and DGGE screening of clone libraries (see Chapter 2).

2.7.2. The impact of antibiotics on the composition of TBZ-degrading consortia

<u>Experiment 1:</u> The impact of a wide range of antibiotics with different antibacterial spectrum on the composition of the TBZ-degrading consortia was investigated in order a) to identify possible members of the consortia which are responsible for the degradation of the fungicide and b) to simplify the composition of the TBZ-degrading consortia without compromising their degrading capacity. The effect of antibiotics was evaluated in both MSM and MSMN supplemented with TBZ (20 μg/ml). In total 11 antibiotics were tested (Table 3.3). In each medium and for each antibiotic, duplicate inoculated and non-inoculated cultures were prepared. In addition, duplicate inoculated cultures which were not amended with antibiotics were also included for comparative reasons. At the day of inoculation (T0) and 2, 4, 7, and 14 days later subsamples from all cultures were collected and used a) for measuring the degradation of TBZ *via* HPLC analysis and

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b) for DNA/ RNA extraction and downstream cDNA synthesis and DGGE fingerprinting. Further clone libraries were established to identify members of the bacterial consortia which showed a banding pattern associated with the degradation of TBZ.

Experiment 2: In order to verify the results of Experiment 1 and further simplify the TBZ-degrading bacterial consortia, selected antibiotic-treated cultures which either maintained or lost their TBZ-degradation capacity were transferred in fresh media containing the same antibiotics. After four subculturing cycles the composition of the cultures which maintained their degrading ability was analyzed *via* DNA/cDNA-DGGE. In addition, aliquots from the same cultures were serially diluted and spread on agar plates of TBZ-amended (20 mg/l) L9 minimal medium (selective for *Sphingomonas*) aiming to pick up and purify the bacterial strain/s responsible for TBZ degradation. Colonies growing on the plates were picked up and their degrading ability was assayed in fresh liquid MSMN or MSM + TBZ as described before. In the same long-term antibiotic exposure experiment, the cultures which lost their degradation capacity during antibiotics exposure were subjected to two biweekly subculturing and they were then transferred to fresh medium in the absence of antibiotic to test if they could recover their TBZ-degrading ability.

Experiment 3: A final experiment was conducted with an MSMN-based TBZ-degrading consortium composed of only two main bacterial members aiming to obtain the TBZ-degrading bacterium in pure culture. Thus, the TBZ-degrading culture was inoculated in fresh MSMN containing 20 mg/l of TBZ which was amended with either colistin (60 μg/ml) or streptomycin (200 μg/ml). Duplicates inoculated and non inoculated samples for each antibiotic were prepared. In addition, inoculated cultures non amended with antibiotics were also included for comparison purposes. The degradation of TBZ was measured *via* HPLC. After two subcultures in the same antibiotic-containing medium DNA-DGGE analysis of the composition of the consortium was performed as described before.

2.7.3. Identification of the role of the different members of the TBZ-degrading consortia via quantitative real time PCR (q-PCR)

Upon identification of the different members of the TBZ-degrading consortia isolated during this study, a genus- or group-specific q-PCR method was employed to follow the dynamics of the different members of the TBZ-degrading consortia during degradation of TBZ. The growth of the members of three TBZ-degrading consortia was determined in: a) the two initially isolated and stabilized consortia (MSMN- and MSM-based) and b) an MSM-based consortium obtained

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after treatment with the antibiotic colistin which was comprised of fewer members. The population of the members of the TBZ-degrading consortia were determined in absolute numbers (*via* q-PCR with group-specific primers) and also relatively to the total bacteria population in the consortia (*via* q-PCR with universal bacterial primers). It was hypothesized that bacteria showing an increasing absolute and relative abundance during the rapid degradation phase of TBZ will be those that are responsible for the degradation of the parent compound.

Primer pairs design: Specific primers for the 16S rRNA sequences of certain bacteria or bacterial groups present in the **two initially isolated and stabilized TBZ-degrading consortia** were designed. The sequences of the 16S rRNA genes of the different bacteria of interest were aligned with the MegAlign™ software (Lasergene®, DNASTAR) aiming to identify target regions for the design of bacterium-specific or if not possible, group-specific primers. Primers were designed to amplify a short product (200-250 bp) appropriate for q-PCR analysis. Initially designed primers were analyzed with the program PrimerSelect™ (Lasergene®, DNASTAR) for secondary structures formations including hairpins, self-dimers, and cross-dimers in primer pairs. The specificity of the primers designed was checked *in silico* with the online tool Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The best possible pair of primers was then chosen. The optimal melting temperature (Tm) of each primer was calculated with the following formula:

$$Tm=81.5+ 16.6*\log M+ 0.41*(\%GC)-500/n$$

Where: M = salt concentration of PCR buffer

%GC = GC content of the primer

N = primer length.

The annealing temperature for each PCR reaction was set to be 2-3°C below the lowest Tm of the two primers in each pair. PCR thermocycling conditions were optimized upon testing and the specificity of each primer pair was evaluated as follows: for each primer set the amplified PCR product was purified using appropriated PCR-clean up kit, ligated to the p-GEM plasmid vector, and the ligation product used to transform competent *E. coli* DH5a cells. Five white colonies containing an insert were selected, the plasmid was extracted, and the insert was sequenced. The sequences obtained were analyzed with the on-line tool BLAST and versus the original sequence which was used for their design and all showed high homology to the target 16S rRNA gene verifying the specificity of the primer sets designed.

The sequences of the designed primers and their target bacterial group used for the assessment of the initial MSMN- and MSM-based consortia are given in Tables 3.5 and 3.6 respectively. Except from Pseudomonads members of the MSM consortium, it was not possible

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to design group-specific reverse primers. For this reason a common universal reverse primer was used. Similarly, it was not possible to discriminate between the members of the α-proteobacteria group and of the different *Pseudomonas* spp. due to their highly conserved 16S rRNA gene sequences. Thus, a common set of primers was designed for all α-proteobacteria in MSMN (*Bosea / Sphingomonas / Shinella* sp.) and MSM (*Bosea / Sphingomonas / Oligotropha*) and in both media for the *Pseudomonas* spp.

Table 3.5. A list of the group-specific primers designed for the specific amplification of the different members of the TBZ-degrading consortia in Experiment 1. Primers' sequences and melting temperatures (Tm) are given.

Primer Name	Target Group	Sequence (5' – 3')	Tm (°C)
B12MSMN_f	*Methylobacillus* / *Aminomonas* sp.	AGG AAA GAA AAC TTA TCT AC	47.9
Methylib_f	*Methylibium* sp.	CTA ATA CCC CGA ACT AAT G	51.5
X4MSMN_f	*Achromobacter* sp.	GGC TAA TAC CCC GTG AAA C	56.0
α-Proteobact_f [a]	*a-proteobacteria*	TAC CCG GGA AGA TAA TGA C	53.5
PseudomMSMN/MSM_f	All *Pseudomonas*	AGT TGG GAG GAA GGG CAG	57.8
E6hydrocMSM_f	*Hydrocarboniphaga* sp.	TGG CTA ATA TCC AAA GCC T	51.5
PseudomMSM_r	All *Pseudomonas*	CTC GCC AGT TTT GGA TGC	55.8
Unico_r	All bacteria	CTA CGC ATT TCA CCG CTA C	56.0

[a] This primer was designed to amplify all α-proteobacteria that were present in MSMN- and MSM-based TBZ-degrading consortia

Table 3.6. The set of primers used for the amplification of the different members of the TBZ-degrading consortia in Experiment 1. The size of the amplicon and the annealing temperature (Ta) are also given.

Target Group	Primers	Amplicon size (bp)	Ta (°C)
Methylobacillus sp.	B12MSMN_f - Unico_r	221	47
Methylibium sp.	Methylib_f - Unico_r	203	48
Achromobacter sp.	X4MSMN_f - Unico_r	204	54
α-proteobacteria	αProteobact_f - Unico_r	201	51
Pseudomonas spp. MSMN	PseudomMSMN/MSM_f - Unico_r	226	54
Hydrocarboniphaga sp. MSM	E6hydrocMSM_f - Unico_r	204	47
Pseudomonas spp. MSM	PseudomMSMN/MSM_f -PseudomMSM_r	173	60

In the same way, a new set of primers (ColC_f and ColC_r) was designed to determine *via* q-PCR the dynamics of a *Sphingomonas* ribotype present in a MSM-based TBZ-degrading culture

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which was simpler in its composition and obtained after continuous cultivation of the initial stabilized MSM-based consortium in the presence of the antibiotic colistin. Based on previous fingerprinting results which will be presented in the Results and Discussion section, this *Sphingomonas* ribotype was suspected to be responsible for the degradation of the fungicide. This set of primers targeted the V3 region of the 16S rRNA gene of the specific ribotype (Table 3.7). The control of the specificity of the designed primers and the preparation of the calibration curve were done as described above and below respectively.

Table 3.7. The sequences and Tm of the *Sphingomonas*-specific primers.

Primer Name	Sequence (5' – 3')	Tm (°C)
ColC_f	ATA ACT AGC TGT CCG GGC A	55.9
ColC_r	TCC ATC TCT GGA AAC CAT AC	54.0

Q-PCR analysis: Quantification of the copies of the target sequence was performed with an external calibration curve generated using a series of dilution of a known concentration of a plasmid containing the target sequence as an insert. For each primer set one transformed *E. coli* colony containing the correct insert was selected for plasmid extraction. A PCR verified the presence of the correct insert and the plasmid concentration was measured using the Qubit® 2.0 Fluorometer (Invitrogen). A series of 10-fold dilutions was prepared and used in the q-PCR for the construction of the calibration curves that allow the quantification of the copies of the target gene. Copy numbers of a gene were then calculated with the following formula [Whelan *et al.*, 2003]:

$$\textbf{DNA copies/}\mu\textbf{l} = \frac{6.02 * 10^{23}\ \text{(copies/mol)} * \text{DNA concentration (gr/}\mu\text{l)}}{\text{DNA length}_{\text{(plasmid+insert)}}\ \text{(bp)} * 660\ \text{(gr*bp/mol)}}$$

The q-PCR mixture was prepared on ice using the Kapa™ SYBR® Fast q-PCR kit (KapaBiosystems). The composition of the q-PCR mix is presented in Table 3.8. 96-wells plates closed with optical adhesive film were used. An Mx 3005P™ q-PCR machine (Stratagene, Agilent Technologies) with the MxPro q-PCR software was used for all measurements. The q-PCR thermocycling programs for every set of primers tested are given in the relevant sections below. Each plate included triplicates for each DNA sample and triplicates for each level of standard used for the preparation of the calibration curve. The amplified products were run on a 1.5% agarose gel to confirm the specificity of the amplification. The starting DNA quantity in

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each sample was determined fluorometrically with Qubit2v in order to normalize the results obtained by q-PCR.

Table 3.8. The composition of the q-PCR reaction mix.

Components	Final concentration
Kit Master mix 2X universal	1X
Forward primer (20µM)	200 nM
Reverse primer (20µM)	200 nM
Template DNA	Variable
Rox low	50 nM
PCR grade water	Up to 20µl

Experiment 1: For each medium (MSMN and MSM), three flasks containing the corresponding media (50 ml) + 50 mg/l TBZ were prepared and inoculated with 2.5 ml of the corresponding **initially isolated TBZ-degrading culture**, while three other flasks with the corresponding medium containing IMZ (instead of TBZ as an alternative C and N source) were also inoculated to act as non-TBZ amended controls. Two non-inoculated flaks per medium were also included to serve as abiotic controls. All samples were incubated in an orbital shaker at 26°C/180 rpm and TBZ degradation was measured *via* HPLC at time 0 and on 12-hour intervals after inoculation and until 2 days after completion of degradation. At each sampling time bacterial pellet was collected and used for DNA extraction which was used as template for q-PCR analysis of the dynamics of the different members of the TBZ-degrading consortia using the group-specific primers described above. Quantification of the copies of the target gene was done *via* external calibration curves prepared as described above. The thermocycling program used for q-PCR analysis of the different bacterial groups was as follows: initial denaturation for 3 min. at 95°C followed by 45 cycles (35 cycles only for *Pseudomonas* MSM q-PCR) of (I) denaturation (15 sec. at 95°C), (II) annealing (20 sec., Ta for the different groups is given in Table 3.6), and (III) extension (11 sec. at 72°C). The fluorescence signal was measured at the end of each extension step at 72°C. After the amplification, a melting curve analysis with a temperature gradient of 0.1^{o}C/s from 45°C (48°C for *Pseudomonas* MSMN, α-proteobacteria, and *Achromobacter* and 50°C for *Pseudomonas* MSM) to 95°C was performed to confirm the specificity of the products amplified.

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Apart from the determination of the absolute population dynamics of the members of the different TBZ-degrading consortia, the dynamics of the different bacterial groups present in the TBZ-degrading consortia relatively to the total bacterial population in the consortia were defined. It was hypothesized that bacterial groups whose relative abundance (group-specific copies of the 16S rRNA gene / total bacteria copies of the 16S rRNA gene) increases during the active degradation phase of TBZ are probably involved in the degradation of the fungicide. Thus, the DNAs extracted were also subjected to q-PCR with the universal primers Eub338_f [Lane, 1991] and Eub518_r [Muyzer *et al.*, 1993] as described by Fierer *et al.* [2005] (Table 3.9). The composition of the q-PCR mix was as shown in Table 3.8 and quantitation of the copies of the 16S rRNA gene was performed as described above. The thermocycling conditions were as follows: initial denaturation for 3 min. at 95°C followed by 35 cycles of 95°C for 15 sec. and 65°C for 15 sec. The fluorescence signal was measured at the end of each step at 65°C. A melting curve analysis with a temperature gradient from 58 to 95°C was performed to confirm amplification only of the specific product (200 bp).

Table 3.9. The set of universal primers used for the amplification of total bacteria in the TBZ-degrading consortia. The size of the amplicon and the annealing temperature (Ta) are also given.

Primer Name	Sequence (5' – 3')	Amplicon size (bp)	Ta (^{o}C)
Eub338_f	ACT CCT ACG GGA GGC AGC AG	200	65
Eub518_r	ATT ACC GCG GCT GCT GG		

Experiment 2: In accordance with the previous experimental set up, triplicate MSM cultures amended with TBZ (25 mg/l) and triplicate MSM cultures amended with IMZ (25 mg/l) serving as non-TBZ inoculated controls were prepared. All flasks were inoculated with fresh cultures of the simpler **MSM-based consortium obtained after treatment with the antibiotic colistin** as described before. Duplicate non-inoculated controls (MSM+TBZ) were also included. All samples were incubated in an orbital shaker at 26°/180 rpm and TBZ degradation was measured at 12-hour intervals. At selected sampling times (12, 36, 24, 48, and 72 hours), bacterial pellet was collected by the TBZ-amended and non-amended samples and was used for DNA extraction and q-PCR analysis using the ColC_f/ColC_r primers which were designed to specifically amplify a *Sphingomonas* ribotype putatively associated with the degradation of TBZ. The q-PCR thermocycling conditions were as follows: initial denaturation for 3 min. at 95°C followed by 35 cycles of 95°C for 15 sec. and 62°C for 20 sec. The fluorescence signal was measured at the end of each step at 62°C. A melting curve analysis with a temperature gradient from 52 to 95°C was performed to confirm the amplification of the specific product (200 bp). Similarly to Experiment

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1, the population of the total bacteria in the degrading consortium was determined *via* q-PCR as described above.

Statistical analysis: The q-PCR results obtained from the two experiments were subjected to two-way analysis of variance (ANOVA). The main independent variables in the experiments were time and treatment (presence/absence of TBZ). The LSD test (p=0.05) was used to identify differences between treatments within time when significant interactions between the main variables were observed.

2.7.4. Growth of TBZ-degrading consortia on group-specific media

After determination of the identity of the different members of the TBZ-degrading consortia *via* cloning, selective media were used to isolate in pure cultures the individual members of the consortia. This would have allowed the identification of their role in TBZ metabolism. For this reason fresh cultures of the isolated MSMN and MSM-based TBZ degrading cultures were serially diluted and spread on agar plates of the following media: a) MSMN/MSM+casamino acids, b) Mandelbaum medium, and c) *Sphingomonas*-selective modified L9 medium. All media were supplemented with TBZ at a concentration of 20 mg/l. Plates were allowed to grow at 26°C and morphologically distinct single colonies were selected and assayed for TBZ degradation in MSMN/MSM liquid cultures.

The whole bacterial biomass growing on the agar plates was also harvested with 0.5 ml of sterile ddH_2O and used for DNA extraction. In addition, its degradation capacity was also tested in fresh liquid cultures. Upon completion of the degradation assay, the bacterial pellet from the different cultures was collected and used for DNA extraction and PCR-DGGE analysis.

2.7.5. Phylogenetic analysis of the TBZ-degrading bacterium

The bacterium identified as responsible for TBZ degradation was subjected to phylogenetic analysis using the full sequence of its 16S rRNA gene as described in Chapter 2.

2.8. Experiments for the characterization of the TBZ-degrading ability of the isolated consortia

2.8.1. The capacity of the isolated consortia to degrade high TBZ concentrations

The capacity of the MSM- and MSMN-based TBZ-degrading consortia to degrade spillage level concentrations of TBZ was evaluated in liquid cultures. Three concentration levels of TBZ were considered: 75, 150, and 600 mg/l. Considering that the two higher concentration levels exceeded the water solubility of TBZ, a filter-sterilized water solution of 30 g/l was prepared

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from the commercial formulation of the pesticides (300 g/l) and 0.26, 0.5, and 2 ml of this were added to 100 ml of autoclaved medium after cooling to obtain the target concentrations of TBZ in the final medium. Three inoculated replicates and two non-inoculated controls for each concentration level were prepared. The samples were placed in an orbital shaker at 26°C/180 rpm and TBZ degradation was measured by HPLC at the day of the inoculation (T0) and 3, 7, 14, and 21 days later.

2.8.2. Cross-feeding studies: assessment of the capacity of the isolated consortia to degrade different compounds of similar chemical structure

Experiment 1: A first experiment was conducted to investigate the capacity of the MSMN and MSM-based TBZ-degrading consortia to degrade compounds of similar chemical structures. It was hypothesized that the isolated TBZ-degrading consortia will be able to actively degrade compounds with similar chemical structures providing information on the initial steps of the metabolic pathway. The compounds included were the fungicides fuberidazole, carbendazim, and imazalil and the metabolite of carbendazim, 2-aminobenzimidazole. TBZ degradation was also measured for comparison purposes. MSMN and MSM containing the appropriate compound at concentration of 25 mg/l were prepared as described in Chapter 2. Carbendazim is photosensitive so the stock solution and the media were incubated in the dark. For every compound, degradation was measured in three inoculated replicates and duplicate non-inoculated controls. All samples were incubated in an orbital shaker at 180 rpm in a constant temperature of 26°C. Aliquots from the liquid cultures were taken at times 0, 2, 4, 7, 10, 21, and 30 days and the concentrations of the different compounds was measured by HPLC as described above.

Experiment 2: In a subsequent experiment, the degrading ability of the two TBZ-degrading consortia towards molecules which have been identified by previous studies as possible metabolites of TBZ [EC, 2013] was assessed. In addition, the effect of those molecules on the composition of the TBZ-degrading consortia was also evaluated. It was hypothesized that prolonged exposure of the TBZ-degrading consortia to those putative metabolites might shape their composition towards dominance of members which have the capacity to degrade those molecules. The tested compounds were 1*H*-benzimidazole-2-carboxylic acid, 1*H*-benzimidazole-2-carboxamide, benzimidazole, and methylamine hydrochloride. The degradation of the first three molecules was assessed in MSMN, while only the degradation of 1*H*-benzimidazole-2-carboxamide and benzimidazole was tested in MSM. The possible degradation of methylamine hydrochloride was not measure since no analytical methods were available. Thus only its effects on the composition of the TBZ-degrading consortia were considered.

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Regarding 1*H*-benzimidazole-2-carboxamide, benzimidazole, and methylamine hydrochloride, filtered water stock solutions (100 mg/l) were prepared and added to the media after autoclaving to establish a final medium concentration of 20 mg/l. 1*H*-benzimidazole-2-carboxylic acid was scarcely soluble in water and it was dissolved in DMSO to a final concentration of 1000 mg/l. After media sterilization and cooling, appropriate amounts of the DMSO stock solution were added to reach a concentration in the media of 10 mg/l and not exceed the presence of 1% of DMSO in the medium. For each compound, one inoculated and one non-inoculated flask were prepared. All samples were incubated in an orbital shaker at 26°C/180 rpm. The degradation of all molecules by the TBZ-degrading consortia was followed for 3 to 4 cycles. Each cycle lasted from 40 to 49 days and degradation was determined *via* HPLC analysis of samples collected at time 0 and at weekly intervals thereafter. Regarding methylamine, samples were sub-cultured following the same scheme as 1*H*-benzimidazole-2-carboxamide. In order to assess the evolution of the composition of the TBZ-bacterial consortia upon continuous exposure to the different compounds, the bacterial pellet from the different cultures was collected at the end of every cycle and it was used for DNA extraction and DGGE analysis.

At the end of the final cycle the cultures were transferred in the corresponding medium + TBZ (20 mg/l) in order to test if the cultures have maintained their degrading capacity against the parent compound after prolonged exposure to other molecules. Degradation of TBZ was assessed *via* HPLC at time 0, 5, 12, and 20 days. In addition, the bacterial pellet was collected at the end of the culture cycle and the DNA extracted was used for DGGE analysis.

Experiment 3: A final experiment was conducted to investigate the formation and subsequent degradation of 1*H*-benzimidazole-2-carboxylic acid, 1*H*-benzimidazole-2-carboxamide, and benzimidazole during the degradation of TBZ by the isolated consortia. Three inoculated replicates and duplicate non-inoculated controls per medium (MSMN/MSM + 20 mg/l TBZ) were prepared. All samples were incubated in an orbital shaker at 26°C/180 rpm. The degradation of TBZ and the formation of metabolites were determined on 12-h intervals *via* HPLC as described before.

2.8.3. Evaluating the degrading ability of TBZ-degrading consortia in rich growth media

The ability of the MSMN- and MSM-based TBZ-degrading consortia to degrade the fungicide when cultivated in a rich medium like LB was investigated. It was hypothesized that TBZ-degrading consortia might lose their degrading ability when cultivated in a nutrient rich medium which offers a wealth of alternative C and N sources apart from the pesticide. LB medium

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supplemented with TBZ (50 mg/l) was prepared and inoculated with a fresh culture of the two TBZ-degrading consortia (MSMN- and MSM-based) as described in Chapter 2. Degradation of TBZ was also measured in the corresponding minimal medium (MSMN or MSM + TBZ, 50 mg/l) for comparative purposes. For each consortium - medium combination triplicate inoculated and duplicate non-inoculated samples were prepared. After inoculation, samples were incubated in an orbital shaker at 180 rpm/26°C. TBZ degradation was measured at 0, 2, 4, 7, and 14 days. At each sampling day the bacterial growth in the LB medium was measured *via* optical density at 600 nm with a spectrophotometer.

2.8.4. Assessment of the degradation capacity of the TBZ-degrading consortia in a range of pH and temperatures

The degrading capacity of the MSMN-based TBZ degrading consortium was evaluated in a range of pH and temperatures to determine its capacity to survive under extreme conditions which might encounter during bioaugmentation of contaminated matrices or under industrial conditions in biofilter systems. Details about the experimental setup are given for all pesticides in Chapter 2.

2.8.5. Assessment of the degradation capacity of the TBZ-degrading consortia in the co-presence of other pesticides and their corresponding pesticide-degrading bacteria

The degrading ability of the isolated bacteria was tested in the presence of *ortho*-phenyl-phenol (OPP) and the corresponding OPP-degrading bacterium (Chapter 5), and diphenylamine (DPA) and the corresponding DPA-degrading bacterium (Chapter 6). Triple mixtures containing TBZ, OPP, DPA and their corresponding degrading bacteria were also tested. It was hypothesized that the co-presence of other pesticides and their corresponding degrading bacteria might have an adverse effect on the degrading capacity of the TBZ-degrading consortia due to possible toxicity exerted by the other pesticides present (OPP, DPA) or *via* competition for nutrients by the other pesticide-degrading microorganisms. TBZ was added in the media by a filter sterilized (0.22 μm, PTFE Syringe Filter, sterilized) DMSO stock solution (30 g/l). The amount of DMSO in the final medium was <0.3%. More details about the experimental setup are given in Chapter 2.

2.8.6. Assessment of the soil bioaugmentation potential of TBZ-degrading consortia

The soil bioaugmentation potential of the TBZ-degrading consortia was tested on an artificially contaminated soil as described in Chapter 2. Soil (200 g) was initially treated with 10 ml of aqueous solutions of TBZ (0.1, 1, and 1 g/l, prepared from the commercial formulation of the fungicide) resulting in three concentration levels: 5, 50, and 500 mg/kg respectively. Both

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MSMN- and MSM-based TBZ-degrading consortia were evaluated. An inoculum of around $2x10^6$ cells/g soil was applied in each soil and the moisture content was adjusted to 40% of the WHC with addition of extra ddH_2O when needed. For every concentration level, three inoculated replicates and two non-inoculated controls were prepared. All samples were separated into subsamples as described in Chapter 2 and incubated at 25^oC in the dark. Immediately after treatment (Time 0) and 3, 7, 10, 15, 20, and 30 days later triplicates from each treatment were removed from the incubator and analysed for TBZ residues as described before.

2.8.7. Assessment of the degradation potential of the TBZ-degrading consortium in Biobed Extract Medium

The ability of the MSMN-based TBZ-degrading consortium to maintain its degradation capacity when inoculated in BEM, a growth medium simulating the composition of an on-farm biobed substrate, was also investigated. This experiment allowed us to assess the capacity of the consortium to degrade TBZ when used to bioaugment biobed systems receiving wastewaters from the fruit-packaging plants [Omirou *et al*., 2012]. A detailed description of the experimental setup for all pesticide-degrading bacteria is given in Chapter 2.

2.8.8. Assessing the survival and maintenance of the degradation capacity of TBZ-degrading bacteria upon incubation at high temperatures simulating composting procedures in biomixtures

The capacity of the MSMN-based TBZ-degrading consortium to survive and maintain its degradation capacity during exposure to a temperature program which resembled the temperature profile of a composting process was assessed. This experiment was conducted in order to investigate if the TBZ-degrading consortia could be used in combination with composting for accelerating the decontamination of spent biobed substrates which have retained high concentrations of TBZ, a possibility which has been observed in previous studies by Omirou *et al*. [2012]. Details on the experimental setup have been given in Chapter 2.

3. Results and Discussion

3.1. Enrichment cultures for the isolation of degrading bacteria

As shown in Figure 3.1 rapid degradation of TBZ was observed from the first enrichment cycle in all three media tested, whereas negligible degradation of TBZ in the non-inoculated controls was observed suggesting that the degradation in the inoculated cultures was purely biotic. The lack of abiotic degradation for TBZ in the aqueous medium is in accordance with the high stability of the fungicide in the water environment [Swann *et al*., 1983].

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In MSM, where TBZ served as both C and N source, from the second enrichment cycle and onwards more than 50% degradation was observed within 5 to 7 days (Figure 3.1a). In MSMN, where TBZ was the sole C source, degradation was slightly faster, with complete TBZ disappearance within 7 days (Figure 3.1b). In SEM, a more nutrient rich but non selective medium, a faster degradation of TBZ was observed in the first two enrichment cycles comparable with the other media, however degradation slowed down thereafter (Figure 3.1c). This could be due to the lower selective pressure exerted by the pesticide in a medium in which other nutritional sources are available. Based on those data it was decided to focus on the isolation of bacteria only from MSM and MSMN cultures.

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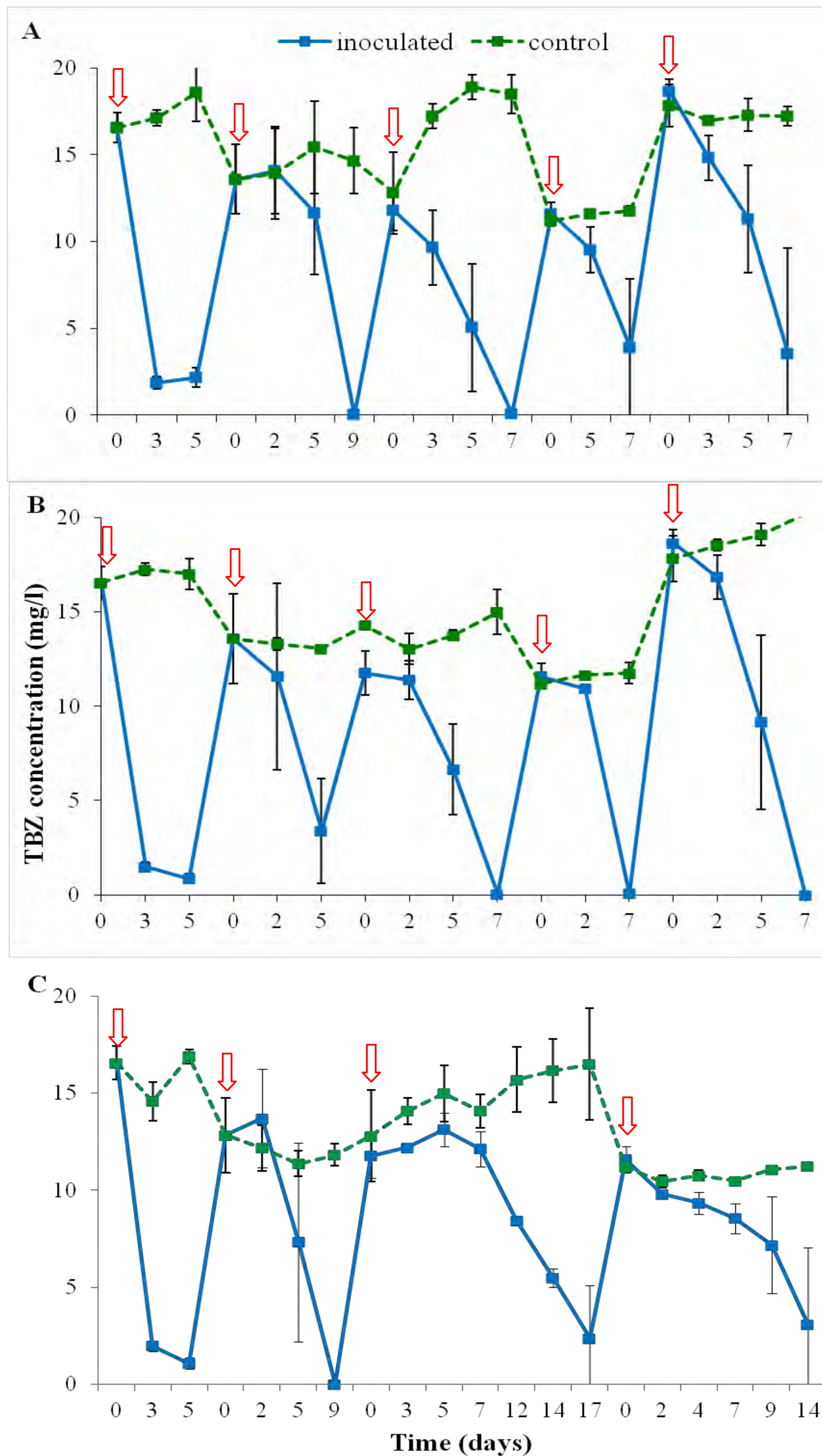

Figure 3.1. TBZ degradation during the enrichment cycles in MSM (A), MSMN (B), and SEM (C). The red arrows indicate the beginning of each enrichment cycle. Each point is the mean of three inoculated or two non-inoculated (control) replicates with error bars representing the standard deviation of the mean.

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3.2. Growing colonies selection and community profiling

Around 90 colonies were randomly selected from MSM and MSMN + TBZ agar plates and screened for TBZ degradation. None of the selected colonies was able to degrade TBZ in 20 days. A molecular fingerprinting approach was then followed to identify the composition of the two enrichment cultures degrading TBZ *via* DGGE which was coupled with clone libraries to allow members identification. The DGGE profile of the bacterial communities in the starting soil and in the first, third, and fifth enrichment cycle were compared (Figure 3.2). The starting soil was the same for both communities. Communities from both media showed high complexity. However, especially in the case of MSMN, the pattern tended to become less complex as the enrichment progressed. It could be noticed that certain bands that were dominant in the soil samples tended to disappear in the fifth enrichment cycle, whereas new bands not initially present in the soil fingerprint became dominant as enrichment progressed. From the two clone libraries developed, the majority of the clones/bands in the MSMN and MSM cultures degrading TBZ showed high sequence homology to uncultured bacterial clones (>98%) (data not shown).

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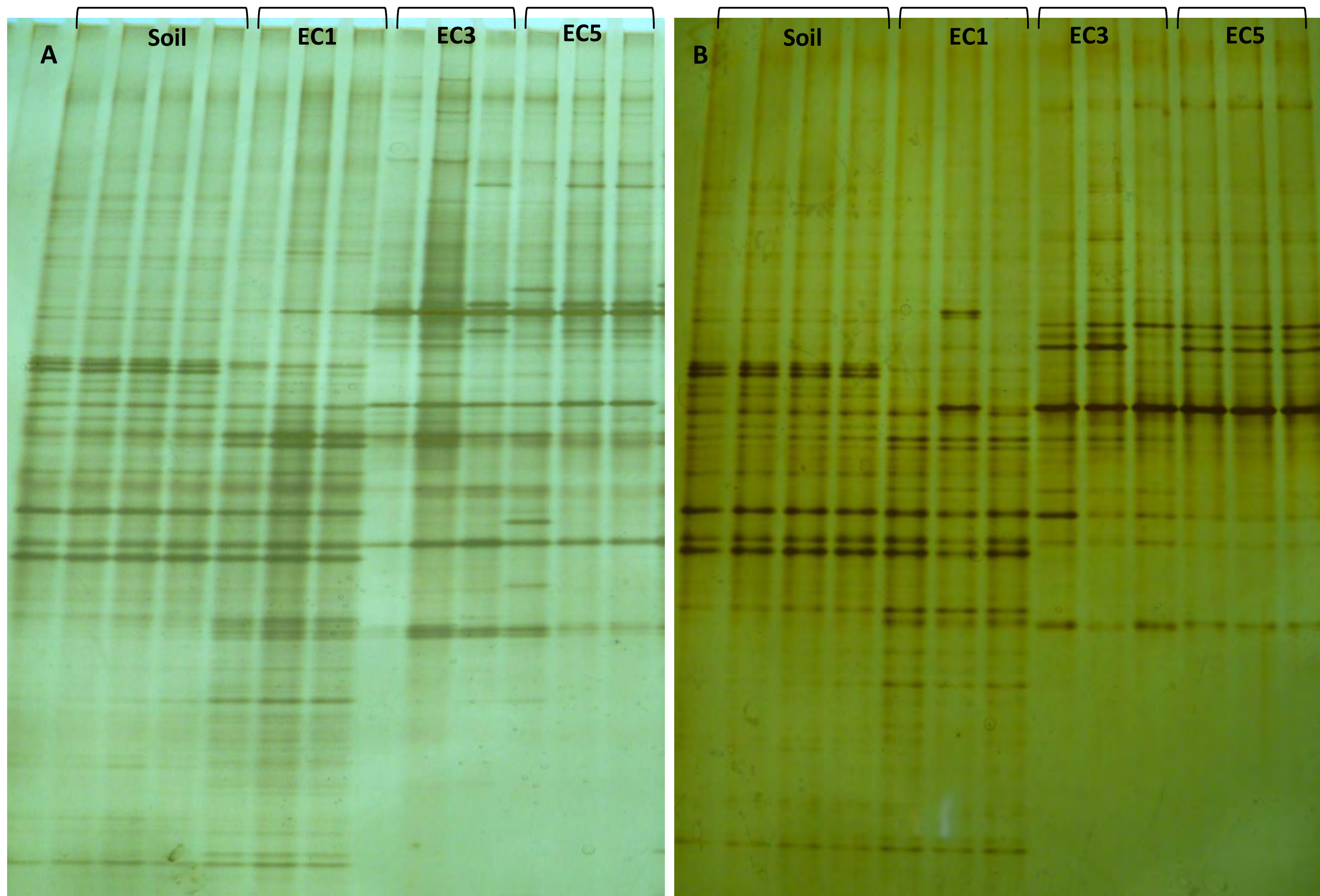

Figure 3.2. The DGGE profile of the bacterial community in the soil used for the isolation of TBZ-degrading bacteria in comparison with the composition of the bacterial community in the first (EC1), third (EC3), and fifth (EC5) enrichment cycle in MSM (A) and MSMN (B).

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3.3. Long-term enrichment

Based on the progressively reduced complexity of the composition of the 5th enrichment cycle in MSM and MSMN, a phase of long-term enrichment consisting of weekly subculturing in fresh media containing TBZ was initiated. It was hypothesized that the continuous selective pressure will gradually eliminate the bacteria which are not directly involved in the degradation of TBZ but probably grow at the expense of contaminants present in the media, allowing to obtain simpler consortia or pure cultures of the bacteria actively involved in TBZ metabolism.

A first effect of the long term enrichment was a progressive increase in the TBZ degradation rates (Figure 3.3). The first refreshed cultures from the fifth enrichment cycle (Figure 3.3a) were completely degrading TBZ in 7 (MSMN) and 14 days (MSM). After an initial 6-month enrichment period of the two TBZ-degrading cultures in MSMN and MSM, faster degrading rates were achieved with complete degradation of TBZ observed in 4 and 6 days respectively (Figure 3.3b). As enrichment was continuing the degradation of TBZ reached the same rates in both consortia with complete degradation observed in 2 days (Figure 3.3c).

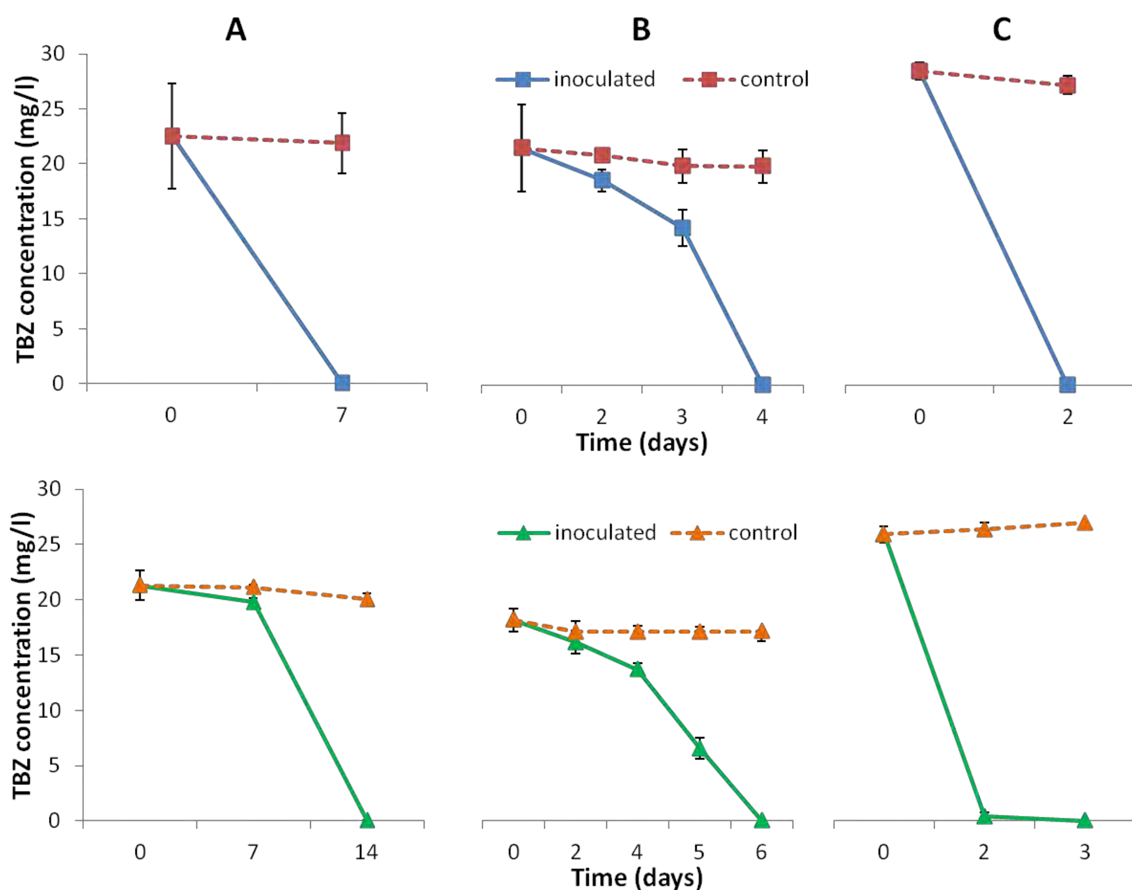

Figure 3.3. The degradation of TBZ by the MSMN- (upper panel) and MSM- (lower panel) based consortia (A): after the fifth enrichment cycle (March 2010); (B): after 6 months of enrichment (September 2010); (C): after 23 months of enrichment (February 2012). Degradation of TBZ in non-inoculated controls is also presented. Each value is the mean of three replicates ± the standard deviation.

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In accordance with the increase in the degradation rates of TBZ, changes in the composition of the TBZ-degrading consortia were shown by DGGE analysis of the bacterial communities at regular intervals (Figure 3.4). In all cases these changes resulted in a gradual simplification of the consortia complexity. This is an expected effect of the enrichment because under a continuous selective pressure the bacteria that can use the pesticide as C and N source, which constituted a small fraction of the original community, progressively become dominant at the expense of other non-degrading members which have no advantage in the selective media. Clone libraries were created to identify the major members of each consortium.

The composition of the starting MSMN-based consortium: In the case of the MSMN-based consortium, the starting community was composed of nine main members (B1, B2, B3, B4, B5, B6, B7, B8, and B10) of which the dominant ones were B1 (not identified), B2 (*Pseudomonas* sp.), B3 (*P. putida*), B4 (*P. putida*), and B8 (*Sphingomonas* sp.) (Lane 1, Figure 3.4). Upon continuous subculturing the consortium became simpler and was composed of four main bands, B12 (*Methylobacillus* sp.), B7 (*P. putida*), X3 (*Bosea*/*Methylibium* sp.), and B13 (*Sphingomonas* sp.) and a few minor ones (Lane 4, Figure 3.4). From there onwards, the composition of the consortium stabilized with no major changes. The effect of the enrichment on the consortium composition was clear: bands B1, B2, B3 that were initially dominant disappeared from the fingerprint of the consortium after prolonged enrichment, while others initially minor bands (e.g. B12, B13, X3) became dominant upon continuous enrichment. The identity of the dominant bands is given in Table 3.10.

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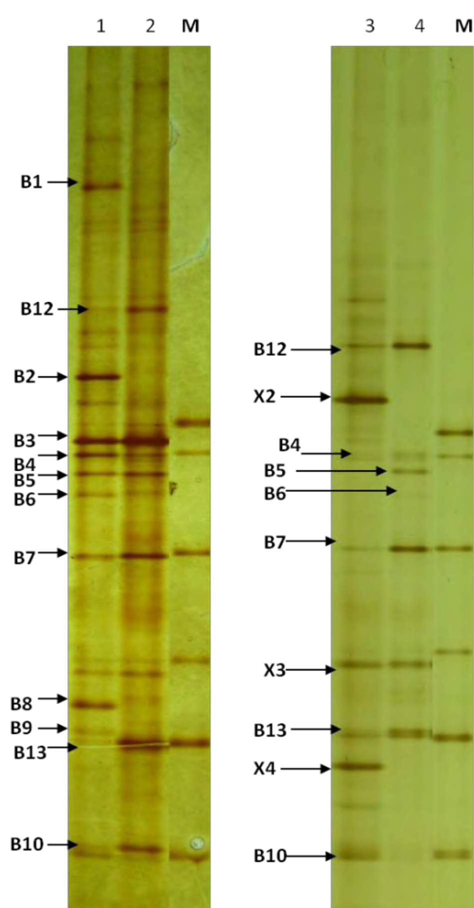

Figure 3.4. The changes in the composition of the MSMN-based TBZ-degrading consortium during prolonged enrichment as determined by DGGE. Major bands are indicated. Lanes 1, 2, 3, and 4: consortium fingerprint in September 2010, November 2010, July 2011, and February 2012 respectively. Lane M: DGGE bacterial marker (for composition see Chapter 2). The presence of the marker allowed the comparison of fingerprints derived from different gels.

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Table 3.10. The identity of the major members of the MSMN-based TBZ degrading consortium determined by clone libraries, DGGE screening, and sequencing. The highest BLAST match based on the homology of the 16S rRNA gene is reported along with % sequence homology and the NCBI accession number of the best match.

Band	Highest match	Homology (%)	Homology (bp)	NCBI Accession number
B2	*Pseudomonas* sp. strain UJ6	99	1012/1016	HM259045.1
B3	*Pseudomonas putida* strain S826	99	999/1013	HM446002.1
B4	*Pseudomonas putida* strain S826	99	1005/1015	HM446002.1
B5	*Pseudomonas* sp. 80GUDO	99	1009/1017	GU911426.1
B6	*Pseudomonas* sp. 80GUDO	99	1003/1016	GU911426.1
B7	*Pseudomonas putida* strain XJ-2	99	1006/1015	HM641753.1
B8	*Sphingomonas* sp. MTR-71	96	951/983	DQ898300.1
B10	*Shinella* sp. LLH-1	99	777/779	FJ548995.1
B12	*Methylobacillus flagellatus* KT	99	838/849	CP000284.1
B13	*Sphingomonas* sp. strain 1-F12	98	833/851	JF309146.1
X2	*Pseudomonas putida* strain PC30	100	847/847	AY918068.1
X3	- *Bosea* sp. R-45681	100	849/849	FR774992.1
	- *Methylibium petroleiphilum* PM1	99	849/850	CP000555.1
X4	*Achromobacter xylosoxidans* strain X96	100	849/849	JN717162.1

Some general considerations on the DGGE technique are needed for understanding the limitations of the method and get a more precise overview of the results. Muyzer *et al.* [1993] suggested that any target DNA that is less than 1% of the total target pool is unlikely to be detected by DGGE. Thus, the appearance of new bands in the community profile, previously undetected, could be attributed to minor members of the bacterial consortium which were initially below the 1% threshold and that, under the selective conditions employed, multiplied thus exceeding the DGGE resolution limit and becoming detectable. On the contrary the disappearance of bacteria bands during enrichment does not necessarily mean that the bacteria died but they might have been reduced in numbers to levels below the limit of detection of the method. This explains the appearance/disappearance of some bands-bacteria in the course of the experiments. A second point is the possible co-migration of DNA molecules of different sequence but same GC content in the DGGE profiles, especially in complex communities. In this case one visible DGGE band could encrypt two or more different ribotypes which becomes apparent when clone libraries are developed and screened.

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The composition of the starting MSM-based consortium: The changes in the composition of the MSM-based TBZ-degrading consortium during the long-term enrichment are shown in Figure 3.5 and the identity of the major bands is listed in Table 3.11. At the beginning of the enrichment (Lane 1) a rather complex consortium composition was observed comprised of a large number of minor bands in the upper part of the gel and five major ones: E1 (not identified), E2 (*Pseudomonas* sp.), E3 (*P. nitroreducens*), E6 (*P. putida*), and E8 (*Sphingomonas* sp.). During the prolonged enrichment, those major bands disappeared (E1, E2, E3) or became minor (E6) and the community became simpler. This was attributed to the progressive disappearance of minor bands which were probably not involved in TBZ degradation. In contrast, new bands like B12, X2, E16 gradually became dominant (Lane 3, Figure 3.5) which might be a first indication of their involvement in the metabolism of TBZ. Since February 2012 (Lane 4, Figure 3.5) no substantial changes in the composition of the bacterial consortium were observed. The main bacterial members of this stabilized consortium were B12/B12b (*Methylobacillus/Aminomonas* sp.), X2 (*P.putida*), E16 (*Bosea/Methylibium* sp.), and E8 (*Sphingomonas* sp.).

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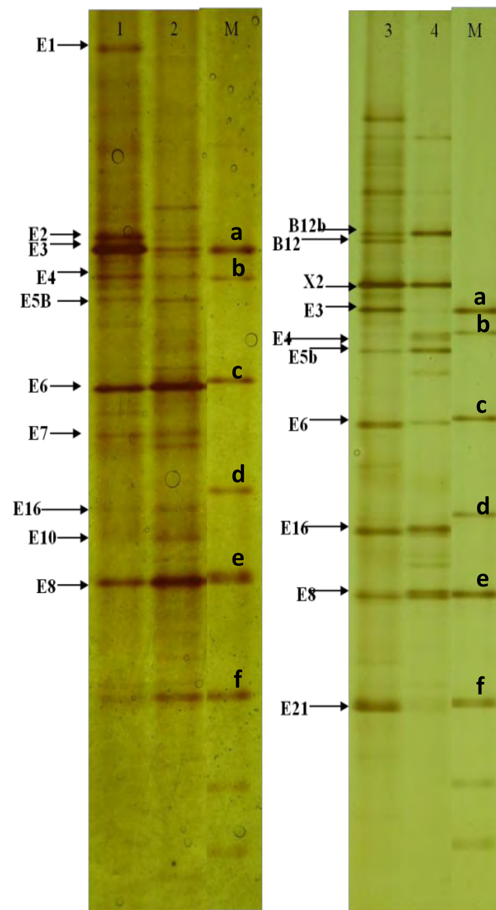

Figure 3.5. The changes in the composition of the MSM-based TBZ-degrading consortium during prolonged enrichment as determined by DGGE. Major bands are indicated. Lanes 1, 2, 3, and 4: consortium fingerprint in September 2010, November 2010, July 2011, and February 2012 respectively. Lane M: DGGE bacterial marker. The bands of the bacterial marker are labelled with letters in order to allow comparison between gels.

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Table 3.11. The identity of the main members of the MSM-based TBZ-degrading consortium. The highest BLAST match based on the homology of the 16S rRNA gene is reported along with % sequence homology and the NCBI accession number of the best match.

Band	Highest match	Homology (%)	Homology (bp)	NCBI Accession number
E2	*Pseudomonas* sp. 418	99	1019/1028	EU841539.1
E3	*Pseudomonas* sp. NBRC 102364	100	850/850	AB681755.1
E4	*Pseudomonas* sp. KT-ql-122	98	1017/1031	FJ611930.1
E5b	*Pseudomonas* sp. SWS3-camc	99	1002/1012	EF151237.1
E6	- *Pseudomonas putida* strain XJ-2	99	907/916	HM641753.1
	- *Hydrocarboniphaga effusa* strain AP103	100	850/850	AY363245.1
E7	*Stenotrophomonas* sp. 2000-18	96	968/998	FJ959380.1
E8	*Sphingomonas* sp. 1-F12	98	834/849	JF309146.1
E10	*Alishewanella* sp. 205	97	978/1005	EU841485.1
B12	*Methylomonas* sp. clone XN1	98	769/788	GU577112.1
B12b	*Aminomonas aminovorus* C2A1	98	863/879	AY027801.1
E16	- *Methylibium petroleiphilum* PM1	100	859/859	CP000555.1
	- *Bosea* sp. R-45681	96	808/839	FR774992.1
E21	*Oligotropha carboxidovorans*	99	829/830	AB099660.1
X2	*Pseudomonas* sp. BM-2(2011)	100	859/859	JN695700.1

Overall the long-term enrichment resulted in the formation of simpler TBZ-degrading consortia in both MSMN and MSM. Despite that, many bands/bacterial members were still present in the TBZ-degrading consortia precluding the determination of the role that each bacteria has in the consortium and mainly the identification of the bacterium responsible for TBZ degradation.

In Figure 3.6 the DGGE profiles of the two TBZ-degrading consortia (MSMN and MSM-based) obtained in February 2012 are compared. The two profiles were very similar, with most of the major bands being present in both consortia. In particular, all bands of the MSMN consortium had a correspondent band in the profile of the MSM consortium. Among the major bands only X2 in MSM did not show a match with a band in MSMN. Moreover, alignment of the sequences of the 16S rRNA gene of the ribotypes with identical electrophoretic mobility in the two consortia showed identical sequences indicating that the two consortia shared common members. Thus, the band B12 in the MSMN-based consortium had identical sequence with the ribotype of band B12/B12b in the MSM-based consortium (*Aminomonas* is now considered as an uncharacterized bacterium placed among *Methylobacillus* species [Hu *et al.*, 2009]). Similarly *P.*

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putida ribotype represented by bands B7 (MSMN) and E6 (MSM), *Bosea* and *Methylibium* sp. ribotypes represented by bands X3 (MSMN) and E16 (MSM), and *Sphingomonas* sp. ribotypes represented by bands B13 (MSMN) and E8 (MSM) showed identical sequences (Table 3.12).

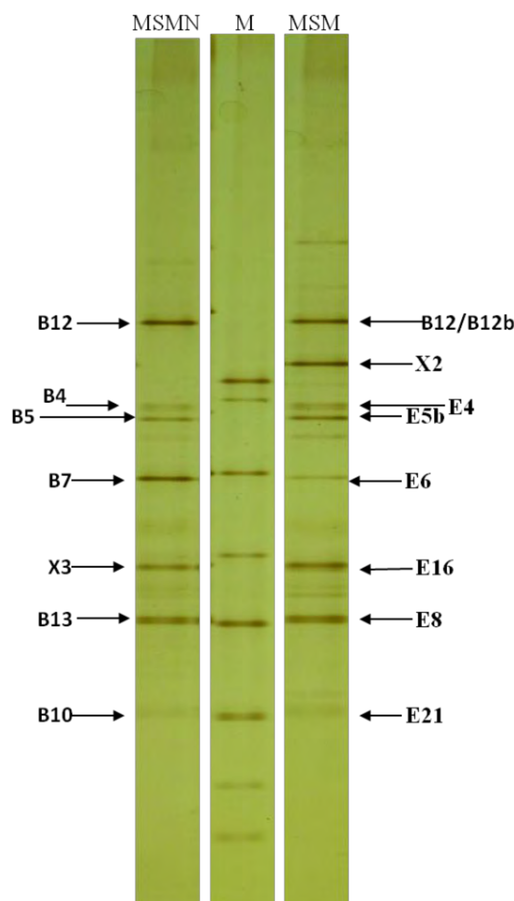

Figure 3.6. Comparison of the DGGE profile of the MSMN- and MSM-based TBZ-degrading consortia obtained after prolonged enrichment (February 2012). Lane M: DGGE bacterial marker.

Table 3.12. The identity of the major bands in the DGGE profile of the MSMN- and MSM-based TBZ-degrading consortia obtained after prolonged enrichment (February 2012). The bacteria which are common in the two consortia based on identical 16S rRNA sequences are indicated with red.

MSMN-based TBZ-degrading consortium		**MSM**-based TBZ-degrading consortium	
Identity	Band No.	Band No.	Identity
Methylobacillus sp.	B12	B12/B12b	*Methylobacillus/Aminomonas*
		X2	*Pseudomonas putida*
P. putida	B4	E4	*Pseudomonas* sp.
Pseudomonas alcaligenes	B5	E5b	*Pseudomonas* sp.
P. putida	B7	E6	*P. putida* / *Hydrocarboniphaga* sp.
Bosea sp. / *Methilibium* sp.	X3	E16	*Bosea* sp. / *Methilibium* sp.
Sphingomonas sp.	B13	E8	*Sphingomonas* sp.
Shinella sp.	B10	E21	*Oligotropha* sp.

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The fact that the two TBZ-degrading consortia which were obtained from the same soil but after independent and prolonged enrichment with TBZ as the sole source of C or C/N showed a similar composition (regarding their major members), combined with the fact that no single isolates were able to metabolize the pesticide provided a first indication of possible synergistic action in the degradation of TBZ. Several previous studies have demonstrated the isolation of bacterial consortia able to degrade different pesticides, and in several cases the role of the different members was elucidated [Dejonghe *et al*., 2003; Smith *et al*., 2005].

A *Methylobacillus* ribotype was present in both consortia (band B12). Members of this genus are known as obligate methylotrophs growing on C1 compounds like methane, methanol, and methylated amines [Yordy and Weaver, 1977; Chistoserdova *et al*., 2007; Bratina *et al*., 1992]. *Aminomonas aminovorus* which was identified as a member of the MSM consortium (band B12b) is an unclassified β-proteobacterium with no cultured representative which has been inserted in the *Methylobacillus* obligate methylotroph group [Ferrari *et al*., 2005; Ginige *et al*., 2004; Taylor *et al*., 2004]. These two bacteria could have a putative role in the utilization of metabolites like methylamine possibly produced during the degradation of TBZ.

A *Methylibium* ribotype was also present in both consortia (bands X3 in MSMN and E16 in MSM). Members of this genus are classified as facultative methylotrophs since they could use methane as a carbon source but they are also able to grow heterotrophically in the presence of a wide range of C substrates including ethanol, toluene, benzene, ethylbenzene, phenol, and dihydroxybenzoates [Nakatsu *et al*., 2006; Kane *et al*., 2007]. The same bands X3 in MSMN and E16 in MSM encrypted the same *Bosea* ribotype. The original description of the genus *Bosea* included thiosulphate oxidation as a phenotypic feature [Das *et al*., 1996]. However subsequent strains assigned to this genus did not appear to possess this phenotype, hence it has to be considered characteristic of *B. thiooxidans* and not of all members of the genus *Bosea* [La Scola *et al*., 2003]. This bacterium could have putative role in thiazole metabolism although this should be further investigated.

Band E6 in the MSM-based consortium showed highest match to a *Hydrocarboniphaga effusa* strain that is the only known species of this genus. Different *Hydrocarboniphaga* strains isolated from different laboratories have demonstrated degrading activity towards alkane and aromatic hydrocarbons [Palleroni *et al*., 2004; Chang *et al*., 2012], suggesting that this ability could be an intrinsic characteristic of the species.

A *Shinella* ribotype (band B10) was present in the MSMN-based consortium. Members of this genus have been shown to metabolize pyridine [Bai *et al*., 2009], nicotine [Ma *et al*., 2013], sulfolane [Matsui *et al*., 2009], and were able to reduce nitrate [Lee *et al*., 2011]. However its possible role in the degradation of TBZ was not obvious.

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Band E21 in the MSM consortium (having similar electrophoretic mobility with band B10 from the MSMN consortium) showed highest homology with an *Oligotropha carboxidovorans* strain. This is the sole species of the genus *Oligotropha*, a chemolithoautotrophic bacterium with the capability to utilize CO, CO_2, and hydrogen as energy source [Debarati *et al*., 2008; Santiago and Meyer, 1996].

In general the upper part of the DGGE gel in both TBZ-degrading consortia was dominated by *Pseudomonas* ribotypes. Numerous studies in the literature have reported the isolation of *Pseudomonas* spp. possessing the ability to degrade aliphatic, aromatic, and/or polyaromatic hydrocarbons [Ting *et al*., 2009; Obayori *et al*., 2009; Zylstra *et al*., 1988; Ma *et al*., 2012]. In addition, several studies have also reported the degradation of different pesticides by *Pseudomonas* strains [Siva and Rajam, 2013; Vijayalakshmi and Usha, 2012; Cain and Mitchell, 1996].

A *Sphingomonas* ribotype (band B13 in the MSMN-based consortium and E8 in the MSM-based consortium) was present in both TBZ-degrading consortia. *Sphingomonas*, similarly to *Pseudomonas*, appears to be involved in the degradation of several different organic pollutants like alogenated-diphenyl ethers, dibenzofuran, polyethylene glycol, toluene, naphthalene, o-xylene, m-xylene, p-xylene, p-cresol, salicylate, and benzoate [White *et al*., 1996]. Degradation of pesticides like carbofuran [Kim *et al*., 2004], diuron [Sørensen *et al*., 2013], and isoproturon [Sørensen *et al*., 2001] by *Sphingomonas* isolates has been also described.

The dominance of *Pseudomonas* and *Sphingomonas* ribotypes in the TBZ-degrading cultures might be a reflection of the prolonged enrichment procedure followed. In fact those two proteobacterial genera encompass copiotrophic bacteria which exhibit fastidious growth under enrichment conditions and could dominate over slow growing bacteria which might drive the degradation of organic pollutant *in situ* [Bastiaens *et al*., 2000; Johnsen *et al*., 2002].

3.4. Isolation of TBZ-degrading bacteria in pure culture

Based on the progressive simplification of the composition of the TBZ-degrading consortia, different attempts were made to isolate *via* plating the bacteria responsible for the degradation of TBZ. In all cases no single colonies able to degrade TBZ were obtained (data not shown). Thus, the total bacterial community that grew on MSMN plates was collected and transferred into the corresponding liquid medium to test its degrading capacity. However no degradation was observed. DGGE fingerprinting analysis of the bacterial community growing on the selective agar plate was compared and contrasted with the fingerprint of the actively degrading consortium in liquid culture. As evident in Figure 3.7, the two communities were largely different. The only exception was band B7 (representing a *P. putida* ribotype) which was present in both

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fingerprints. No other band of the profile of the consortium obtained from the liquid culture (except from B7-*P. putida*) was present in the fingerprint of the bacterial community scrapped off from the plate. These results suggest that the major members of the MSMN-based TBZ-degrading consortium were not growing on the corresponding agar plates. Instead, novel bacterial members that were not visible on the fingerprint of the degrading consortium in the liquid culture grew effectively on the plates.

A clone library was constructed to identify the bacteria which were growing on the MSM and MSMN agar plates. Sequencing of the clones obtained showed that the community in the agar plates was dominated by the same bacteria belonging to *Citrobacter*, *Variovorax*, and *Pseudomonas* (Table 3.13). These results suggested that the failure to isolate pure TBZ-degrading bacteria *via* plating could be the result of the inability of the major members of the TBZ-degrading consortia to grow on agar. This is not surprising considering that only 1-5% of the microorganisms in the ecosystem can be cultivated in currently known media [Torsvik and Øvreås, 2002]. The only member of the TBZ-degrading consortia (as appearing in the liquid culture) which grew in agar plates was *P. putida*-band B7. However when tested in pure culture it was not able to degrade TBZ suggesting that this bacterium does not have a leading role in the degradation of TBZ in the two consortia and its function could be supportive. Because of the limited cultivability of most of the major members of the TBZ-degrading consortia, alternative ways were explored in order to isolate the bacteria responsible for the rapid degradation of the parent compound and to elucidate the role of the other bacterial members in the two consortia.

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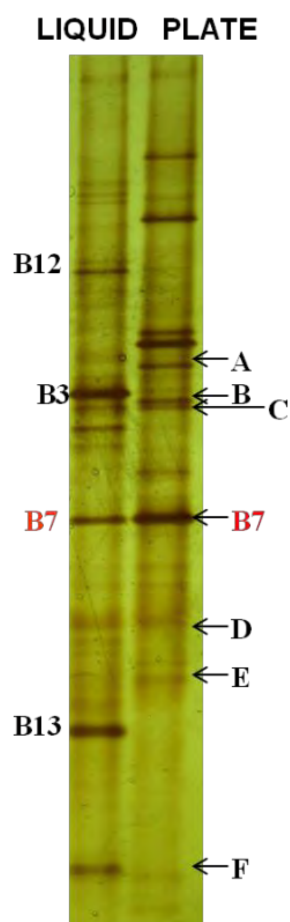

Figure 3.7. The DGGE profile of the MSMN-based TBZ-degrading consortium growing in liquid culture (left) and on MSMN+TBZ agar plates (right). Major bands in the liquid culture growing consortium (for identification see Table 3.9) and in the plates growing consortium (for identification see Table 3.13) are indicated. Band B7 is marked in red since it was the only major band shared by the two communities.

Table 3.13. The identity of the main bands in the DGGE fingerprint of the consortium growing on MSMN+TBZ agar plates. The highest BLAST match based on the homology of the 16S rRNA gene is reported along with % of sequence homology and the NCBI accession number of the best match

Band	Highest match	Homology (bp)	NCBI accession number
A	*Pseudomonas nitroreducens* strain ES-22	733/735 (99%)	JF513159.1
B	*Variovorax paradoxus*	803/804 (99%)	AB680785.1
C	*Pseudomonas stutzeri* strain 3b	691/691 (99%)	JF422069.1
B7	*Pseudomonas putida*	751/754 (99%)	AB680123.1
D	*Citrobacter* sp. S-77	866/872 (99%)	AB668058.1
E	*Citrobacter amalonaticus*	786/789 (99%)	FR870441.1
F	Uncultured *Citrobacter* sp. clone F1Sjun.22	690/694 (99%)	GQ417185.1

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3.5. DNA/cDNA DGGE profile comparison

DNA-based DGGE profiles provide information on the bacteria present in the consortium, but it is difficult to determine if the recovered gene marker (16S rRNA gene) is derived from living or dead cells and if the detected microorganisms are active or dormant [Kielak, 2010; Ogram *et al*., 1987; Coolen and Overmann, 1998]. This limitation can be partially overcome by targeting RNA instead of DNA. In fact, rRNA levels have been taken as a reliable marker of cellular activity [Buckley and Schmidt, 2003; Rosset *et al*., 1966; Kramer and Singleton, 1993; Lee and Kemp, 1994]. Thus, it has been argued that analysis of sequences derived from RNA rather than DNA templates could provide a more representative picture of the active members of a given bacterial community [Wagner, 1994; Pichard and Paul, 1993; Teske *et al*., 1996]. So, it was hypothesized that the DNA-DGGE profiles of the TBZ-degrading consortia show the bacterial members which are just present (regardless of their physiological status), while RNA-DGGE profiles will indicate the predominantly active members of the consortia. Different studies used this approach to obtain information on the bacterial structure and activity [Duineveld *et al*., 2001; Girvan *et al*., 2003; Nogales *et al*., 2001]. Thus cDNA-DGGE bands would indicate bacteria with high transcriptional activity suggesting possible involvement in TBZ degradation.

Based on this, analysis of the composition of the two TBZ-degrading consortia by DNA and cDNA (RNA)-DGGE was performed. In both media TBZ dissipated gradually with a faster degradation rate observed in MSMN (Figure 3.8). The DNA and cDNA-DGGE profiles of the MSMN community are shown in Figure 3.9. Differences in the presence and intensity of DGGE bands in the fingerprints of the MSMN consortium obtained by DNA and cDNA could be seen. On this basis, speculations for the role of the major bacterial members of the consortium could be made. *P. putida* represented by band X2 was present in both DNA and cDNA fingerprints throughout the experiment which suggests a potential role in the degradation of TBZ although this should be clarified further. *P. putida* represented by band B7 showed a similar pattern. However previous results from plate isolation and degradation assays suggested that this bacterium is not involved, at least in the first step, of the metabolism of TBZ (see Section 3.4). *Achromobacter* sp., represented by band X4 did not seem to contribute to TBZ degradation since its band was not apparent in the cDNA fingerprints throughout the study. *Bosea/Methylibium* sp. represented by band X3 and *Shinella* sp. represented by band B10 showed to be active right from the beginning of TBZ degradation, with evident bands in the cDNA profile, but rather faint bands in the DNA profile. Their activity increased progressively as it is evident by the increasing intensity of their bands in both cDNA and DNA profiles from 7 days onwards in the case of band X3 and from day 4 onwards in the case of band B10. Interestingly, *Sphingomonas* sp. represented by band B13 became visible only at day 4, when the degradation of TBZ

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commenced (Figure 3.8) and remained apparent in both fingerprints until the end of the incubation.

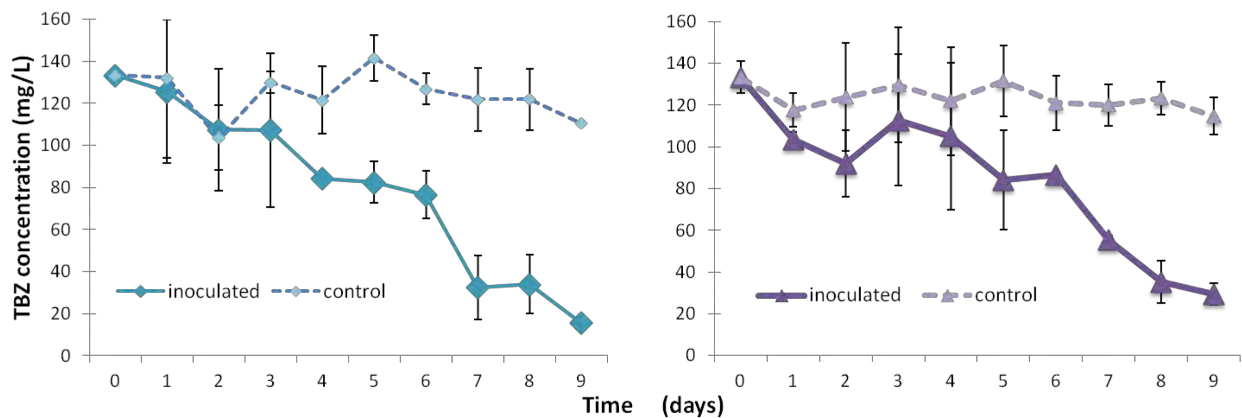

Figure 3.8. The degradation of TBZ in MSMN (left) and MSM (right) inoculated or non inoculated (control) with the respective consortia. Error bars represent the standard deviation

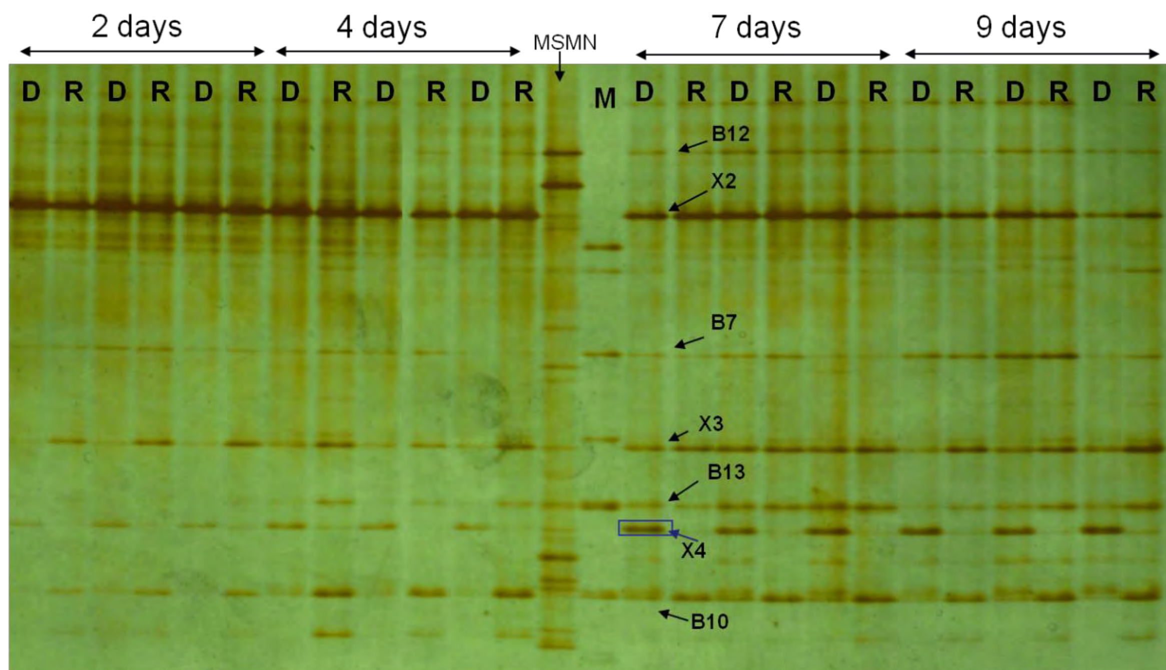

Figure 3.9. DNA (D) and cDNA (R) DGGE profiles of the MSMN consortium obtained at 2, 4, 7, and 9 days after inoculation. Three replicates per sampling day are presented. Lane M: bacterial DGGE marker. All major bands are labeled. For bands identification see Table 3.10.

The DNA and cDNA fingerprints of the MSM-based TBZ-degrading community are shown in Figure 3.10. *Methylobacillus* represented by band B12, *P. putida* represented by band X2, *Pseudomonas* sp., represented by band E3, and *P. putida/Hydrocarboniphaga* sp., represented by band E6 did not manifest differences in their presence and intensity between

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DNA and cDNA patterns during TBZ degradation. This provides a first indication that those bacteria were not directly involved in TBZ metabolism. An uncultured unclassified α-proteobacterium represented by band Z1 showed a high band intensity in the cDNA fingerprints during the first days of TBZ degradation (4 and 7 days), compared to their DNA fingerprints indicating a putative role in the degradation of TBZ. *Oligotropha* sp., represented by band E21 was equally present in the two DGGE profiles. *Sphingomonas* sp., represented by band E8 showed the same banding pattern as *Sphingomonas* sp. B13 (in MSMN) and this is not surprising considering that these two bands belong to the same microorganism which is present in both TBZ-degrading consortia. Overall, these results provide preliminary evidence for the putative involvement of more than one bacterium in TBZ degradation with *Sphingomonas* sp. B13/E8 being the most prominent.

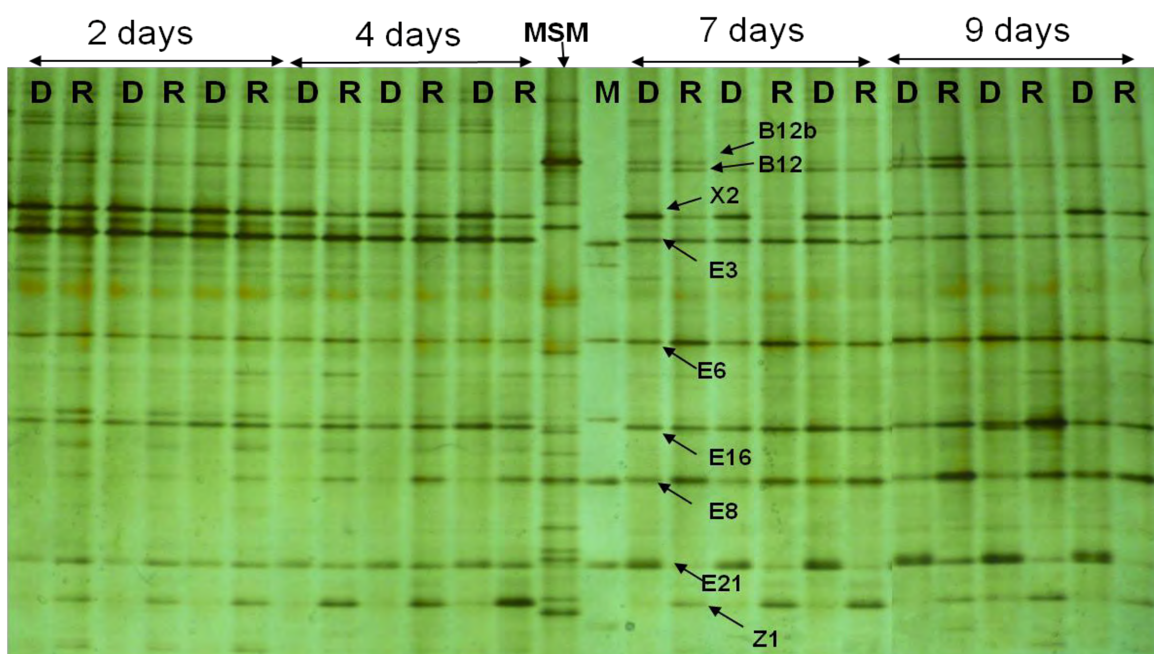

Figure 3.10. DNA (D) and cDNA (R) DGGE profiles of the MSM consortium obtained at 2, 4, 7, and 9 days after inoculation. Three replicates per sampling day are presented. Lane M: DGGE bacterial marker. All major bands are labeled. For bands identification see Table 3.11. Z1 (not listed in the Table) showed highest match (>98%) to an "uncultured unclassified α-proteobacterium".

3.6. Identification of the role of the different members of the TBZ-degrading consortia *via* q-PCR

In order to further verify the preliminary findings obtained by fingerprinting analysis (described above) regarding the role of different members of the consortia on the degradation of TBZ, a group-specific q-PCR assay was set up. It was supposed that it would allow us to determine the growth dynamics of the individual members of the two consortia during TBZ degradation. Q-

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PCR has gained popularity as a technique to detect and quantify a specific group of target microorganisms from various environmental samples [Lim *et al*., 2011]. This technique has been also used in medical studies in which 16S rDNA -targeted species- and group-specific primers were selected for accurate q-PCR identification and quantification of bacteria from fecal samples [Matsuki *et al*., 2004; Rinttilä *et al*., 2004] or from gastrointestinal mucosa [Huijsdens *et al*., 2002]. The hypothesis tested was that if a member of the consortium is involved in the degradation of TBZ it is expected to actively proliferate thus increasing the abundance of its 16S rRNA gene copies in absolute and also in relative (to the total bacteria) numbers.

The primers designed for specific amplification of the 16S rRNA sequences of the different bacterial members of the two consortia showed high specificity as depicted from both *in silico* and *in vivo* tests. In fact, for each primer set, all the transformed colonies that were randomly screened presented high homology to the target 16S rRNA gene after sequencing and BLAST analysis (Table 3.14).

Table 3.14. *In vivo* testing of the specificity of the group-specific primer sets designed to amplify *via* q-PCR the 16S rRNA of the different bacteria present in the TBZ-degrading consortia. The identity of the randomly selected clones for each primer set is reported. The BLAST highest match is presented along with the % of sequence homology and the NCBI accession number of the best match.

Target Group	Highest match	Homology (bp)	NCBI Accession number
Methylobacillus sp. (MSMN and MSM)	*Methylobacillus* sp. LF-1	218/221 (99%)	FR691489.2
Methylibium sp. (MSMN and MSM)	*Methylibium* sp. BAC115	203/203 (100%)	JN125621.1
Achromobacter sp. (MSMN)	*Achromobacter* sp. T-19	205/205 (100%)	JX130404.1
Bosea, Sphingomonas, Shinella, Oligotropha (α-proteobacteria) (MSMN and MSM)	*Shinella granuli* strain R1-702 *Sphingomonas* sp. TES7	201/201 (100%) 211/216 (98%)	JQ659575.1 AY466432.1
Pseudomonas spp. (MSMN)	*Pseudomonas putida* strain zol-13 *Pseudomonas stutzeri* strain KJ-W22	226/226 (100%) 225/225 (100%)	JQ968690.1 JQ799118.1
Pseudomonas spp. (MSM)	*Pseudomonas mendocina* TSM4 *Pseudomonas putida* strain P090	174/174 (100%)	JX025167.1 EF011017.1
Hydrocarboniphaga sp. (MSM)	*Hydrocarboniphaga effusa* P103	205/205 (100%)	NR_029102.1

TBZ was completely degraded within 96 hours in both media (Figure 3.11). In every group-specific q-PCR run, the final melting curve had a single peak, confirming the production of only one PCR product. In the total bacteria q-PCR run each final melting curve had a single

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peak, but a shift on the right was observed for the calibration curve peaks due to the different origin of the plasmid insert. Agarose gel electrophoresis confirmed the amplification of a single product of the expected size (Annex I, Figure A.13). The dissociation, amplification, and standard curves of the different q-PCR assays performed are shown in Annex I, whereas the upper and lower detection limits of the standard curves, r^2 value, and the amplification efficiency for each q-PCR assay developed are shown Table 3.15.

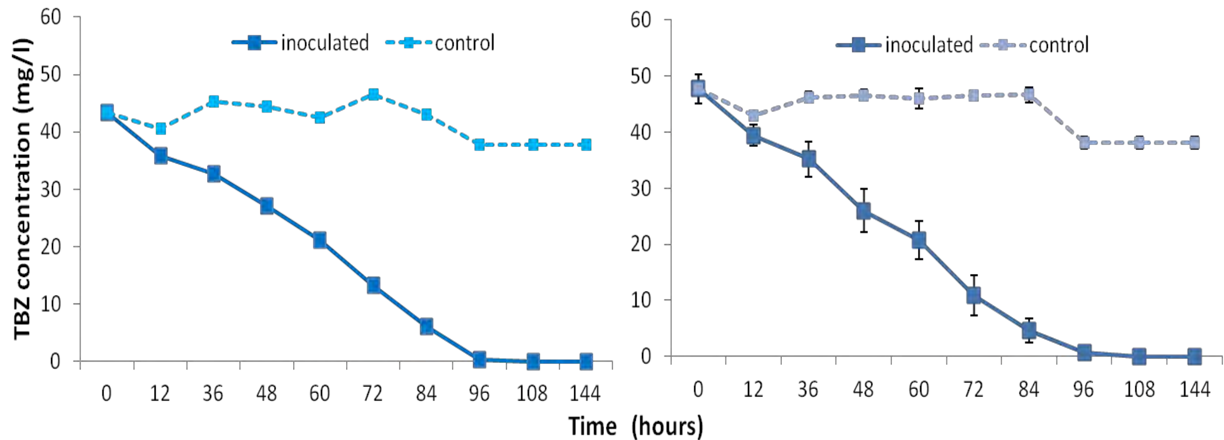

Figure 3.11. The degradation of TBZ in MSMN (left) and MSM (right). The degradation of TBZ in non-inoculated cultures (control) is also presented. Each value is the mean of three replicates ± the standard deviation of the mean.

Table 3.15. The standard curves limits, r^2 values, and amplification efficiency for each q-PCR assay run.

Target bacterial group	Calibration curve limits (copies/µl)	r^2	Amplification efficiency (%)
Achromobacter (MSMN)	$1.2x10^3$-$1.2x10^9$	0.994	113.6
Methylibium (MSMN)	10^3- 10^9	0.999	100.5
Methylobacillus (MSMN)	$5.8x10^4$- $5.8x10^8$	0.997	112.9
A-proteobacteria (MSMN)	$5.2x10^2$- $5.2x10^8$	0.998	104.6
Pseudomonas (MSMN)	$9x10^2$- $9x10^8$	0.994	114.3
Hydrocarboniphaga (MSM)	10^3- 10^9	0.997	99.7
Methylibium (MSM)	10^3- 10^9	0.997	98.4
Methylobacillus (MSM)	$5.8x10^2$- $5.8x10^8$	0.996	103.6
A-proteobacteria (MSM)	$8x10^3$- $8x10^8$	0.992	108.5
Pseudomonas (MSM)	$7x10^2$- $7x10^8$	0.998	110.2
Total bacteria MSMN	$3.6x10^2$- $3.6x10^9$	0.999	105.4
Total bacteria MSM	$3.6x10^3$- $3.6x10^9$	0.999	96.8

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The number of copies of the 16S rRNA gene of the different members of the TBZ-degrading consortia was considered as a measure of the population of the specific bacteria. Statistical analysis of the q-PCR data obtained suggested no significant changes in the population of *Achromobacter* sp. X4 in MSMN (Figure 3.12A), *Hydrocarboniphaga* sp. E6 in MSM (Figure 3.12B), and *Methylibium* sp. X3-E16 in both media (Figure 3.12C and D) in the presence *vs* in the absence of TBZ. These results suggest no involvement of those bacteria in the degradation of TBZ. The same trend was evident when the results were presented relatively to the total bacterial numbers (Figure 3.13) as relative abundances (Figure 3.14A, B, C, D). The population of *Methylobacillus/Aminomonas* sp. B12 in MSM showed a significant increase in the presence *vs* in the absence of TBZ at the later stages of the experiment and certainly after TBZ was completely degraded (108 hours) (Figure 3.12F). The same trend was evident when the results were presented as relative abundance of these bacteria (Figure 3.14F). However no such pattern was evident for *Methylobacillus* in MSMN (Figure 3.12E and 3.14E). The different response in the two growth media might be attributed to the activity of the *Aminomonas* sp. that was present only in the MSM consortium. The late increase in the population of *Methylobacillus/Aminomonas* sp. B12 could be an indication of their involvement in the utilization of simpler metabolic products produced at the later stages of TBZ metabolism. This is conducive with the methylotrophic nature of those bacteria that are known to grow on C1-molecules [Ferrari *et al*., 2005; Chistoserdova *et al*., 2007] like methylamine which might be produced during transformations of TBZ. This hypothesis might explain the different response of B12 in the two media; the lack of extra N sources in MSM might have induced the late increase in the population of those bacteria since they were using C1 compounds produced during metabolism of TBZ. Whereas in the presence of extra N source it is possible that this pathway might not be functional and thus methylamine or other C1 acting as C and N sources are not produced preventing the later proliferation of those bacteria.

Q-PCR assays for α-proteobacteria and *Pseudomonas* in the two consortia showed a significantly increase in their population in the presence *vs* in the absence of TBZ only at 36 h after inoculation. This increase coincided with the active degradation phase of TBZ (Figure 3.11). However, this difference was statistically significant only in the MSMN consortium (Figure 3.12G and I). Although the two consortia were composed of the same bacteria with only slight differences, the common members of the two consortia, especially α-proteobacteria and Pseudomonads appeared to respond differently upon exposure to TBZ. The nutritional differences of the two minimal media (extra N source in MSMN whereas TBZ acted as both C and N source in MSM) could be a reason for the different response of the common bacterial members of the two consortia during TBZ degradation. However, the not significant different

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increase of the specific bacterial populations relatively to the total population in all cases (Figure 3.14G, H, I, L), did not allow to conclude positively about the role of the different bacterial members and further investigations were needed.

Overall, this experiment provided only indications regarding the role of certain bacteria or bacterial groups in the degradation of TBZ. The complex composition of the two consortia might have been the reason for our failure to get conclusive evidence for the role of the different bacterial members of the TBZ-degrading consortia. Similar studies with simpler TBZ-degrading consortia might shed light into the role of individual α-proteobacteria or Pseudomonads which were responsive during degradation of TBZ.

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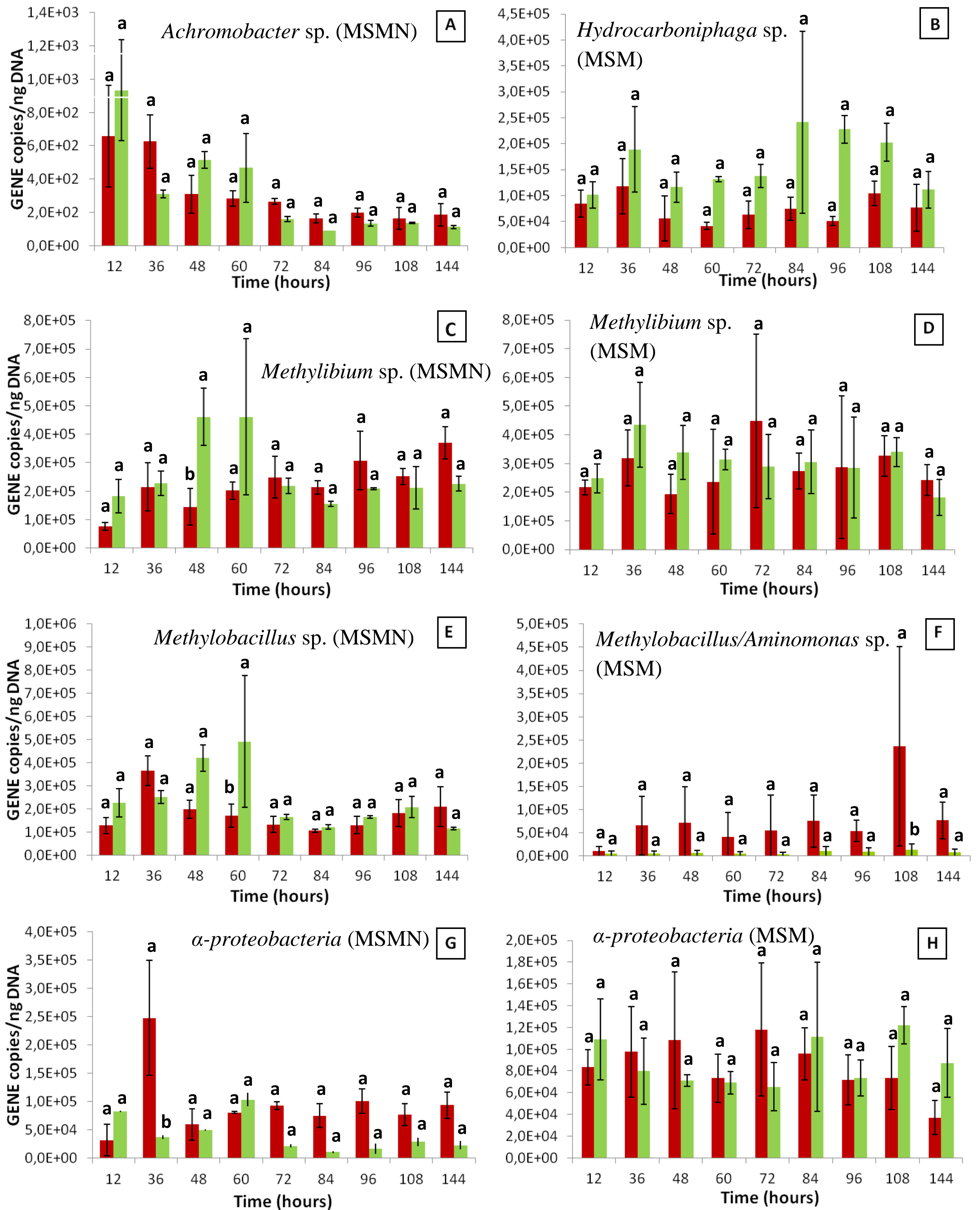

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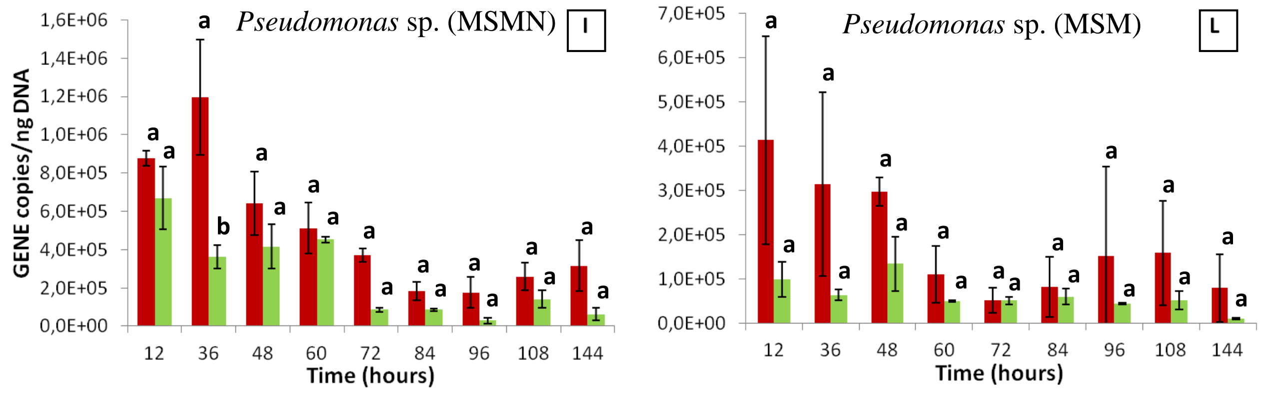

Figure 3.12. The dynamics of the population of the different members of the two TBZ-degrading consortia (expressed as copy numbers of their 16S rRNA gene) when growing in the presence (red bars) or in the absence (green bars) of TBZ. Within each time point bars designated with the same letter are not significantly different at 5% level.

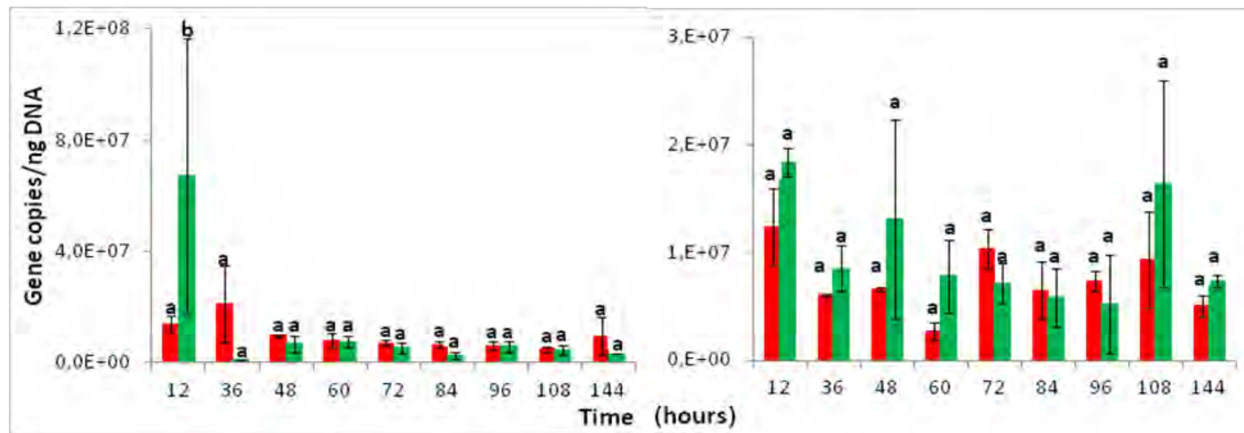

Figure 3.13. The population dynamics of the total bacteria (expressed as copy numbers of the 16S rRNA gene) in the MSMN (left) and MSM (right) degrading consortia estimated *via* q-PCR in the presence (red) or in the absence (green) of TBZ. Within each time point bars designated with the same letter are not significantly different at 5% level.

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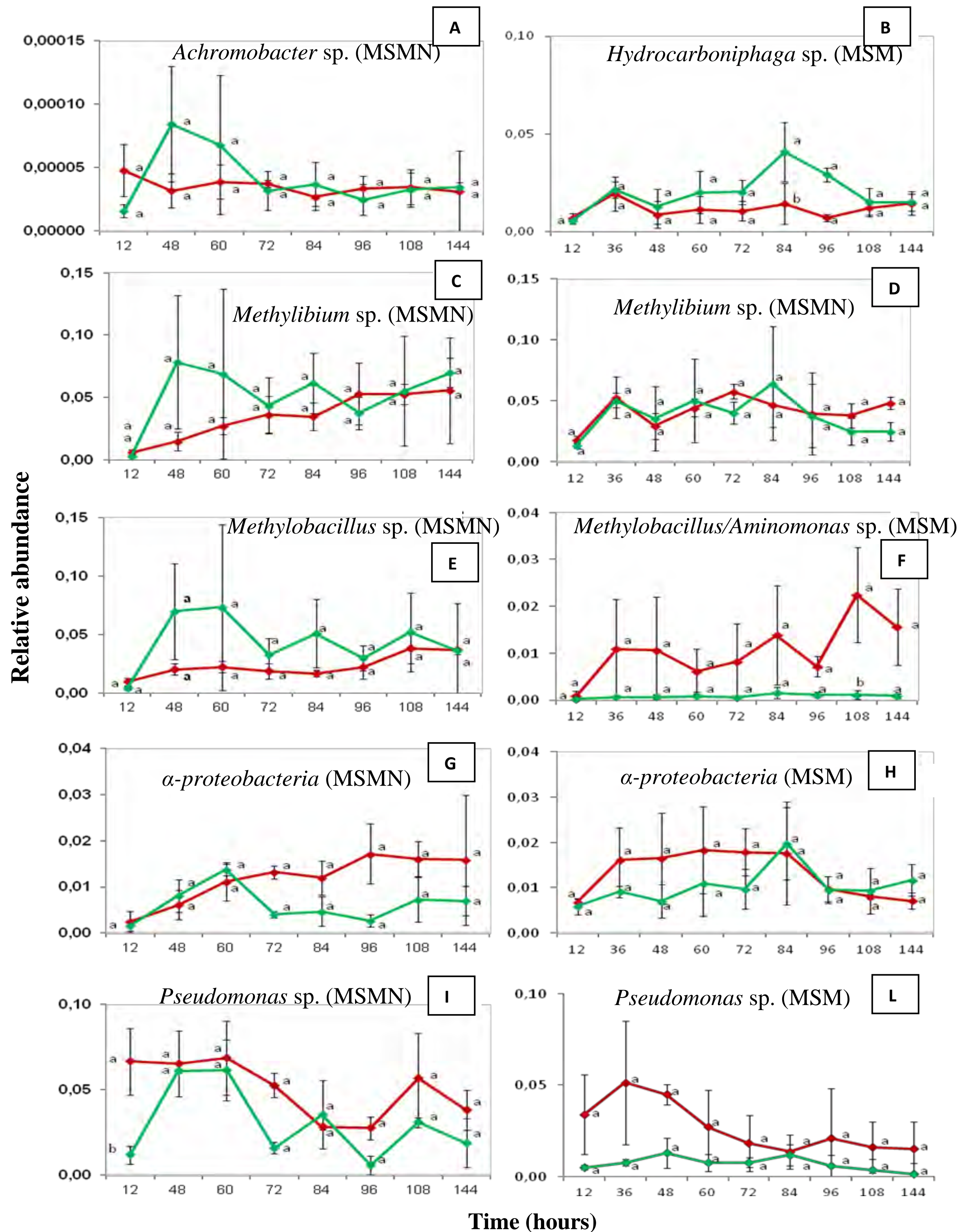

Figure 3.14. Changes in the relative abundance (number of copies of the 16S rRNA gene obtained by group-specific qPCR assay/number of copies of the 16S rRNA gene obtained by qPCR assay for total bacteria) of the different members of the two TBZ-degrading consortia when grown in the presence (red lines) or in the absence of TBZ (green lines). Within each time point same letter indicates no significant difference at 5% level.

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3.7. Effect of different antibiotics on the degrading capacity and composition of the TBZ-degrading consortia

3.7.1. The effect of antibiotics on the degrading capacity of the TBZ-degrading consortia

Based on the outcome of the q-PCR experiment which provided preliminary evidence that α-proteobacteria and *Pseudomonas* might play a role in the degradation of TBZ, a new approach for the understanding of the role of the different bacteria in the TBZ-degrading consortia was followed. It was hypothesized that, upon exposure of the TBZ-consortia to a wide range of antibiotics with variable spectrum, parallel TBZ degradation measurements and fingerprinting analysis of the two consortia *via* DNA/cDNA-DGGE would provide further insights on the role of certain α-proteobacteria or *Pseudomonas* in the degradation of TBZ.

<u>MSMN-based TBZ-degrading consortium:</u> Exposure of the MSMN-based TBZ-degrading consortium to nalidixic acid, trimethoprim, and streptomycin did not affect its degrading capacity, with degradation of TBZ proceeding at rates similar to the non-antibiotic inoculated control (Figure 3.15a). In those treatments complete degradation was observed in 4 days. Exposure to colistin resulted in a longer lag phase with complete degradation of TBZ observed after 7 days (Figure 3.15a). Thus, these antibiotics at the concentrations used seemed not to eliminate the TBZ-degrading bacteria of the consortium. Ciprofloxacin and amikacin treatments partially inhibited the degradation of TBZ with only 50 and 60% degradation observed respectively at 14 days (Figure 3.15b). It is possible that those two antibiotics exerted a delayed effect on the degrading bacteria, eliminating them slowly and allowing degradation at the beginning of the culture. Piperacillin + streptomycin, doxycylin, ampicillin, and vancomycin + penicillin completely halted the degradation of TBZ suggesting an effect on the viability of the bacteria involved in the degradation of the fungicide (Figure 315b).

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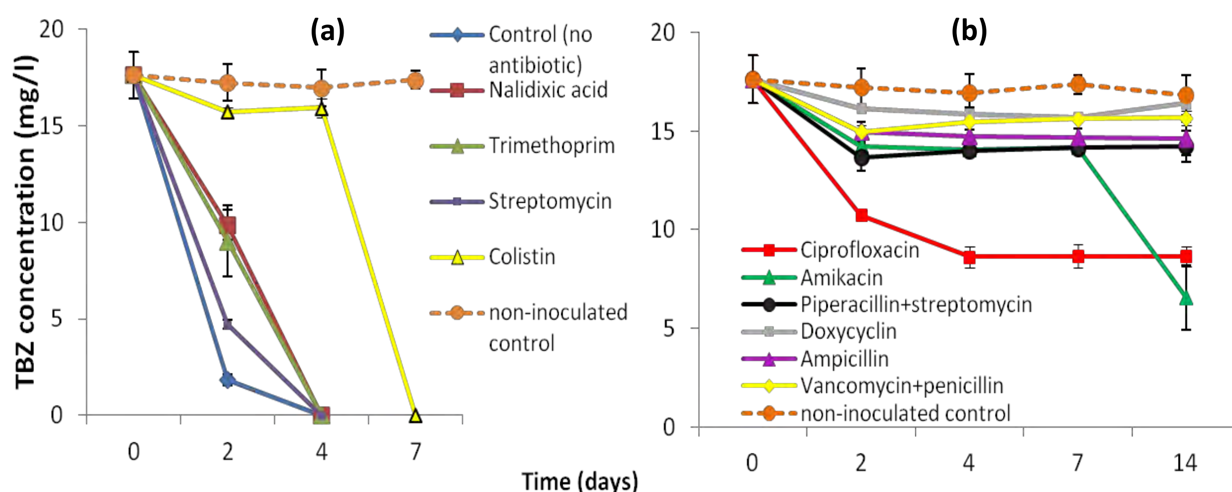

Figure 3.15. The degradation of TBZ by the MSMN-based consortium in the presence of different antibiotics. **(a)** Antibiotic treatments that did not affect the degradation capacity and **(b)** antibiotic treatments that partially or completely halted the degradation capacity of the consortium.

MSM-based TBZ-degrading consortium: The effect of antibiotics on the degradation capacity of the MSM-based TBZ-degrading consortium is showed in Figure 3.16. Similar patterns with the respective MSMN consortium were observed which is not surprising considering the similar composition of the two consortia. Complete pesticide degradation within 4 to 7 days was observed in the presence of nalidixic acid, trimethoprim, and streptomycin, while colistin again imposed a delayed but almost complete degradation of TBZ at 14 days (Figure 3.16a). No degradation of TBZ was measured in the presence of the other antibiotics tested including ciprofloxacin and amikacin which in the MSMN consortium induced a partial inhibition of degradation (Figure 3.16b).

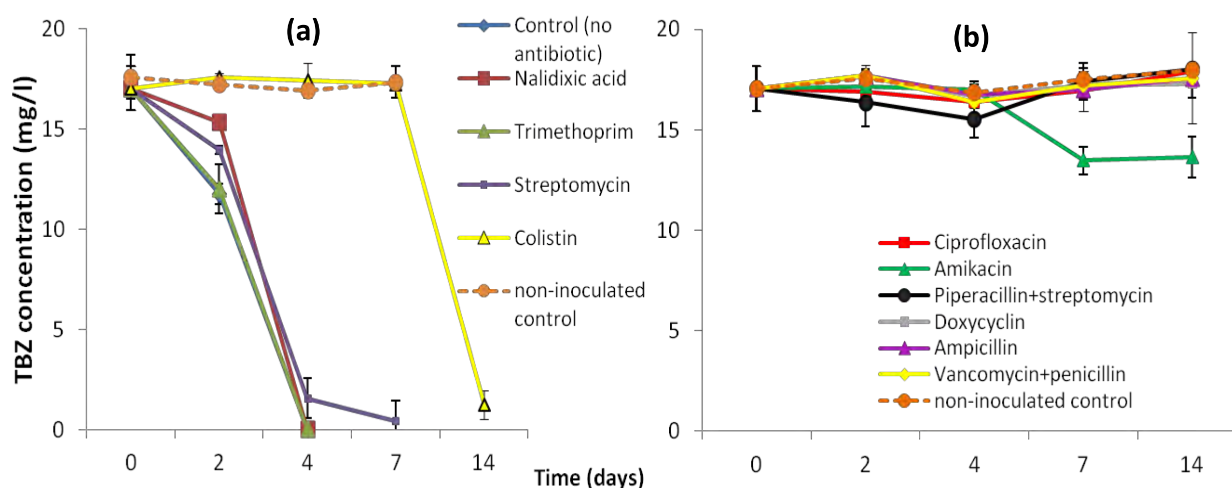

Figure 3.16. The degradation of TBZ by the MSM-based consortium in the presence of different antibiotics. **(a)** Antibiotic treatments that did not affect the degradation capacity and **(b)** antibiotic treatments that partially or completely halted the degradation capacity of the consortium.

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3.7.2. The effect of antibiotics on the composition of the TBZ-degrading consortia

In both media, DGGE analysis showed substantial changes in the community due to the inhibitory effect of the different antibiotics to different members of the bacterial consortia. Some bands disappeared and some new appeared as a response to antibiotics exposure (Figures 3.17 and 3.18). Clone libraries of the MSMN treatments were created to identify the new members and confirm the identity of the old ones (Table 3.16). The obtained sequences of the main bands were registered in the GenBank database (http://www.ncbi.nlm.nih.gov/). Accession numbers and sequences are reported in Annex 1, Section 3.1. Visual comparison between degrading and not degrading treatments suggested the constant presence of bands B13 (MSMN) / E8 (MSM), identified as *Sphingomonas* sp., in all treatments where active degradation of TBZ was observed. In contrast, the same band/ribotype was absent in the fingerprints of all the non-degrading treatments. This was more clearly demonstrated in the cDNA-DGGE fingerprints that represent active and probably proliferating members of the consortium. The presence of *Sphingomonas* sp. band B13 in all the cultures exhibiting active degradation of TBZ and its concurrent absence in the non-degrading cultures was observed in both media (Figures 3.17 and 3.18). For example, band B13 became dominant and intense in the fingerprints of the MSMN-based consortium exposed to nalidixic acid, trimethoprim, streptomycin, and control between days 2 and 4 (Figure 3.17) which concurs with the rapid degradation phase of TBZ (Figure 3.15a). In contrast, the same band was not visible in the fingerprints of the MSMN cultures exposed to piperacillin + streptomycin, doxycycline, ampicillin, and vancomycin + penicillin where TBZ was not degraded (Figure 3.15b). Overall these data provided strong evidence for the involvement of this bacterium in the degradation of TBZ.

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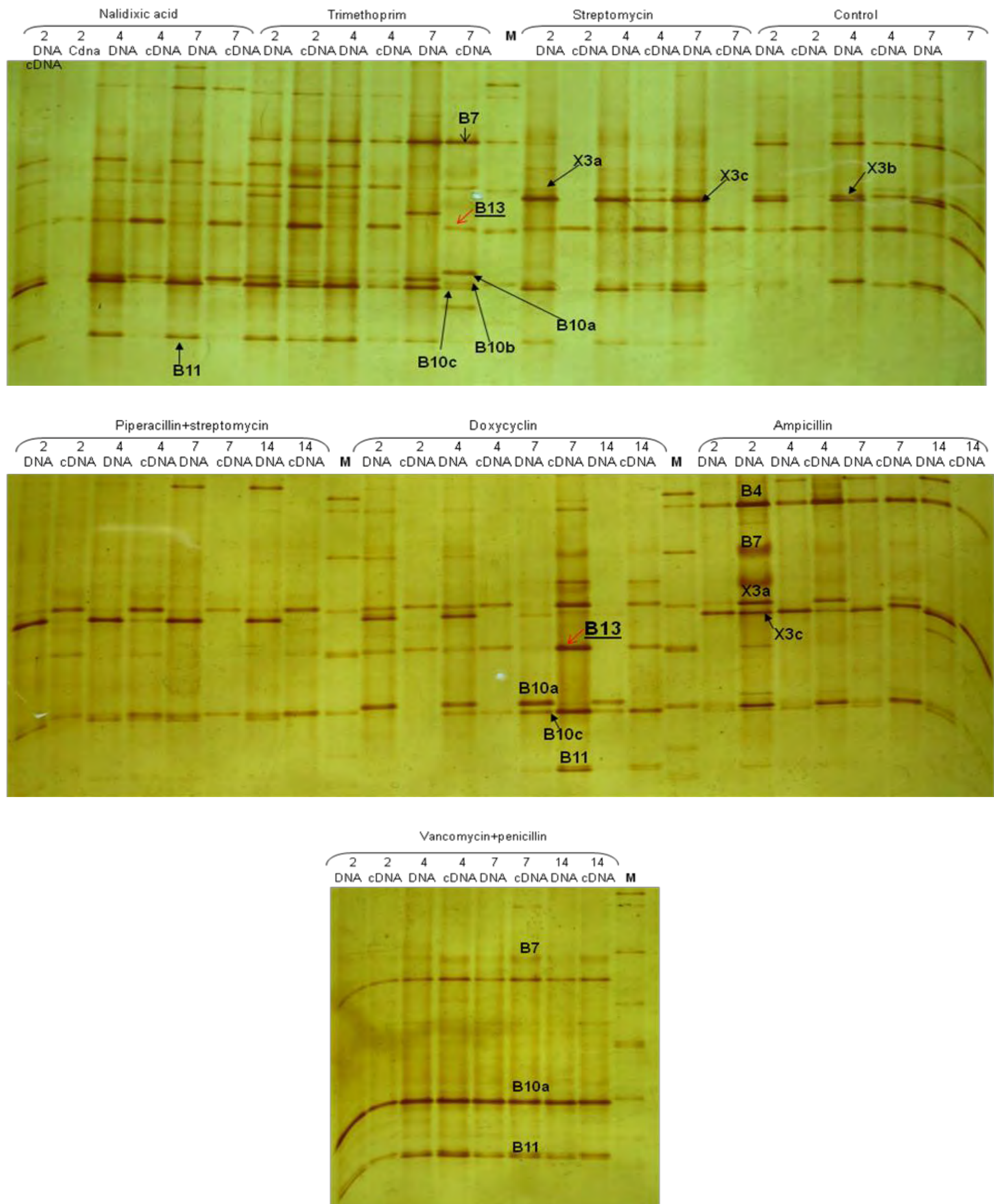

Figure 3.17. DNA/cDNA-DGGE profiles of the MSMN-based TBZ-degrading consortium in the presence of different antibiotics. **Upper panel**: fingerprints of the consortium exposed to nalidixic acid, trimethoprim, streptomycin, and in the non-antibiotic control (treatments positive for degradation of TBZ). **Middle and Lower panel:** fingerprints of the consortium exposed to piperacillin+streptomycin, doxycyline, ampicillin, and vancomycin+penicillin (treatments negative for degradation of TBZ). Bands names are labeled and bacterial identities are listed in Table 3.16.

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Table 3.16. The identity of the main members of the MSMN-based TBZ-degrading consortium after antibiotics treatment. The highest BLAST match based on the homology of the 16S rRNA gene is reported along with % of sequence homology and the NCBI accession number of the best match.

Band	Highest match	Homology (%)	Homology (bp)	NCBI Accession number
B4	*Variovorax paradoxus* strain: DSM 30162	100	1460/1460	AB622223.1
B7	*Hydrocarboniphaga effusa* strain AP103	99	1449/1451	NR_029102.1
X3a	*Sphingopyxis terrae* strain: BSN20	99	1413/1414	AB675372.1
X3b	*Methylibium petroleiphilum* PM1	99	1455/1456	CP000555.1
	Bosea thiooxidans	100	849/849	AJ250798.1
X3c	*Hydrogenophaga* sp. Gsoil 1545	99	1453/1454	AB271047.1
B13	*Sphingomonas* sp. strain 1-F12	97	1370/1413	JF309146.1
B10a	*Oligotropha carboxidovorans* strain: S28	99	1410/1414	AB099660.1
B10b	*Shinella granuli* strain R1-702	99	1402/1410	JQ659575.1
B10c	*Achromobacter* sp. Ko	99	1454/1456	HQ200411.1
B11	*Alcaligenes* sp. Yen	99	1452/1456	AY744384.1|

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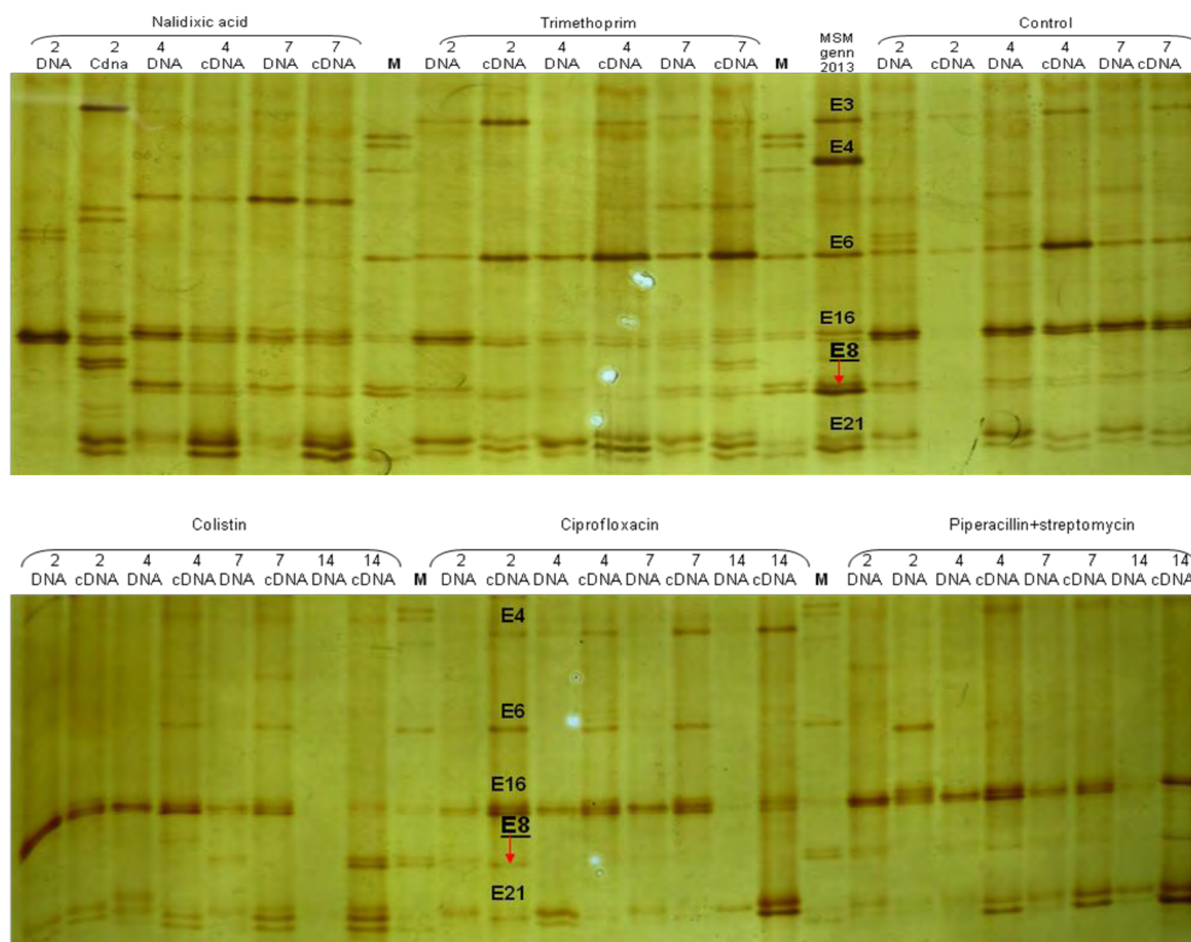

Figure 3.18. DNA/cDNA DGGE profiles of the MSM-based TBZ-degrading consortium when grown in the presence of different of antibiotics. **Upper panel**: fingerprints of the consortium exposed to nalidixic acid, trimethoprim, and in the non-antibiotic control (treatments positive for degradation of TBZ). **Lower panel**: fingerprints of the consortium exposed to colistin, ciprofloxacin, and piperacillin+streptomycin (treatments negative for TBZ degradation). Bands names are labeled and bacterial identities are listed in Table 3.11.

Based on the results of the antibiotics-exposure experiment, an attempt was made to isolate the *Sphingomonas*-like bacterium represented by band B13-E8 that appears to have a role in the degradation of TBZ. Thus the TBZ-degrading cultures that kept their degrading ability regardless of antibiotics exposure (nalidixic acid, trimethoprim, colistin, and streptomycin) were maintained in the presence of the corresponding antibiotics for three further subculturing cycles. In addition a culture supplemented with a mixture of nalidix acid, trimethoprim, and streptomycin was also prepared aiming to eliminate more effectively the undesirable members of the consortium and facilitate isolation of the *Sphingomonas* strain B13.

Subculturing in the presence of the four antibiotics individually did not affect the degrading capacity of the consortia. Moreover, in the colistin-amended cultures an enhancement in the TBZ degradation rates compared to the initial culture was observed (Figure 3.19). After a

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rapid degradation of TBZ in the first cycle of the cultures exposed to the mixture of antibiotics, the MSMN-based TBZ-degrading consortium lost its degrading capacity, compared to the corresponding MSM-based consortium where degradation was retarded (complete degradation in 17 days) but not halted completely (Figure 3.20). This was reflected in the DNA/cDNA DGGE analysis of the final subculture cycle (Figure 3.21), where band B13 representing the putative degrading ribotype *Sphingomonas* was not present in the fingerprint of the MSMN-based consortium exposed to the antibiotics mixture. In the other treatments, the constant exposure to antibiotics resulted in simpler consortia and in many cases the cDNA profiles were composed of only a few bands suggesting the presence of a limited number of active bacteria (e.g. MSM-based consortium exposed to colistin composed of three bands, E6, E8, and E21).

The cultures which lost their degrading capacity during continuous cultivation with the antibiotics were not able to recover their TBZ degradation capacity after transfer into fresh media without antibiotics. This finding suggests that the specific antibiotics had a non reversible effect (killed) on the bacterial members responsible for the degradation of TBZ.

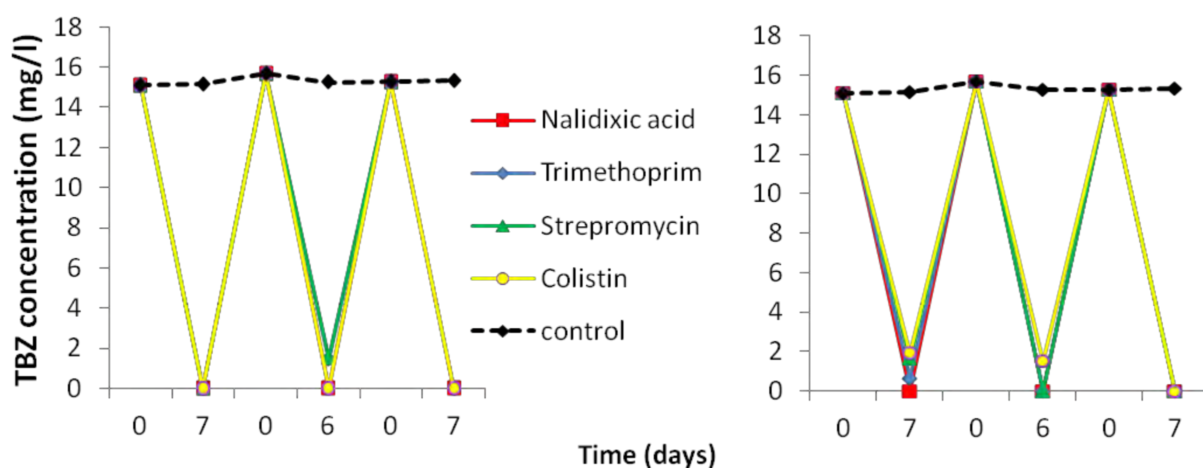

Figure 3.19. The degradation of TBZ in the three successive subculturing cycles of the MSMN- (left) and MSM- (right) based consortia in the presence of the antibiotics nalidixic acid, trimethoprim, streptomycin, and colistin. Degradation of TBZ in non-inoculated cultures (control) is also presented. Three subculture cycles were followed and 0 indicates the start of each subculture cycle.

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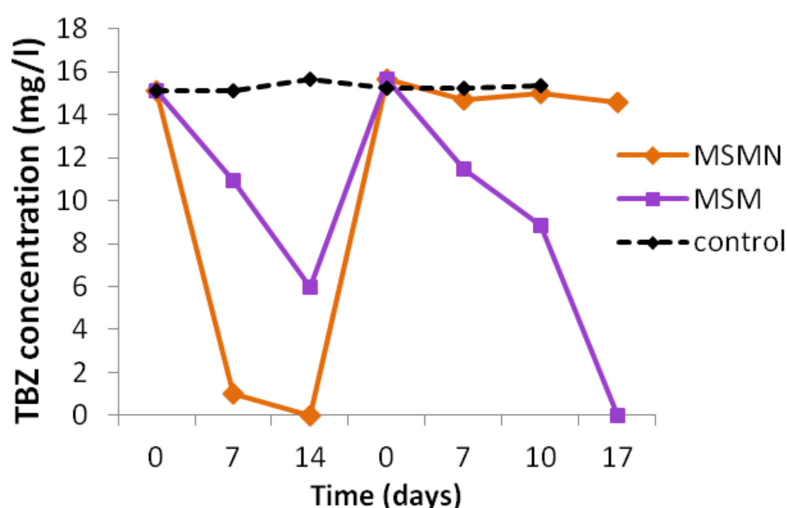

Figure 3.20. The degradation of TBZ in two successive sub-culturing cycles of the MSMN- and MSM-based TBZ consortia exposed to a mixture of the antibiotics nalidixic acid, trimethoprim, and streptomycin. Degradation of TBZ in non inoculated cultures (control) is also presented. 0 indicates the start of each cycle.

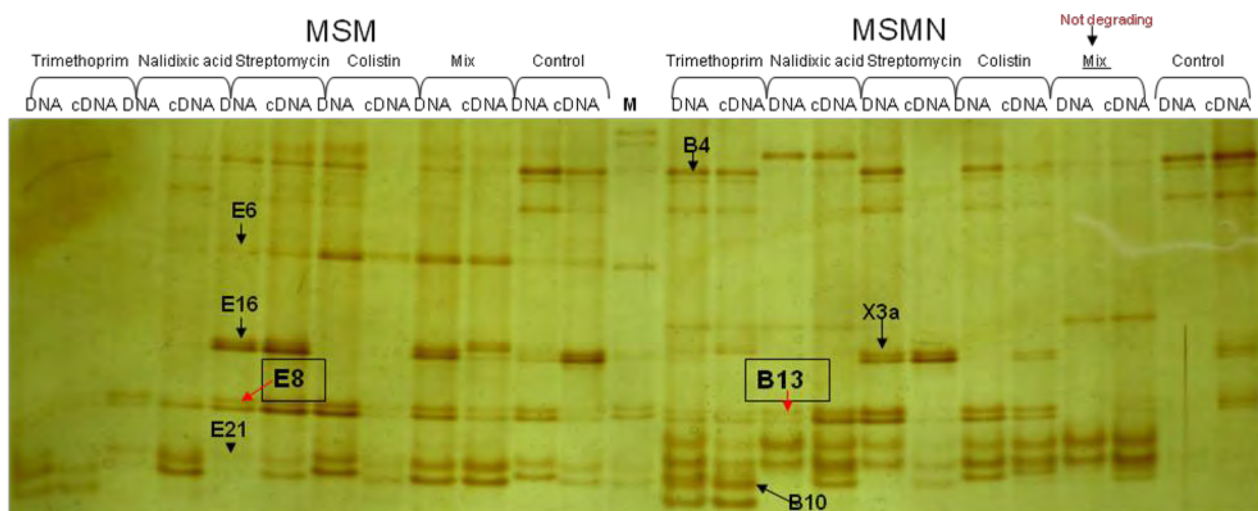

Figure 3.21. DNA/cDNA-DGGE profiles of the MSM- (left) and MSMN- (right) based consortia after three sub-culture cycles in the presence of the antibiotics trimethoprim, nalidixic acid, streptomycin, colistin, and a mixture of the first three (Mix). The composition of the corresponding consortia grown in the absence of any antibiotics is also shown (Control). Bands names are labeled and bacterial identities are listed in Tables 3.11 (MSM) and 3.16 (MSMN). Band B13/E8 representing the *Sphingomonas* sp. ribotype is indicated with red arrow. Lane M: bacterial DGGE marker.

The consortia obtained after the fourth sub-culturing cycle were serially diluted and spread on agar plates of the *Sphingomonas*-selective medium L9 amended with 20 mg/l TBZ plus the respective antibiotic or mixture of antibiotics. Growing colonies of different morphology were selected and checked for degradation, with the aim to isolate the strain represented by band B13-E8 in pure culture. A colony selected from the MSM-based consortium amended with colistin (colony MSM Col1) degraded 84% of TBZ within 19 days. The culture showed a uniform

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morphology on LB (Figure 3.22) and MSM+TBZ plates suggesting purity. However this was not confirmed by DGGE fingerprinting analysis which showed that the culture was composed of six main bands (Figure 3.22). The identity of those bands was defined by clone library analysis (Table 3.17). Bacterial sequences were registered at the GenBank database (http://www.ncbi.nlm.nih.gov/). Accession numbers and sequences are reported in Annex 1, Section 3.1. Four of those bands had same DGGE running profile with bands of the original MSM consortium and two of them gave the same identification, as showed in Table 3.17. The presence of the putative degrading ribotype of band B13-E8 was confirmed (band Col C). Allignment of the sequences obtained in the current clone library for the band B13/E8 (ColC) and the sequence of clones from the initial clone libraries of the two consortia showed a perfect match, indicating that it was effectively the same bacterium.

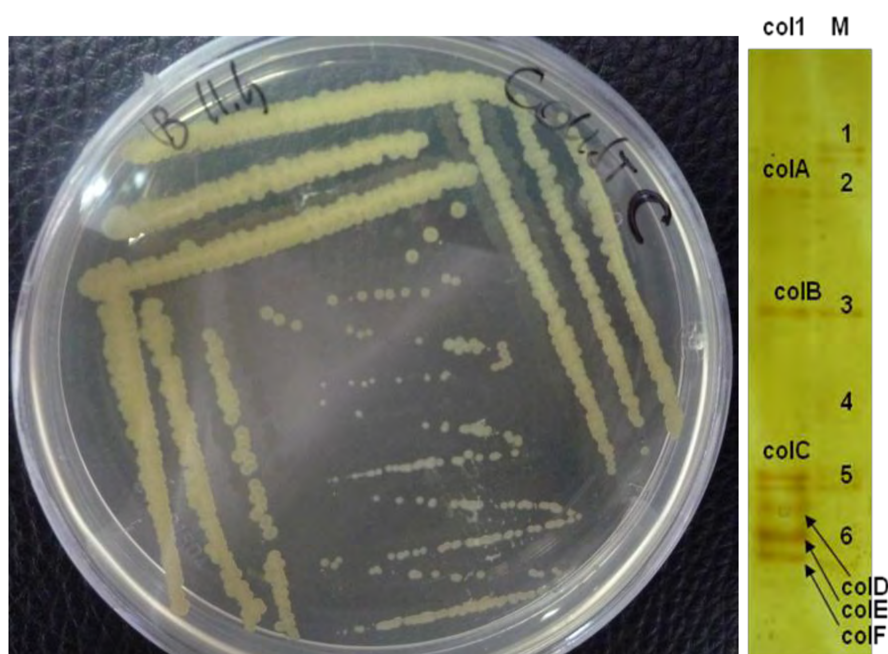

Figure 3.22. Left: MSM Col1 culture on LB plate. **Right**: DGGE profile of the degrading culture MSM Col1. The identities of the ribotypes represented by each band are given in Table 3.17.

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Table 3.17. Identification of the MSM Col1 culture main bands. The best match according to BLAST, its % of sequence homology, and its NCBI accession number are shown. Corresponding bands in the original MSM consortium are also reported.

Corresponding MSM band	Band	Highest match	Homology (%)	NCBI Acces. No.
E4 (*Pseudomonas* sp.)	**Col A**	No clones		
E6 (*P.putida*/*Hydrocarboniphaga* sp.)	**Col B**	*Novosphingobium* sp. HU1-AH51	99	FJ177534
E8 (*Sphingomonas* sp.)	**Col C**	*Sphingomonas* sp. 1-F12	97	JF309146
-	**Col D**	*Cupriavidus* sp. WBF7	100	DQ777737
E21 (*Oligotropha* sp.)	**Col E**	*O. carboxidovorans* S28	99	AB099660
-	**Col F**	*Hyphomicrobium* sp. P2	99	AF148858

Morphologically distinct colonies of the MSM Col1 culture were selected from MSM + TBZ plates but none was able to degrade TBZ within 25 days (data not shown). DGGE profiling of the non-degrading colonies and of the whole bacterial biomass which grew on the plates revealed that most of the members of the consortium were able to grow on agar plates with the exception of the strain represented by the band ColC (same as band B13/E8) (Figure 3.23). The lack of degradation and the concurrent absence of ColC from the fingerprint of the consortium further supports the suggestion that the bacterium represented by this band (*Sphingomonas* sp.) is directly involved in the degradation of TBZ. Subculturing of the MSM Col1 consortium in liquid cultures resulted in an acceleration in the degradation rates of TBZ with complete degradation observed in 3 days (Figure 3.24).

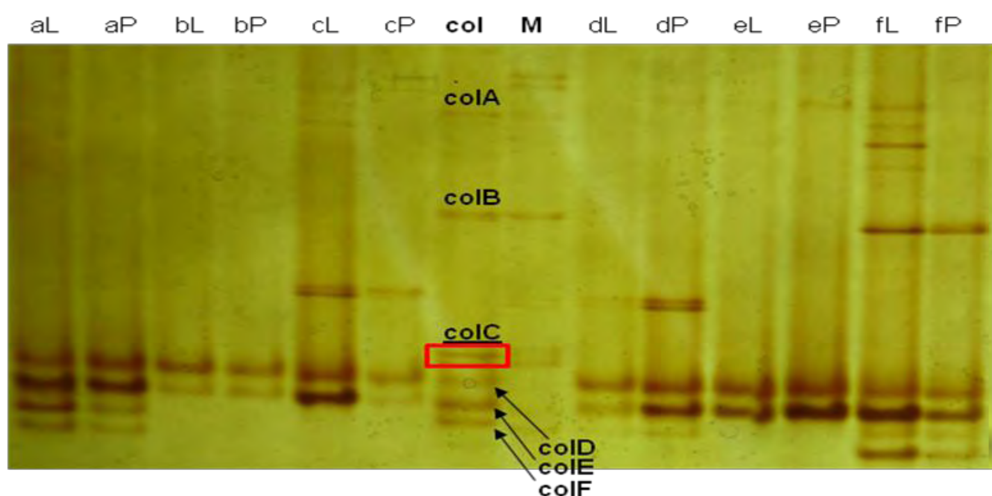

Figure 3.23. DGGE profile of the single colonies selected from the MSM + TBZ plates spread with the MSM Col1 consortium. A, b, c, d, e, and f indicate the number of the single colonies selected, while L and P indicate if the fingerprint was obtained from bacteria grown in liquid culture or in agar plate respectively. Lane col represents the fingerprint of the starting MSM Col1 consortium.

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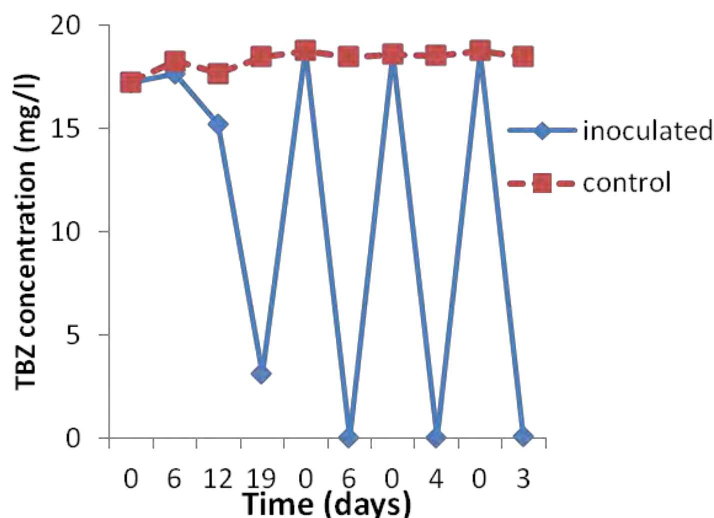

Figure 3.24. The degradation of TBZ by the MSM Colistin 1 consortium upon successive subculturing in MSM + TBZ + colistin. Degradation of TBZ in non-inoculated cultures (control) is also reported. 0 indicate the beginning of each cycle.

3.8. Isolation of the *Sphingomonas* strain ColC/E8/B13 *via* plating on selective media

Considering all previous results showing the inability of the *Sphingomonas* strain represented by band ColC/E8/B13 to grow on the growth media tested, the MSM Col1 consortium was spread on a wider variety of selective media with TBZ always serving as the sole C and N source. The selective media tested were a) the *Sphingomonas* selective medium L9, b) the Mandelbaum medium which provides vitamins and micronutrients, and c) MSM supplemented with casamino acids which contain a mixture of aminoacids and small peptides. The last two media were selected based on the hypothesis that the *Sphingomonas* strain might require extra vitamins, microelements, or amino acids to grow. Previous studies have also suggested that pesticide-degrading Sphingomonads required casamino acids to effectively grow and degrade MCPA [Sorensen *et al*., 2002; Onneby *et al*., 2010]. In addition the MSMN-based TBZ-degrading consortium was also plated but only on MSMN + casamino acids.

None of the selected colonies was able to degrade TBZ within 21 days and the DGGE profile of the colonies tested and of the total bacteria growing on the plates from where the colonies were selected revealed the absence of band ColC/B13/E8 (Figure 3.25). When the total pellet of the bacteria growing on the three media was harvested and used for the inoculation of liquid cultures of MSM + TBZ, their degrading ability was recovered (Figure 3.26). In contrast, this was not possible for the MSMN-based consortium which did not show degradation upon transfer in the liquid culture (Figure 3.26). Interestingly, this distinct behaviour showed by the two degrading consortia was reflected in their DGGE profiles where the band corresponding to the *Sphingomonas* strain ColC/E8/B13 was constantly present in all MSM-based liquid cultures

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(positive for degradation of TBZ) but not in the non-degrading MSMN-based consortium (Figure 3.27), providing further evidence in support of the role of this specific strain in the degradation of TBZ.

The *Sphingomonas*-like bacterium ColC/E8/B13 which appears to have a key role in the degradation of TBZ by the two isolated consortia was not succesfully isolated *via* plating in a range of media and further DGGE analysis showed that it was not actively growing on the agar plates. However the fact that bacterial pellets harvested from the agar plates recovered the degrading capacity when put back in liquid culture and that the specific *Sphingomonas* ColC/E8/B13 was again present in the liquid culture suggest that it is probably not actively growing on the agar plates but could survive in a dormant form. The inability of this strain to grow on agar plates could not be attributed to nutritional deficiencies since the same bacteria grow actively in the corresponding liquid media. A possible cause could be a toxic effect exerted by impurities of the agar, as demonstrated for the sulfate-reducing bacterium *Acidithiobacillus thiooxidans* syn. *Thiobacillus thiooxidans* that grows poorly on agar media, due to the toxicity of agar hydrolysis products [Kelly and Harrison, 1989]. Also Tamaki *et al.* [2009] have shown that agar may influence the culturability of microorganisms. Further studies should be undertaken to clarify this issue.

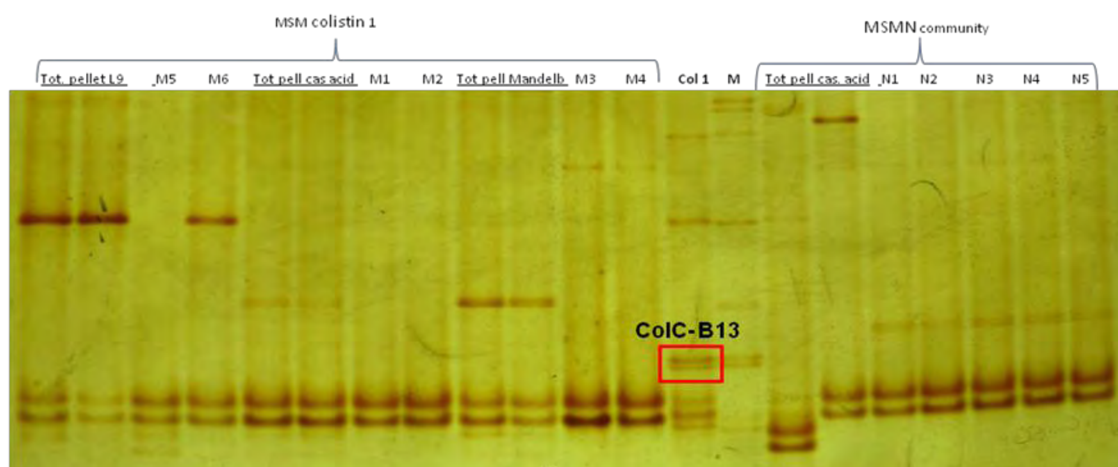

Figure 3.25. DGGE profile of the bacterial community growing on selective agar media (total pellet L9, cas acid, Mandelb) and of the respective selected colonies (indicated with M for MSM and N for MSMN) which did not degrade the fungicide. Lane Col1: the fingerprint of the starting degrading consortium obtained from liquid culture and used as inoculum for plating; Lane M: DGGE bacterial marker.

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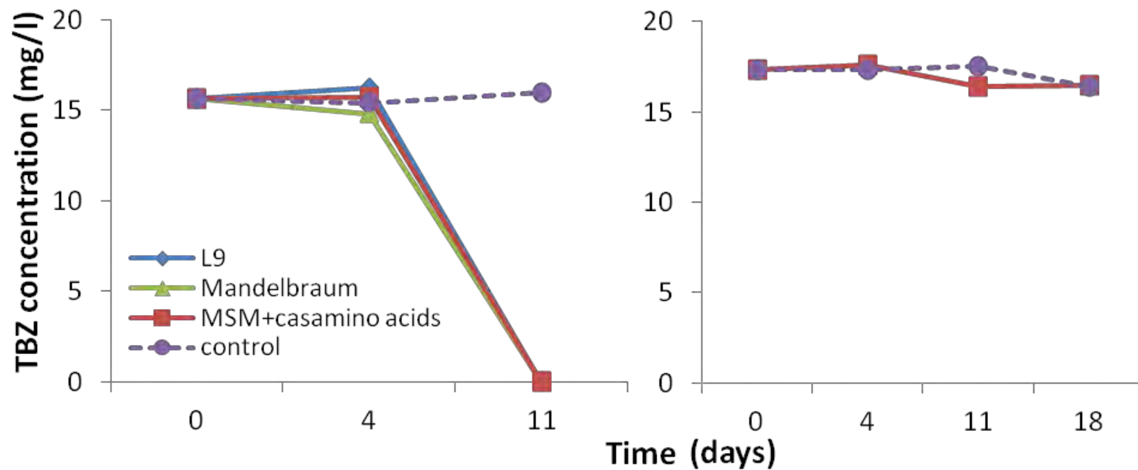

Figure 3.26. The degradation of TBZ in liquid MSMN/MSM cultures inoculated with the total bacterial pellet growing on selective agar media: L9, Mandelbaum, and MSM+casamino acids media for the MSM Col1 consortium (left) and MSMN+casamino acids for the MSMN consortium (right). The degradation of TBZ in non-inoculated cultures (control) is also reported.

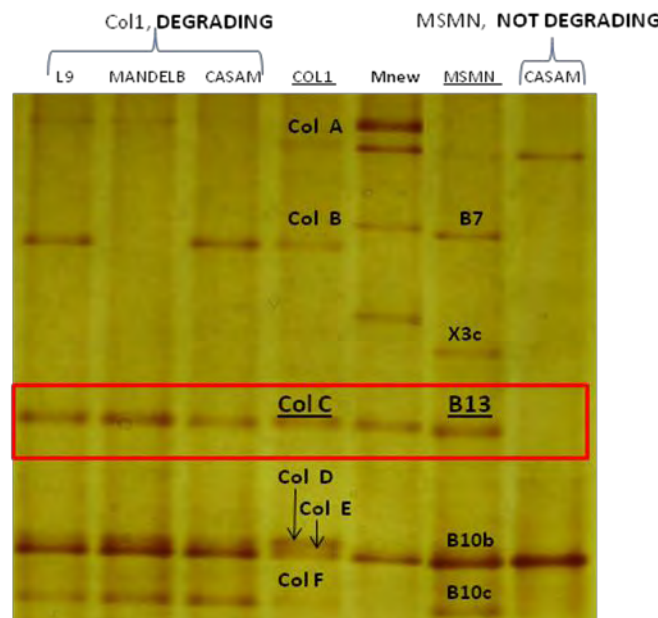

Figure 3.27. DGGE profile obtained from liquid cultures (MSMN/MSM + TBZ) inoculated with the total bacterial mass that grew on the agar plates of the different selective media tested (L9, Mandelbaum, MSM+casamino acids plus TBZ). Lanes Col1 and MSMN indicate respectively the MSM Col1 TBZ-degrading consortium and the initial stabilized MSMN-based TBZ degrading consortium. Major bands names are indicated. Lane Mnew: DGGE bacterial marker.

3.9. Assessing the role of *Sphingomonas* B13/E8/ColC in MSM Col1 consortium *via* q-PCR

Based on the results of all the previous studies regarding the role of the *Sphingomonas* ribotype rerpresented by band B13/E8/ColC on the degradation of the fungicide, a new set of primers was designed (ColC_f and ColC_r) to specifically amplify the 16S rRNA gene of this bacterium. This

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set of primers was used to determine the population dynamics of this specific bacterium during degradation of TBZ *via* q-PCR. This was possible since the MSM Col1 consortium was simpler in its composition compared with the original MSM-based TBZ-degrading consortium thus allowing the specific amplification of the 16S rRNA of this *Sphingomonas* strain. The population dynamics of this bacterium was determined in absolute numbers but also relatively to the total bacterial population also determined *via* q-PCR.

The MSM Col1 consortium completely degraded 15 mg/l TBZ within 48 hours (Figure 3.28).

The primer set designed was tested for specificity *in silico* and *in vivo* and showed the expected specificity. In the *in vivo* test all selected clones showed 99% homology with *Sphingomonas* sp. TNR-2 (NCBI accession number HF544321) and the alignment of their sequences with the sequence of the *Sphingomonas* ribotype represented by band B13/E8/ColC showed a perfect match. For both q-PCR assays (*Sphingomonas*-specific and total bacteria), the calibration curve obtained had a good linearity within the limits of 10^2 to 10^8 copies/μl, r^2 = 0.997 and 0.990, and amplification efficiencies of 88% and 98.6% respectively. The standard, amplification, and dissociation curves are shown in Annex I, Figure A.14 and A.15 respectively.

The q-PCR results showed a significant increase in the copy numbers of the 16S rRNA gene of the *Sphingomonas* sp. B13/E8/ColC growing in the presence of TBZ (Figure 3.28) compared to the numbers obtained when the same bacterial consortium was grown in the absence of TBZ (and in the presence of IMZ). The same trend was evident in the data of the q-PCR assay relatively to the total bacterial population, where the population of the *Sphingomonas* B13/E8/ColC grew disproportionally to the total bacteria population (which was stable during the incubation) in the liquid culture, as determined by qPCR (Figure 3.29). The results of this experiment provide strong evidence for the involvement of the *Sphingomonas* sp. B13/E8/ColC in TBZ degradation.

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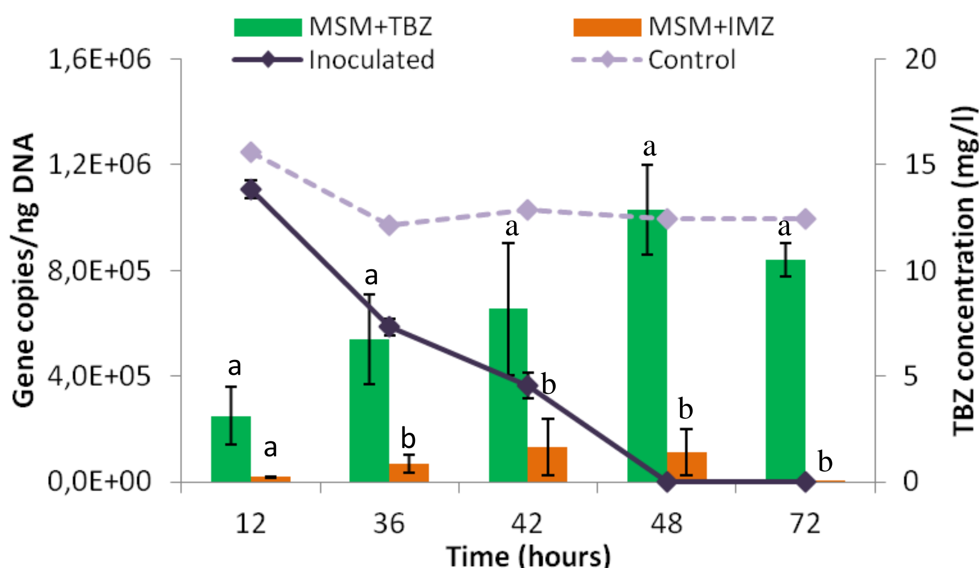

Figure 3.28. The population dynamics of *Sphingomonas* sp. B13/E8/ColC strain determined *via* q-PCR in MSM+TBZ or MSM+IMZ which were inoculated with the MSM Col consortium. Within each sampling time, bars designated with the same letter are not statistically different at 5% level. The degradation of TBZ in inoculated and non-inoculated (control) cultures is also presented.

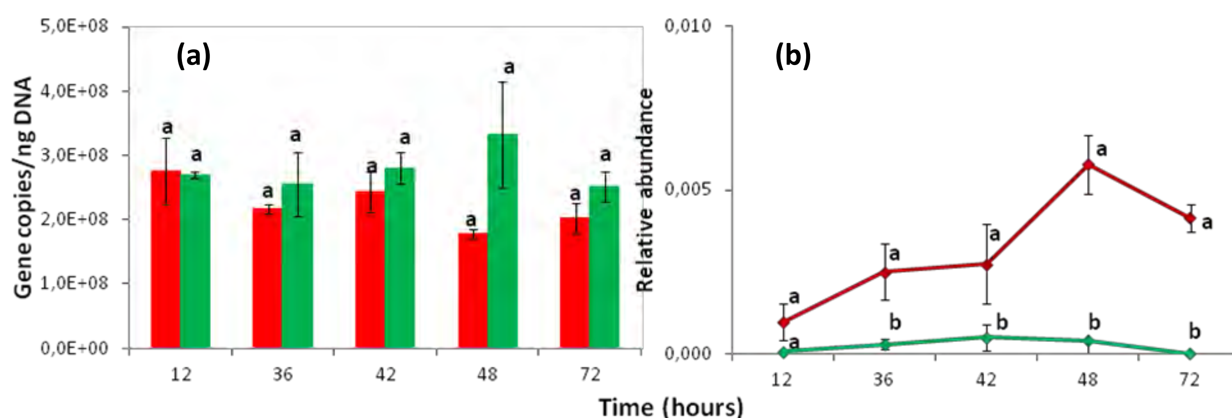

Figure 3.29. (a) The population of total bacteria, expressed as copies of the 16S rRNA gene determined *via* q-PCR, in MSM+TBZ (red bars) or MSM+IMZ (green bars) inoculated with the MSM Col1 degrading consortium. **(b)** The changes in the population of the *Sphingomonas* sp. B13/E8/ColC relatively to the total bacterial population (relative abundance) in MSM + TBZ (red line) or + IMZ (green line). Within each sampling time, bars/points designated with the same letter are not statistically different at 5% level.

3.10. Effect on the composition of the TBZ-degrading consortia upon prolonged feeding on putative metabolites of TBZ

As it will be further described in Section 3.12.2., the two TBZ-degrading consortia, the initial stabilized MSMN-based and the MSM Col1, were sub-cultured for a period of several months in the presence of different compounds which were identified by previous studies [EC, 2013] as

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potential metabolites of TBZ: 1*H*-benzimidazole-2-carboxylic acid (acid), 1*H*-benzimidazole-2-carboxamide (amide), benzimidazole, and methylamine (for MSM Col1 only amide and benzimidazole were tested). At the end of the experiment, the final culture of every treatment was used to inoculate fresh media amended with TBZ to assess if their pesticide degradation capacity was maintained. The MSMN-based consortium which was subcultured in acid and methylamine maintained its degradation capacity with complete degradation of TBZ observed in 12 days (Figure 3.30). In contrast, the consortia of both MSMN and MSM Col1 maintained in amide-amended cultures exhibited a slower degradation capacity, while both consortia subcultured in the presence of benzimidazole lost their degradation ability (Figure 3.30).

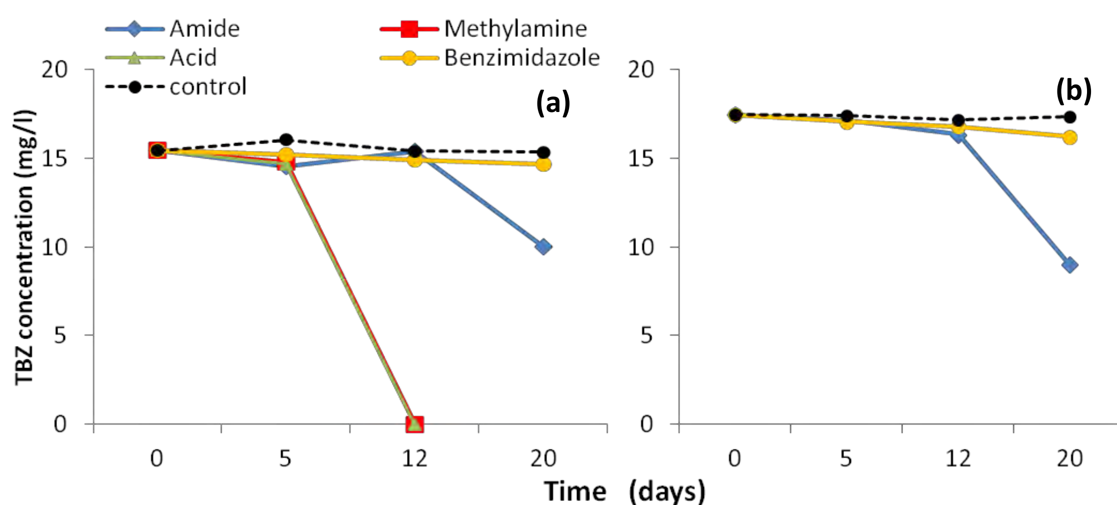

Figure 3.30. Recovery of the TBZ-degrading ability of the MSMN-based (a) and the MSM Col1 (b) consortia after prolonged subculturing in MSMN supplemented with amide, acid, methylamine, and benzimidazole, or MSM supplemented with amide and benzimidazole instead of TBZ. Degradation of TBZ in non-inoculated cultures (control) is also presented.

The DGGE profiles of the two TBZ-degrading consortia which were maintained in prolonged enrichment on putative metabolites and subsequently transferred to the corresponding liquid medium + TBZ are shown in Figure 3.31. Prolonged sub-culturing on metabolites and transfer into TBZ-amended media resulted in simpler consortia composed of two to three main members among which the *Sphingomonas* band ColC-B13 being always present only in the cultures which maintained their degrading capacity (MSMN-consortium in amide, acid, and methylamine and MSM-consortium in amide). The derived consortia were dominated by α-proteobacteria (*Shinella* sp., *Oligotropha* sp., *Hyphomicrobium* sp.) and β-proteobacteria of the order Bulkholderiales (*Achromobacter* sp., *Cupriavirus* sp.), the latter represented by bands at the lower part of the DGGE gel (Tables 3.15 and 3.16).

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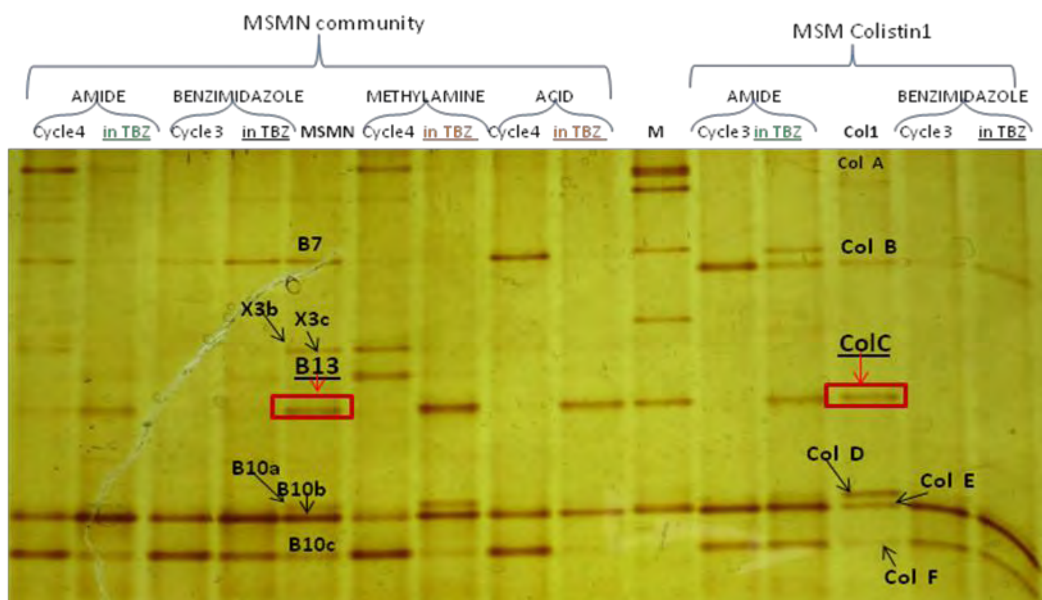

Figure 3.31. DGGE profile of the MSMN-based and MSM Col1 consortia after prolonged feeding on different potential metabolites of TBZ (1*H*-benzimidazole-2-carboxylic acid (acid), 1*H*-benzimidazole-2-carboxamide (amide), benzimidazole, and methylamine) and subsequent transfer on the corresponding TBZ-amended media. The coloration of *in TBZ* is an indicator of the degradation capacity of the different cultures (black = no TBZ degradation; Green= slow degradation; Red = rapid degradation). Cycle number indicates the number of sub-culturing cycles that were followed (see Section 3.12.2). The names of the major bands are highlighted. For identification see Tables 3.15 (MSMN) and 3.16 (MSM Col1). The *Sphingomonas* sp. B12/E8/ColC band is indicated with red arrows. Lane M: DGGE bacteria marker; Lanes MSMN and Col1: the fingerprints of the starting consortia.

In particular, the MSMN-based consortium fed on acid which maintained its TBZ-degrading capacity was found to be composed of only two members represented by bands B12/E8/ColC (*Sphingomonas* sp.) and band B10b (*Shinella* sp.). An attempt was made to eliminate *Shinella* sp. and obtain the *Sphingomonas* B12/E8/ColC strain in pure form *via* exposure to antibiotics like colistin (60 mg/l) and streptomycin (200 μg/ml) which our previous observations and previous studies [Vaz-Moreira *et al*., 2010; An *et al*., 2006] have indicated to be able to eliminate *Shinella* spp. without having a toxic effect on the *Sphingomonas* strain. Amendment with colistin resulted in elimination of the *Sphingomonas* sp. strain and loss of TBZ-degrading capacity. Amendment with streptomycin did not affect the two bacteria and the consortium maintained its TBZ-degrading capacity (Figure 3.32). Those results suggest that the *Shinella* strain represented by band B10b is not directly involved in the degradation of TBZ, and further reinforce the suggestion about the direct involvement of the *Sphingomonas* sp. (band B12/E8/ColC) in the degradation of TBZ.

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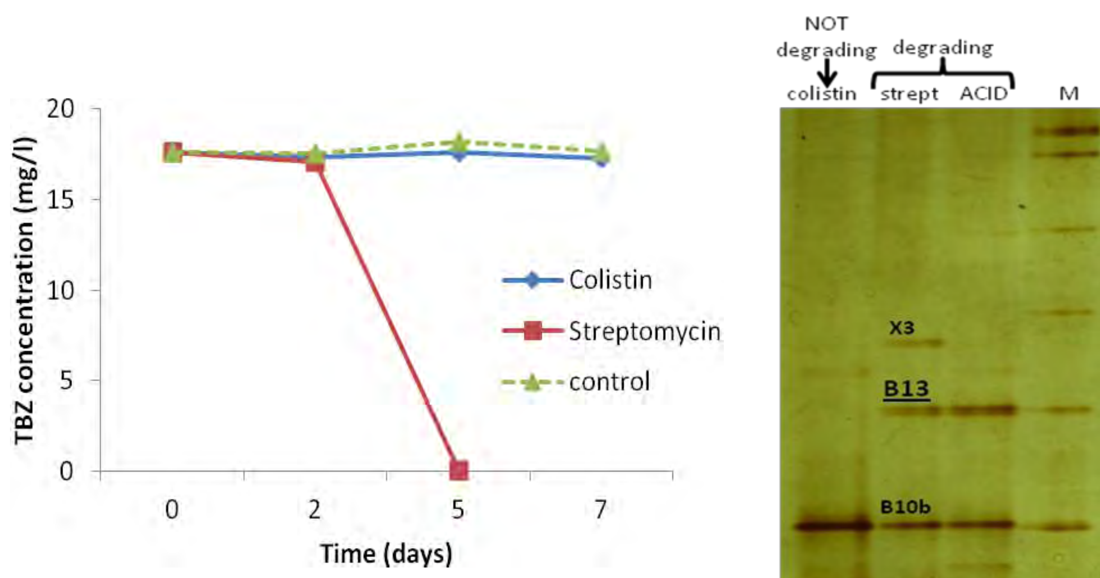

Figure 3.32. Left: The degradation of TBZ by the MSMN-based TBZ-degrading consortium obtained after prolonged cultivation with 1*H*-benzimidazole-2-carboxylic acid (acid) instead of TBZ and final transfer into MSMN+TBZ amended or not amended with the antibiotics colistin (60 mg/l) or streptomycin (200 mg/l). Degradation of TBZ in non-inoculated cultures (control) is also presented. **Right:** DGGE profile of the MSMN-based TBZ-degrading consortium after exposure to colistin and streptomycin. Band names are indicated (for identification see Table 3.16).

These final experiments confirmed that in both MSMN and MSM TBZ-degrading consortia the main responsible for TBZ degradation is the *Sphingomonas* strain B12/E8/ColC. It was not yet possible to isolate it in pure culture because of its incapacity to grow on the agar media used. This bacterium is probably responsible for the first step in the metabolism of TBZ. However, the involvement of other members of the community on the consumption and further degradation of metabolites formed in further metabolic steps cannot be excluded. Although the capacity of different *Sphingomonas* strains to degrade aromatic compounds such as 4(3',5'-dimethyl-3'-heptyl)-phenol [Corvini *et al*., 2004], benzo[a]pyrene [Rentz *et al*., 2008], phenanthrene, naphthalene, fluoranthene, toluene, benzoic acid, and others [Story *et al*., 2004] has been described, no reports of degradation of benzimidazole, imidazole, or thiazole rings that are present in TBZ molecule have been notified. Moreover, this is the first study reporting the isolation of bacteria able to efficiently and rapidly degrade the particularly persistent fungicide TBZ.

3.11. Phylogenetic characterization of the TBZ-degrading bacterium

Based on the full length sequence of the 16S rRNA gene of the identified TBZ degrader *Sphingomonas* B13/E8/ColC a phylogenetic analysis was conducted to taxonomically characterize this bacterium (Figure 3.33). The given strain was confirmed to belong to the genus *Sphingomonas* and it resulted most closely related to a *Sphingomonas wittichii* strain. Among

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this species, the strain *Sphingomonas wittichii* RW1 is known as a potent degrader of dioxins, as it completely mineralizes the dibenzo-*p*-dioxin structure and co-oxidize a large number of chlorinated congeners of both dibenzo-*p*-dioxin and dibenzofuran [Wittich *et al*., 1992; Wilkes *et al*., 1996; Hong *et al*., 2002].

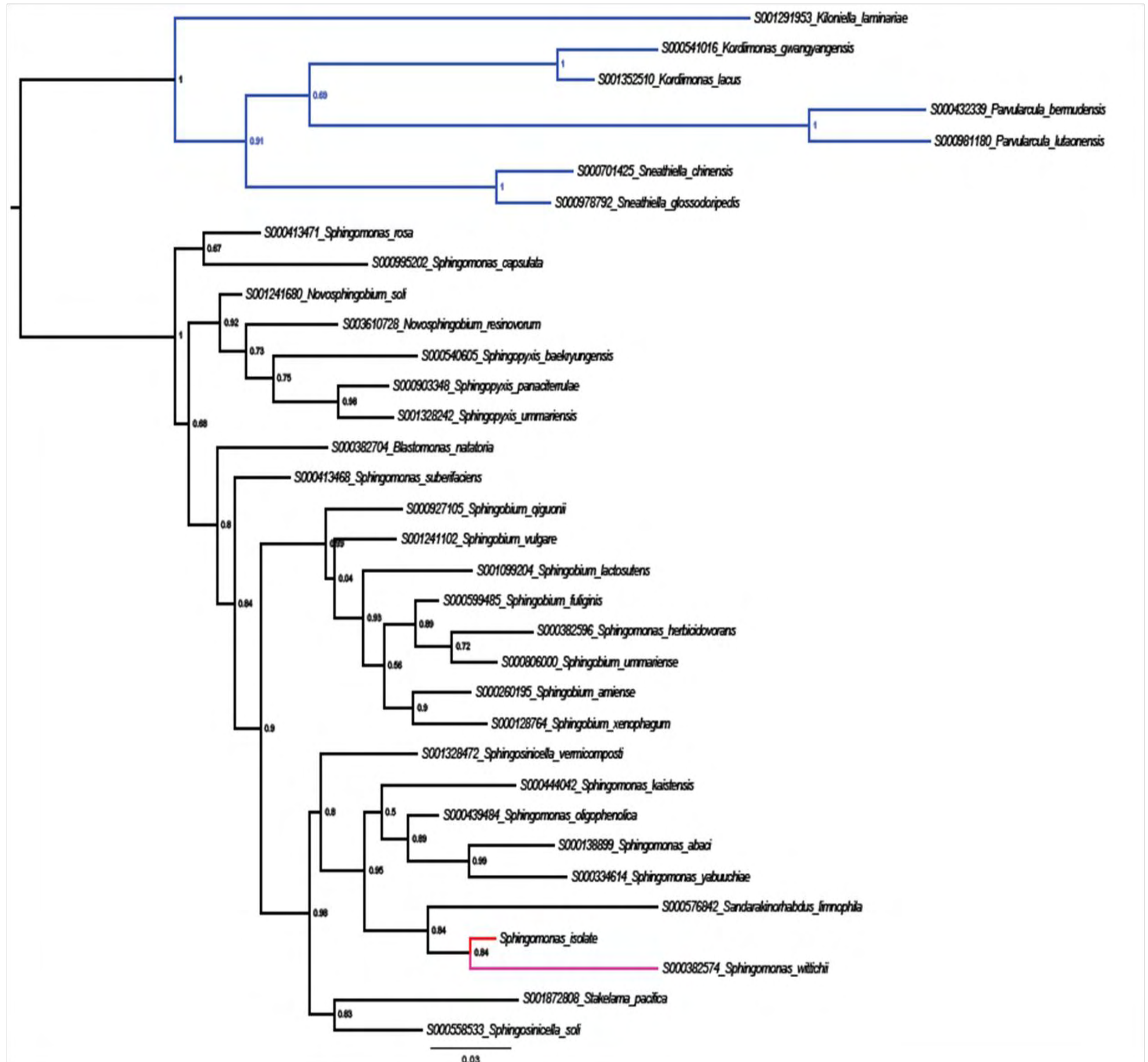

Figure 3.33. Phylogenetic analysis of the TBZ-degrading bacterium *Sphingomonas* sp. strain B13/E8/ColC based on the complete 16S rRNA gene sequence. 1000 bootstrap replicates were run with PhyML (maximum likelyhood method). The bootstrap support is expressed in scale from 0 to 1. The strain B13/E8/ColC is colored in red, the closest relative in rose, and the out-group sequences in blue.

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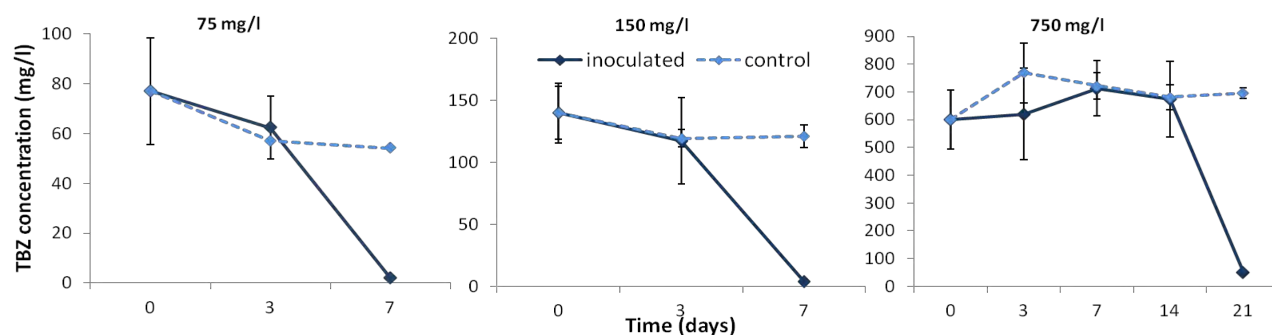
3.12. Characterization of the degrading ability of the two consortia.

3.12.1. Ability of the TBZ-degrading consortia to degrade high TBZ concentrations

Both MSMN- and MSM-based stabilized consortia were able to degrade high concentrations of TBZ in liquid cultures. Both consortia degraded 75 and 150 mg/l of TBZ within 7 days (Figure 3.34) and degraded even the highest concentration level tested (750 mg/l) at a slower rate with differences observed between the two consortia. Thus, this high concentration level was completely degraded within 21 days by the MSMN-based consortium, compared to 35 days which were need for the degradation of TBZ by the MSM-based consortium. The longer lag phase observed in the degradation of the highest concentration of TBZ by the MSM-based consortium was not surprising due to the poorer nutritional nature of the MSM medium in which not only extra C but also extra N is not supplied.

TBZ is considered as non-biodegradable compound and the only report of its biotic degradation refers to the ligninolytic fungus *Trametes versicolor* which managed to partially degrade a lower concentration of TBZ (50 mg/l) within 30 days [Karas *et al.*, 2011]. Considering the persistent nature of TBZ ($t_{1/2}$ soil field > 2 years [US EPA, 2002]) and the high concentrations of TBZ expected in wastewaters from the fruit packaging industry, the isolated bacterial consortia appear highly effective in the degradation of this molecule.

A



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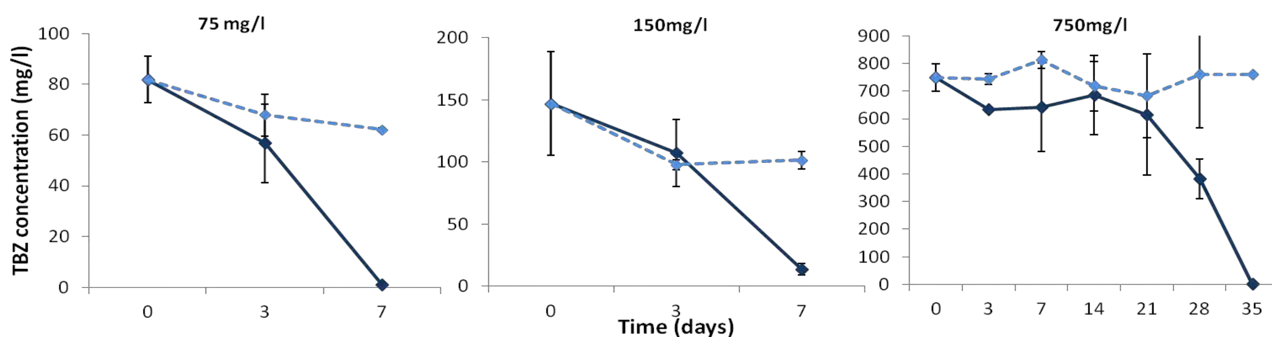

Figure 3.34. The degradation of 75, 150, and 750 mg/l of TBZ by the MSMN-based (A) and the MSM-based (B) TBZ-degrading consortia. Each value is the mean of three replicates ± the standard deviation of the mean. TBZ degradation in non inoculated samples for each concentration level and medium is also shown (control).

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3.12.2. Assessment of the degradation capacity of the TBZ-degrading consortia against different molecules

The capacity of the isolated TBZ-degrading consortia to degrade structurally related compounds (fuberidazole, carbendazim, and 2-aminobenzimidazole) or relevant pesticides regarding their practical-application aspect (imazalil) was evaluated in both media. TBZ, fuberidazole, carbendazim, and 2-aminobenzimidazole (2-AB) share a common benzimidazole structure with different substituent in position 2: a thiazole ring (TBZ), a furanic ring (fuberidazole), a methyl carbamate (carbendazim), and an amino group (2-AB) (Figure 3.35).

Both consortia showed the same degradation patterns (Figure 3.36). TBZ and fuberidazole were completely degraded in 2 and 4 days respectively. In contrast, neither imazalil nor 2-AB were degraded during the 30-day duration of the experiment, whereas 30-40% degradation of carbendazim was observed in the first week of incubation, but degradation was ceased thereafter. A relationship between chemical structure of the three benzimidazole compounds and the enhancement of microbial degradation was pointed out. In fact, fuberidazole that has the most similar structure to TBZ regarding the substituent of the benzimidazole moiety (thiazole ring *vs* furan ring) was rapidly degraded, while the other two compounds that have short (carbendazim) and very short (2-AB) substituents were not substantially degraded. Therefore TBZ degradation it is probably not initiated by the benzimidazole moiety that is common to all four compounds tested, but from the side moiety which is bulky (thiazole and furan rings) and rich in electrons. This is further supported by the limited capacity of the isolated consortia to degrade benzimidazole demonstrated below (Figure 3.38).

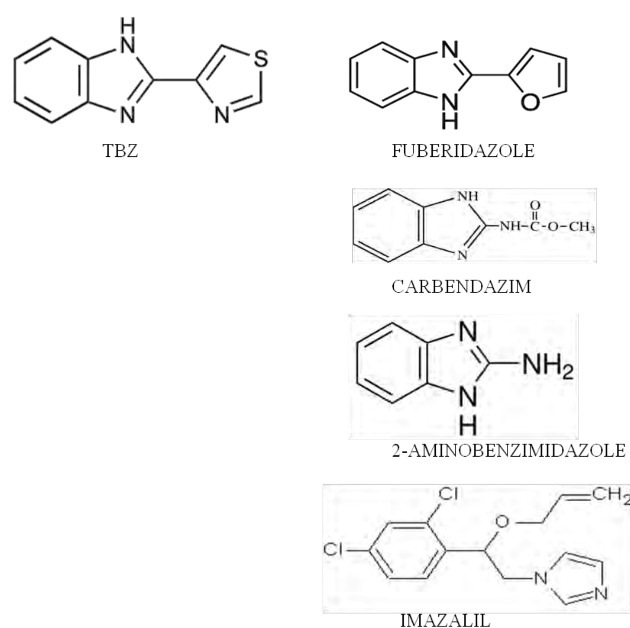

Figure 3.35. The chemical structures of the pesticides tested.

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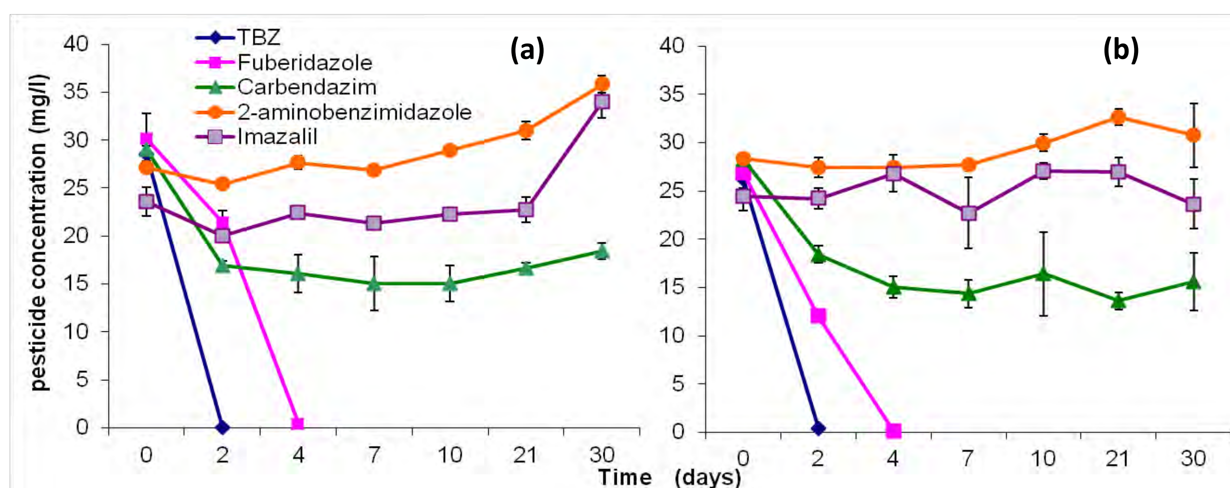

Figure 3.36. The degradation of fuberidazole, carbendazim, 2-AB, and imazalil by the MSMN- (a) and MSM- (b) consortia. The degradation of the compounds in non-inoculated controls was negligible and is not presented for clarity. Each value is the mean of three ± the standard deviation of the mean.

Effectively the thiazole and the furan rings constitute the most vulnerable part of the molecule, as demonstrated in previous photodegradation studies. In fact, TBZ was photodegraded to 1*H*-benzimidazole-2-carboxamide (~10%), a polar fraction (8.6%), while low levels of benzimidazole and 1*H*-benzimidazole-2-carboxylic acid were detected [FAO, 1997]. In addition, 1*H*-benzimidazole-2-carboxylic acid was identified as a major metabolite of fuberidazole [EFSA, 2007]. Similarly, Yarden *et al*. [1990] demonstrated that the cyclic moiety linked to benzimidazole, as in TBZ, does not cross-enhance carbendazim degradation and that, among the benzimidazoles tested, only those substituted *via* an amino group at position 2 (as 2-aminobenzimidazole and carbendazim) showed mutual cross-enhanced degradation. These results provided a first indication that the isolated bacterial cultures degrade TBZ by attacking initially the side heterocyclic ring linked to the benzimidazole moiety. Further analytical efforts are expected to shed light into the metabolic pathway of TBZ by the isolated consortia.

Based on the results above a possible pathway for the metabolism of TBZ was proposed (Figure 3.37). TBZ degradation occurs *via* cleavage of the thiazole ring which produces 1*H*-benzimidazole-2-carboxamide that is further transformed to 1*H*-benzimidazole-2-carboxylic acid. This molecule would undergo loss of CO_2 to give benzimidazole. The production of benzimidazole from TBZ is considered the most likely metabolic route affected by soil microorganisms, since the thiazole ring in TBZ represents the most labile component of the compound [Zbozinek, 1984].

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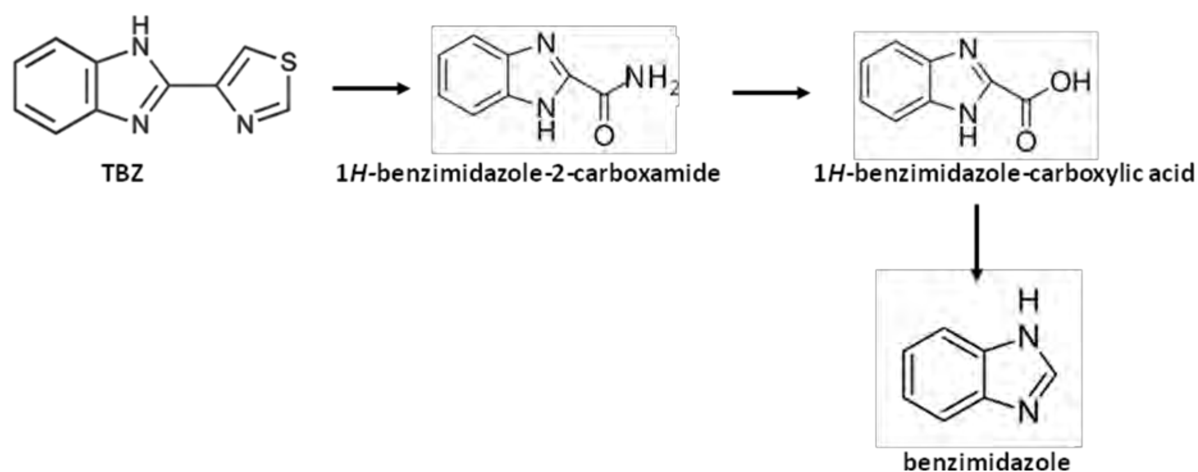

Figure 3.37. A proposed pathway for the metabolism of TBZ by the isolated consortia

Based on this proposed degradation pathway of TBZ, the degradation capacity of the MSMN-based and of the MSM Col1 consortium against 1*H*-benzimidazole-2-carboxamide (amide), benzimidazole, and 1*H*-benzimidazole-2-carboxylic acid (acid) was tested (MSM Col1 consortium only amide and benzimidazole). The degradation of these compounds was followed for three to four successive subculture cycles. The MSMN-based consortium showed a progressively reduced capacity to degrade the amide with approximately 85% degradation occurring in the first cycle, compared to a 70% degradation achieved for the same compound by the MSM Col1 consortium (Figure 3.38). However, the capacity of the MSM Col1 consortium to degrade the amide was halted in the second subculture cycle. The acid was not metabolized by the MSMN-based consortium (MSM Col1 was not tested), while benzimidazole was only partially degraded (50% degradation in 40 days) and only by the MSMN consortium.

Further studies assessed the formation of 1*H*-benzimidazole-2-carboxamide, benzimidazole, and 1*H*-benzimidazole-2-carboxylic acid during TBZ degradation by the two consortia. However no formation of those molecules was evident (data not shown). The lack of detection of those metabolites during TBZ degradation could not be attributed to their instability and transient formation/degradation since the same metabolites appear to be degraded at slow rates by the TBZ-degrading consortia indicating that if they were produced they would have persisted in the medium and thus detected. Overall those data are not in support of the proposed metabolic pathway and suggests an alternative route for the metabolism of TBZ by the isolated consortia. This contrasts to most of the reports published so far on other benzimidazolic compounds such as carbendazim that is usually hydrolyzed to 2-aminobenzimidazole and benzimidazole which is not further transformed and accumulates [Xu *et al.*, 2006]. The lack of benzimidazole formation, which is considered the most recalcitrant part of the benzimidazolic compounds to microbial degradation [Fuchs and Devries, 1978a and 1978b; Sisler, 1982], during

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the degradation of TBZ by the isolated consortia might be a first indication for the complete mineralization of the parent compound, which is desirable from an application point of view. However, on-going studies with ^{14}C-labelled TBZ will further verify this initial hypothesis.

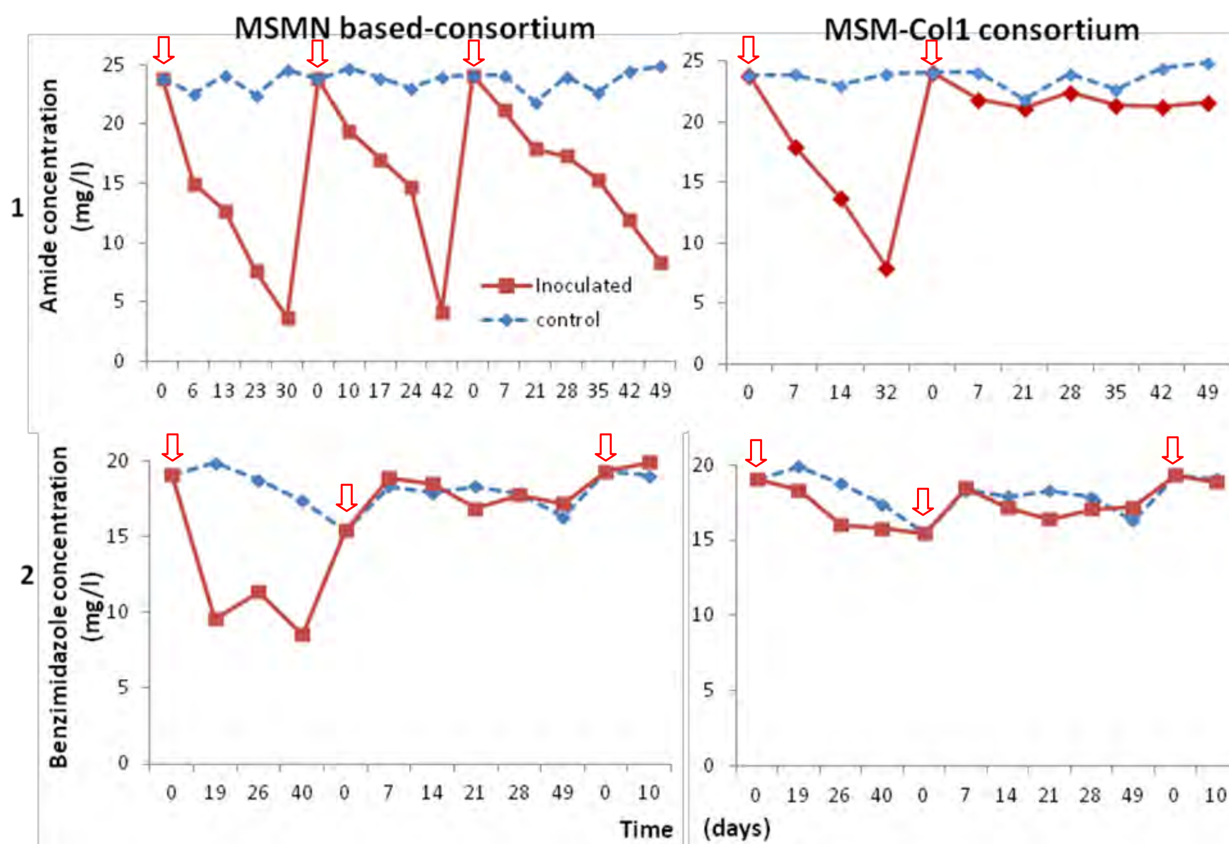

Figure 3.38. Degradation of 1*H*-benzimidazole-2-carboxamide (Lane 1) and benzimidazole (Lane 2) by the MSMN-based and the MSM Col1 consortia. Degradation of TBZ in non-inoculated cultures (control) is also presented. Red arrows indicate the beginning of each subculturing cycle. Data for 1*H*-benzimidazole-2-carboxylic acid are not reported because no degradation occurred.

3.12.3. The degradation of TBZ by the isolated consortia in nutrient rich media

The two consortia were not able to degrade TBZ when grown in LB medium. This suggests that the degradative enzyme(s) are not constitutive but are induced in the presence of TBZ. Thus, the use of TBZ as energy source could be considered facultative and the bacteria synthesize the respective enzymes when TBZ is offered as a C and N source. This was reported also in other studies, in which the pesticides degradation rates slowed down or were completely halted in the presence of other energy sources [Chaudhry and Ali, 1988; Singh *et al*., 2004; Malghani *et al*., 2009]. In contrast, in other cases degradation seemed strictly connected with the co-presence of extra C and N sources in the medium [Ellegaard-Jensen *et al*., 2013], suggesting a co-metabolic degradation process.

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3.12.4. The effect of pH and temperature on the degradation capacity of the TBZ-degrading consortia

The degradation capacity of the MSMN-based TBZ-degrading consortium in a range of pH and temperatures was investigated. The fastest degradation rates of TBZ were measured at neutral to moderately acidic pH (5.5 to 6.5) (Figure 3.39). A slower degradation of TBZ was observed at more acidic (pH=4.5) and more alkaline (pH =7.5) pH, while degradation was completely halted at pH 9. TBZ was stable to abiotic hydrolysis at all the pH levels tested which is in agreement with previous studies [EC, 2001].

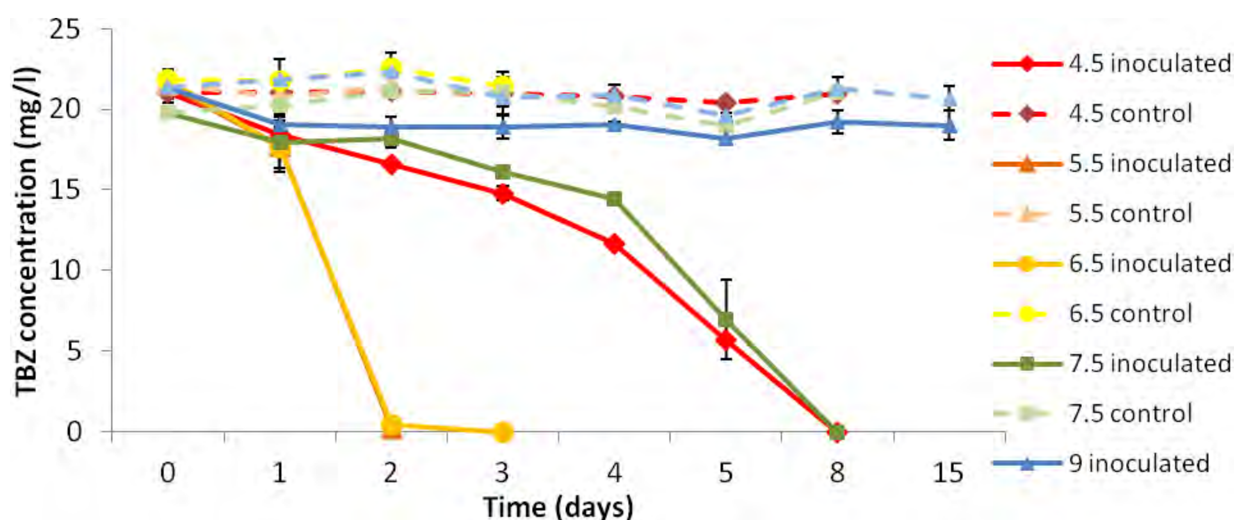

Figure 3.39. The degradation of TBZ by the MSMN-based consortium in a range of pH. The degradation of TBZ in non-inoculated cultures (control) is also reported.

The effect of temperature on the degradation activity of the MSMN-based consortium is shown in Figure 3.40. The highest degradation rates were evident at 26°C and 37°C with almost complete degradation observed in 2 days. Incubation at lower temperatures retarded (15°C) or halted (4°C) the degradation of TBZ. Degradation of the pesticide was negligible in all non-inoculated controls.

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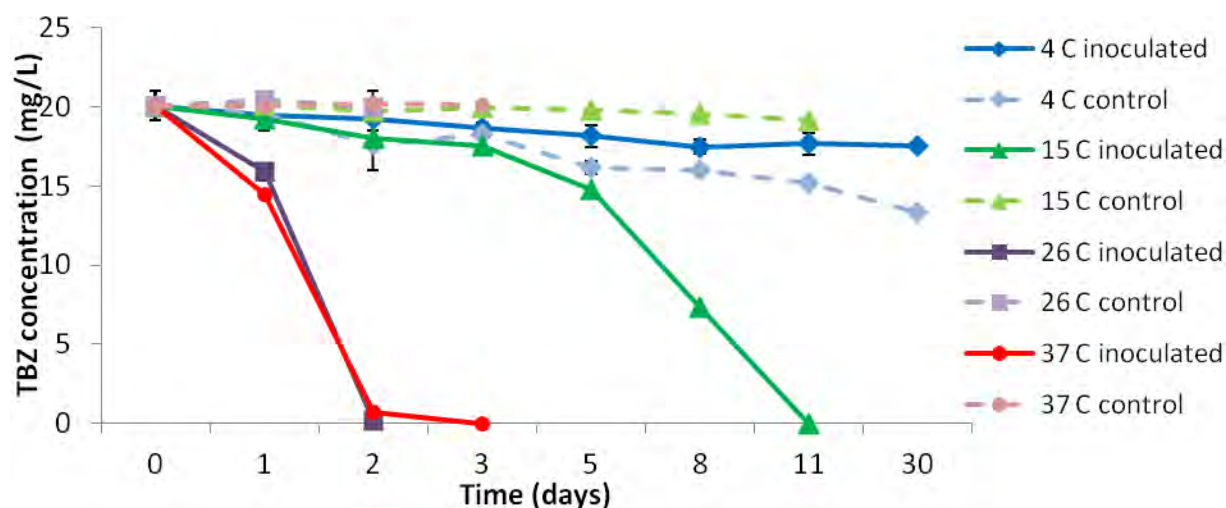

Figure 3.40. The degradation of TBZ by the MSMN-based consortium in a range of temperatures. The degradation of TBZ in non-inoculated cultures (control) is also reported.

These results showed that the isolated TBZ-degrading consortium was able to degrade effectively TBZ in a broad range of pH and temperatures which makes it attractive for further bioremediation and industrial applications.

3.12.5. Assessment of the effect of co-presence of other pesticides and their corresponding degrading bacteria on the degradation capacity of the TBZ-degrading consortium

The degradation of TBZ by the MSMN-based consortium in the presence of diphenylamine (DPA), *ortho*-phenyl-phenol (OPP) and their respective degrading bacteria was evaluated in all possible double and triple combinations (TBZ, TBZ+OPP, TBZ+DPA, TBZ+DPA+OPP). As shown in Figure 3.41, the co-presence of other compounds and their respective degrading-bacteria resulted in changes in the degradation rates of TBZ. Thus in the presence of DPA or OPP (double mixtures) TBZ was completely degraded in 3 and 4 days respectively, compared to 2 days which were needed for the complete degradation of the fungicide when it was added individually in the media. The parallel presence of all three pesticides and their degrading-bacteria slowed down the degradation of TBZ which showed a longer lag phase resulting in complete degradation in 6 days. The evident retardation in the degradation of TBZ by the MSMN-consortium in the presence of other compounds and their respective degrading bacteria could be attributed either to higher competition between the different bacteria inocula in the media (the higher the number of inocula the slower the degradation of TBZ) or to an initial toxicity of OPP or DPA to the TBZ-degrading bacteria which was alleviated upon degradation of those compounds by their respective degraders (DPA and OPP were completely degraded in 1 and 2 days respectively).

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Overall, the MSMN-based TBZ-degrading consortium was able to completely degrade TBZ in the presence of other pesticides and their corresponding degrading microorganisms which is a desirable asset for future bioremediation applications where composite inocula might be applied for the decontamination of wastewaters containing more than one pesticide. In contrast to our TBZ-degrading bacterial consortium, the only previous study regarding biotic degradation of TBZ reported that the *fungus Trametes versicolor* failed to degrade TBZ when exposed to a similar mixture of substances [Karas *et al*., 2011].

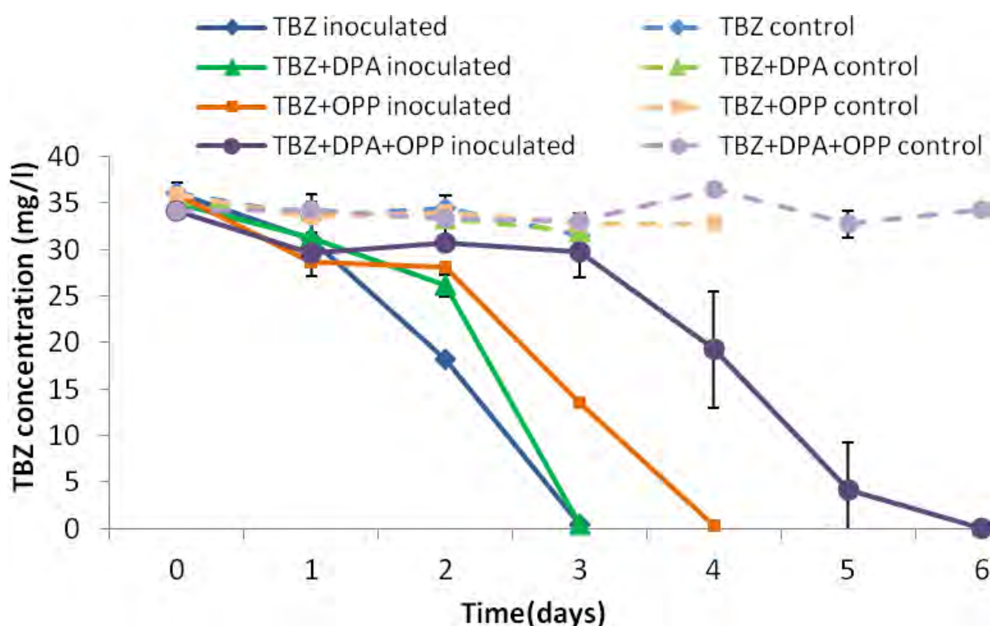

Figure 3.41. The degradation of TBZ by the MSMN-based consortium in the co-presence of diphenylamine (TBZ+DPA), *ortho*-phenyl-phenol (TBZ+OPP), and DPA and OPP (TBZ+DPA+OPP). Apart from the different pesticides, cultures were co-inoculated with the respective pesticide-degrading microorganisms in each case (*Pseudomonas monteilii* for DPA and *Sphingomonas haloaromaticamans* for OPP). The degradation of TBZ in non-inoculated cultures (control) is also reported.

3.12.6. Assessment of the bioaugmentation potential of the TBZ-degrading consortia in soil

The ability of the two consortia to degrade TBZ in soil contaminated with different levels of TBZ (5, 50, and 500 mg/kg) which have been aged before inoculation was evaluated. Both consortia were able to rapidly degrade the lower concentration level (5 mg/kg) of TBZ within 3 days of inoculation, while >80% degradation of TBZ was evident in the inoculated samples amended with the 50 mg/kg dose level at the same time (Figure 3.42). The two consortia managed to degrade 80% of the highest dose rate (500 mg/kg) in 7 days. In the non inoculated soil samples (controls) TBZ dissipation was negligible which is in accordance with the high persistence of this fungicide in soil [EC, 2013; US EPA, 2002]. Considering the high persistence of TBZ in soil [EC, 2013; US EPA, 2002], the isolated consortia showed a great potential for

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their future use in bioaugmentation strategies for the decontamination of soils from disposal sites which exhibit high contamination levels of such persistent fungicides.

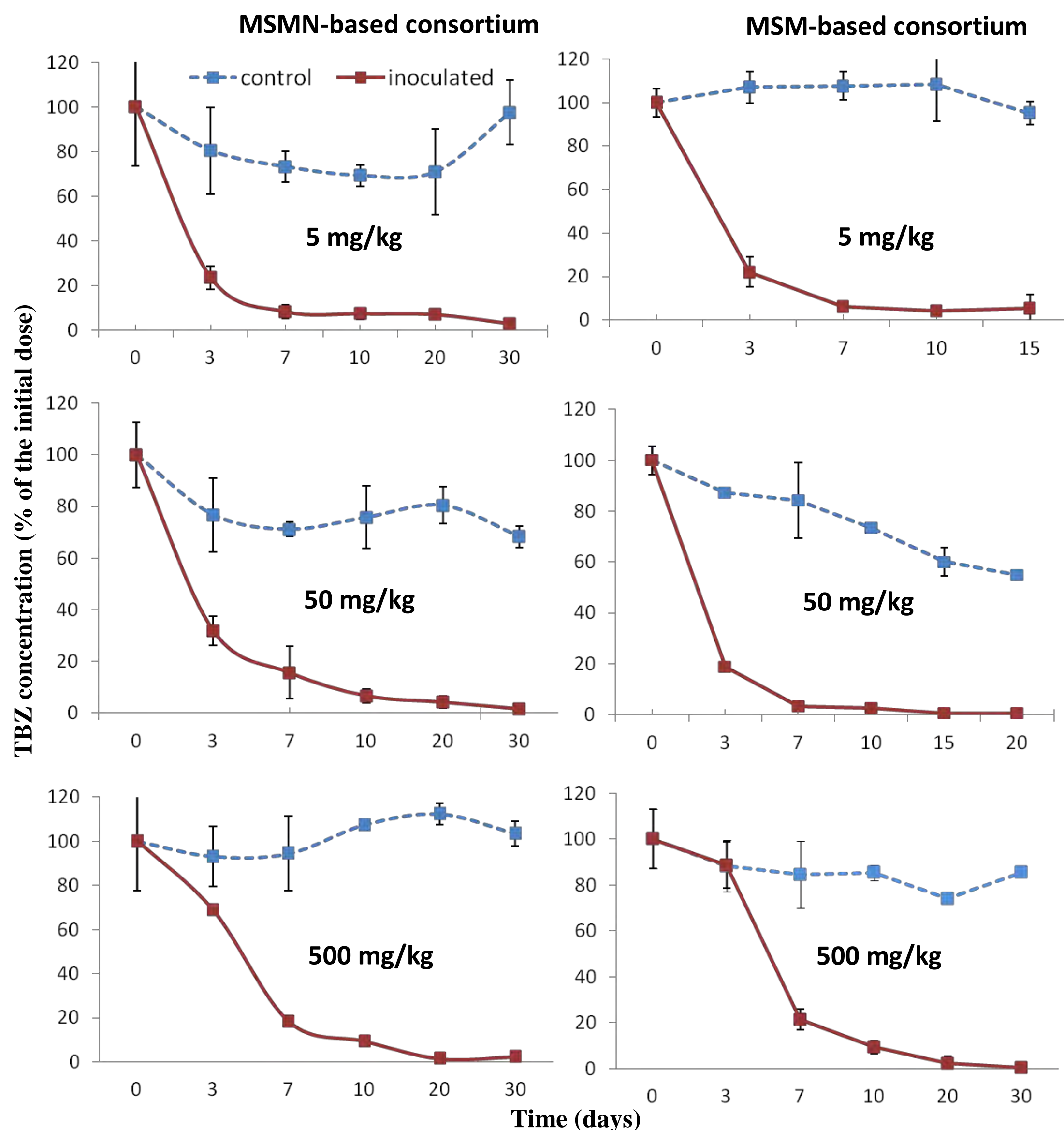

Figure 3.42. Degradation of 5, 50, and 500 mg/kg of TBZ in soil after inoculation with the MSMN- or the MSM-based consortium. The degradation of TBZ in non-inoculated soils (control) is also reported. Each value is the mean of three replicates ± the standard deviation.

3.12.7. Assessing the degrading ability of the TBZ-degrading consortia in BEM and when exposed to composting temperatures

The potential application of the TBZ-degrading consortia in biobed-like systems aiming to accelerate the degradation of persistent molecules like TBZ led us to investigate the degradation capacity of those consortia in liquid media whose nutritional composition resembled those of biobed substrates. For this purpose degradation of TBZ by the isolated consortia was tested in BEM (Biobed Extract Medium) which is a non-selective media containing a range of C, N, and

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energy sources simulating the biobed environment. As shown in Figure 3.43, the MSMN-based consortium degraded TBZ in BEM and MSMN at similar rates, with almost complete degradation observed in 5 days. This result provides a first indication that the TBZ-degrading consortium could be potential used as bioaugmentation carrier for the acceleration of the degradation of TBZ residues retained by biobeds. Indeed previous studies by Omirou *et al.* [2012] have shown that significant amounts of TBZ were retained at the top 30 cm of an on-farm biobed system. The application of TBZ-degrading inocula in that case could accelerate their dissipation precluding any potential release of those residues by the biobeds drainage system.

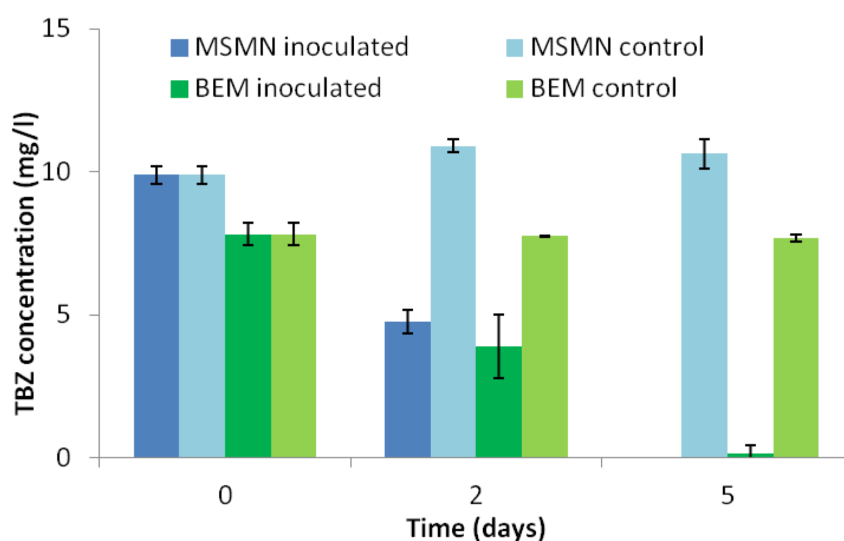

Figure 3.43. The degradation of TBZ in BEM and MSMN inoculated with the MSMN-based consortium. The degradation of TBZ in non-inoculated cultures (control) is also reported.

Upon the end of the life cycle of a biobed system, the biobed substrate commonly contains high pesticide loads which have been retained by the biobed matrix and could be effectively considered as hazardous material requiring decontamination [Castillo *et al.*, 2008]. This is particularly true for persistent chemicals like TBZ. Indeed Omirou *et al.* [2012] showed that considerable amounts of TBZ had been retained and persisted for >120 days in an on-farm biobed system which received wastewaters from fruit-packaging industry. Possible ways of handling this material include dispersal, landfill disposal, or incineration. Some of these techniques can lead to complete depuration but are rather expensive (incineration) or they cannot be considered as terminal processes as they include transfer of the contaminant to another environmental medium. In a pioneering study Torstensson [2000] showed that an 8-month storage period in a protected area of the farmyard could be enough for the reduction of pesticide residues to levels below the detection limits. A more recent study by De Wilde *et al.* [2010] showed that tunnel-composting resulted in a substantial dissipation of most pesticides except

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bifenthrin. The heat generated during the composting process could effectively lead to the degradation of heat-labile compounds contained in the biobed substrate. At the same time, the temperatures that might develop during the thermophilic phase of composting (40-60°C) could be challenging for the survival of mesophilic bacteria. Alternatively, bioaugmentation of those substrates with specific pesticide-degrading bacteria like the TBZ-degrading consortium isolated in our study could be used either as a stand-alone practice or in combination with composting to decontaminate spent biobed substrates and allow their safe environmental disposal.

Based on this an experiment was conducted to investigate the degradation potential and mostly the survival of the MSMN-based TBZ-degrading consortium upon exposure to temperatures prevailing during the composting process. The MSMN-based TBZ-degrading consortium was inoculated in MSMN and BEM liquid cultures which were then incubated for a 28-day period under a temperature profile simulating composting of spent biobed substrate performed by P. Karas (PhD student, Group of Plant and Environmental Biotechnology, University of Thessaly, Greece). As shown in Figure 3.44 temperatures ranged from 25 to 50°C and the thermophilic phase of composting lasted for 4 days. TBZ was completely degraded in both MSMN and BEM before the thermophilic phase was reached (complete disappearance within 7 days). After the 28-days of incubation, cultures were refreshed in the corresponding media which were subsequently incubated in the working temperature of 26°C. However, no degradation of TBZ was observed within the next 28 days (data not shown). The loss of degradation capacity upon exposure to a composting temperature scheme could be attributed to bacterial death. This is in agreement with the general mesophilic nature of our TBZ-degrading consortium. In any case, bioaugmentation of contaminated biobed substrates could be an effective measure by itself. However, if bioaugmentation with the TBZ-degrading consortium is applied in combination with composting the degrading bacteria should be applied in advance in order to effectively perform their task before the thermophilic phase of composting prevails and eliminates them.

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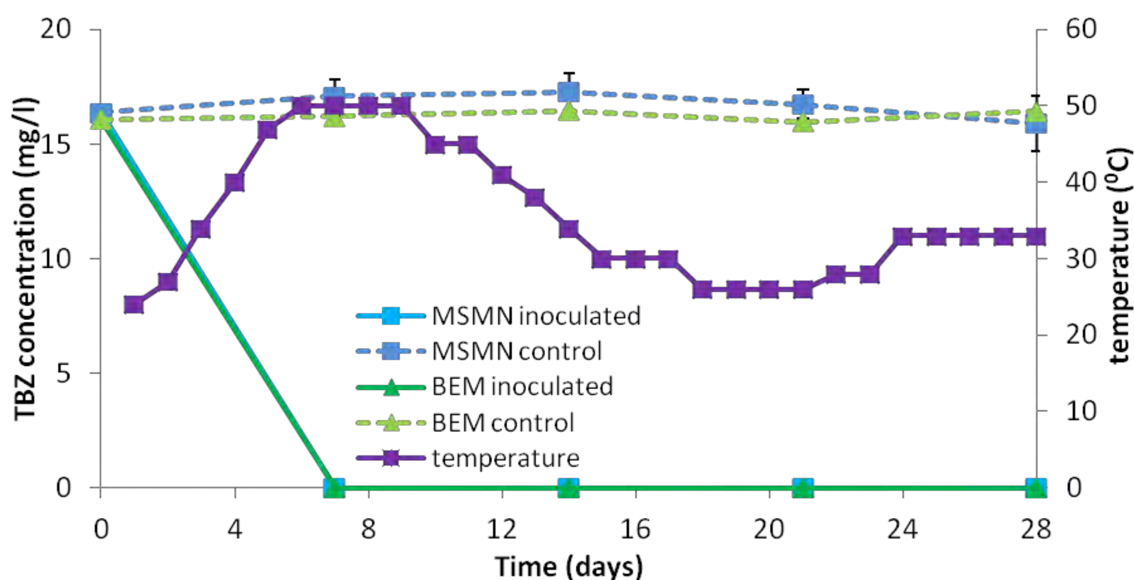

Figure 3.44. The degradation of TBZ in MSMN and BEM inoculated with the MSMN-based consortium and incubated under fluctuating temperatures (z axis) simulating a composting temperature scheme. The degradation of TBZ in non-inoculated cultures (control) of the same media is also shown.

4. Conclusions and Future work

TBZ is a fungicide widely used in the fruit packaging industry to protect fruits from post-harvest fungal infestations. Due to their water-based mode of application, large volumes of wastewaters containing high concentrations of TBZ are produced and their direct environmental release constitutes a major threat for natural resources including soil (considering the long persistence of TBZ in soil) and surface water systems (considering the high toxicity of TBZ to aquatic organisms). The need for the development and implementation of an economic, efficient, and sustainable method for the treatment of those wastewaters is pointing towards a biological treatment method based on tailored-made inocula with specialized degrading capacities against the few pesticides currently in use in fruit packaging plants including TBZ. Our work resulted in the isolation of the first microorganisms able to rapidly degrade the non-biodegradable fungicide TBZ. The first task of the work was to isolate and identify bacteria able to rapidly degrade TBZ and it was achieved through the following steps:

- Two TBZ-degrading bacterial consortia were isolated from soil collected from a disposal site receiving wastewaters from the fruit packaging industry. The composition of the two consortia was stabilized upon prolonged enrichment and was very similar with the major members belonging to α- (*Bosea, Shinella, Oligotropha, Sphingomonas*), β- (*Methylobacillus, Methilibium*) and γ-proteobacteria (*Pseudomonas).*
- Continuous selection and subculturing in the presence of different compounds and antibiotics resulted in a two-member consortium composed of two α-proteobacteria (*Shinella* sp. and *Sphingomonas* sp.), which was able to rapidly degrade TBZ.

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- Molecular approaches (DGGE, cloning, q-PCR) revealed that the bacterial member responsible for the degradation of TBZ in both consortia was a *Sphingomonas* sp. strain (phylogenetically closer to *S. wittichii*) although various attempts to obtain it in pure culture were not successful due to its limited capacity to grow on the agar media tested.

The second task was the characterization of the degrading capacity of the isolated bacteria and it was achieved through the following steps:

- The bacterial consortia a) were able to effectively degrade up to 750 mg/l of TBZ in liquid cultures, b) showed high degradation capacity in a wide range of pH (4.5-7.5) and temperatures (15-37°C), c) degraded TBZ in the presence of other pesticides (OPP, DPA) and their respective degrading bacteria, and d) effectively degraded spillage level concentrations of TBZ (500 mg/kg) when inoculated in a soil containing aged residues of TBZ. All those assets suggest that the isolated bacteria had a high potential for future application in the biological treatment of wastewaters from the fruit packaging industry but also in the bioremediation of pesticide-contaminated disposal sites nearby fruit-packaging plants.
- The metabolic pathway of TBZ by the isolated consortia was not elucidated although our results provided first indications that the metabolism of TBZ is initiated *via* cleavage of the thiazole ring.

There are still questions unanswered regarding the microbial degradation of TBZ and future work aims to clarify those issues:

- The purification of the *Sphingomonas* strain which is responsible for the degradation of TBZ *via* treatment of the two-member consortium with a range of different antibiotics hoping to obtain the degrading bacterium in pure culture. Alternatively, other solidified agents instead of agar (e.g. gellan gum) will be tested.
- The functional analysis and identification of the genes/enzymes controlling the microbial degradation of TBZ. Comparative proteomic and genomic analysis of the TBZ-degrading bacterium (preferably in pure form) will shed light into the genetic mechanism of TBZ degradation and will facilitate the elucidation of the metabolic pathway of TBZ which is mostly unknown. This will be further verified by advanced analytical tools (LC-MS/MS) aiming to clarify the detoxification nature of the degradation imposed by the isolated bacteria

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- Advanced molecular techniques (Stable Isotope Probing of ^{13}C-labelled TBZ or methylamine) will explore the role of the other members of the TBZ-degrading consortia on TBZ metabolism.
- Considering the urgent need for the development of a biological treatment system for the depuration of wastewaters from the fruit-packaging industry, the development and optimization of inocula based on the already available TBZ-degrading bacteria and their application in pilot treatment systems would constitute an important future task.

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Chapter 4

Isolation and characterization of bacteria able to degrade imazalil

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1. Introduction

Imazalil (IMZ) is a systemic fungicide mainly used in fruit-packaging plants for the control of postharvest infestations by fungi like *Penicillium digitatum* and *Penicillium italicum*, *Diaporthe citri*, *Diplodia* spp., *Alternaria citri*, *Botrytis* spp., and *Phomopsis* spp. IMZ is particularly effective against green mold (*Penicillium digitatum*), including benzimidazole-resistant strains [EC, 2009; Wardowski and Brown, 1991] and it is currently authorized for use in USA [US EPA, 2003] and EU [EC, 2009].

During fruit packaging processes, IMZ can be applied on fruits by dipping, drenching, spraying, or waxing. At EU level, the recommended application doses of IMZ on citrus fruits range between 100 to 2000 mg/l depending on the mode of application [EFSA, 2010]. After its application, large amounts of IMZ-containing wastewaters are produced and their direct disposal into municipal sewers or natural water resources entails a serious risk for the receiving ecosystems. This risk was recognized by the EC which granted authorization to IMZ under the clause that "*appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste, are put in place. Conditions of authorisation should include risk mitigation measures, where appropriate*" [EC, 2010]. So far different physicochemical treatments of IMZ-containing wastewater have been tested at lab scale including ozonation [Genena *et al*., 2011], heterogeneous photocatalysis with different photocatalysts [Santiago *et al*., 2013; Hazimea *et al*., 2013], and electrochemical degradation by anodic oxidation [Errami *et al*., 2012]. Degradation of IMZ by laccases in the presence of natural mediators has also been demonstrated [Maruyama *et al*., 2007]. However, the only treatment method that has reached the application level is the CONTROL TEC ECO® system which achieves pesticide removal *via* adsorption onto granular activated carbon and a pre-treatment filtration of the contaminated wastewater through bags of mesh filled with a diatom soil for eliminating solids. [Garcia Portillo *et al*., 2004]. However its high establishment and maintenance cost has precluded its wider uptake by the fruit-packaging industry. Despite legislation and in the absence of appropriate treatment systems to detoxify those wastewaters, effluents from many fruit-packaging plants are still discharged directly onto adjacent fallow fields or into nearby creeks, evaporation ponds, and sewage treatment plants. Therefore an economic, simple to operate, and sustainable method for treating those wastewaters is required.

Biological treatment of agroindustrial effluents is considered an appealing method. In a pioneering study Omirou *et al*. [2012] demonstrated that discharge of IMZ-containing wastewater in modified on-farm biopurification systems like biobeds resulted in a successful

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depuration of the fungicide-contaminated effluents (TBZ, OPP, and IMZ). However, more than 40% of IMZ contained in the effluents was still detected at the upper layer of the biobed at the end of its operation indicating a general resistance to microbial degradation. Indeed, previous studies have shown that IMZ is classified as not readily biodegradable with $t_{1/2}$ in a loam soil of 166 days [US EPA, 2003]. In a recent study Karas *et al*. [2011] showed that the white rot fungi *Trametes versicolor*, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium* were able to degrade a concentration of 10 mg/l of IMZ within 10 days. However those fungi were not equally effective in degrading IMZ at concentration levels relevant to wastewaters detoxification (50 mg/l). Despite their high versatility in degrading organic pollutants, white rot fungi are characterized by slow growth, limited competitivity in natural and industrial environments, and require particular conditions (N limitation) in order to activate their lignolytic enzymatic system. Thus bacteria could be a good alternative for the degradation of IMZ [Gao *et al*., 2010].

Based on these considerations, the main aim of this study was to isolate and characterize bacteria able to degrade IMZ. Such bacteria could be used a) as starting inocula in biofiltration systems for the depuration of wastewaters from the fruit packaging industry, b) for the remediation of the soil of disposal sites contaminated with high levels of IMZ, and c) for the bioaugmentation of biobed systems which have shown a good potential for the treatment of those wastewaters.

2. Materials and methods

2.1. Pesticide

Imazalil analytical standard (Pestanal®, 99.8% purity) was purchased from Fluka, Sigma-Aldrich. A methanol stock solution of 1000 mg/l of IMZ was initially prepared and appropriate dilutions in methanol were used for the construction of the calibration curve used for the quantitation of IMZ residues by HPLC-UV (see Chapter 2). A 100 mg/l aqueous solution of IMZ was used for the preparation of growth media amended with IMZ. More details are given in Chapter 2.

2.2. Pesticide extraction from liquid media and soil

The extraction of IMZ from liquid media was as described in Chapter 2. For the extraction of IMZ from soil 5 g of soil were mixed with 1 ml of NaOH 1N and 10 ml of methanol (HPLC gradient grade). Samples were shaken for 30 min. at 26°C and centrifuged at 11000 rpm for 5 min., the supernatant was collected in a glass bottle, and the soil resuspended in another 10 ml of methanol. After 30 min. shaking and centrifugation, the supernatant was recovered and

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combined with the supernatant from the first extraction cycle resulting in a final extraction volume of 20 ml. The extract was passed through a syringe filter (0.45μm PTFE Syringe Filter) and kept at -20°C until HPLC analysis.

2.3. Analysis of IMZ residues in HPLC

The residues of IMZ in the final extract were determined in an HPLC-UV system described previously. In particular, IMZ residues were detected at 204 nm using a mobile phase of 80:20 methanol: NH_3 solution 0.25% (by volume). Under those conditions the retention time of IMZ was 5 min.

2.4. Enrichment culture for the isolation of IMZ degrading bacteria

In total three attempts were made to isolate IMZ-degrading *via* enrichment cultures in the selective minimal media MSMN and MSM and in the non-selective medium SEM. In the former, IMZ served as the sole C or C and N source respectively, whereas SEM constitutes a non-selective medium which simulates as much as possible the nutritional status of soil.

2.4.1. First Enrichment Culture

In the first attempt, soils from a wastewater disposal site in Cyprus (the same used for the isolation of thiabendazole-degrading bacteria, Chapter 3) and a soil collected from a similar disposal site in Agià, Larissa, Greece, were used as inocula of the enrichment cultures. Soils were stored at 4°C for more than 2 months and for this reason they were subjected to repeated (seven in total) laboratory IMZ additions (20 mg/kg IMZ) on 20-day intervals to stimulate the IMZ-degrading fraction of the soil microbial community (see Chapter 2). At the end of the 7th treatment cycle, enrichment was initiated by inoculating fresh IMZ-containing media (20 ml) with 0.5 g of soil. Three inoculated replicates and two not-inoculated controls were prepared and incubated in an orbital shaker at 26°C/160 rpm. Immediately prior to inoculation and at regular intervals thereafter aliquots from the media were removed aseptically and the concentration of IMZ was determined by HPLC. At the end of the first enrichment cycle an aliquot of the culture (1 ml) was used to inoculate a fresh culture of the same medium and the bottles were incubated again under the same conditions. The same procedure was repeated once more resulting in the establishment of three enrichment cycles.

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2.4.2. Second enrichment culture

In the second enrichment attempt fresh soils collected from the same disposal sites from Cyprus and Agià were utilized aiming to minimize storage time and get access to a non-stressed microbial community in the soil. After sieving, soils were subjected to a single application of IMZ (10 mg/kg soil) and they were incubated at 25°C for a period of 15 days. Subsequently the two soils were used to inoculate fresh IMZ-containing media exactly in the same way described in the first experiment. Three enrichment cultures were established and pesticide degradation was determined *via* HPLC at different time after inoculation.

2.4.3. Third enrichment culture

In a third enrichment attempt aerobic activated sludge obtained from the municipal sewage treatment plant of the city of Larissa was used as a source for isolating IMZ-degrading bacteria. This time enrichment cultures were only established in MSMN medium+20 mg/l of IMZ. Three replicates were inoculated with an aliquot of the liquid sludge (1 ml) and degradation was measured by HPLC at 0, 7, 14, 21, and 28 days. A second enrichment cycle was established following the same procedure as described above and the degradation of IMZ was followed for further 14 days. In all enrichment steps two non-inoculated controls were always included.

3. Results and Discussion

No appreciable degradation of IMZ was observed in the first enrichment cycles in the three attempts to isolate IMZ-degrading bacteria. Despite the absence of degradation in the first cycle, the cultures were passed into subsequent enrichment cycles to eliminate any possible inhibitors that might be contained in the soil inoculated in the first cycle and hampered the degradation of IMZ. However, no appreciable degradation of IMZ was observed (Figures 4.1, 4.2, and 4.3).

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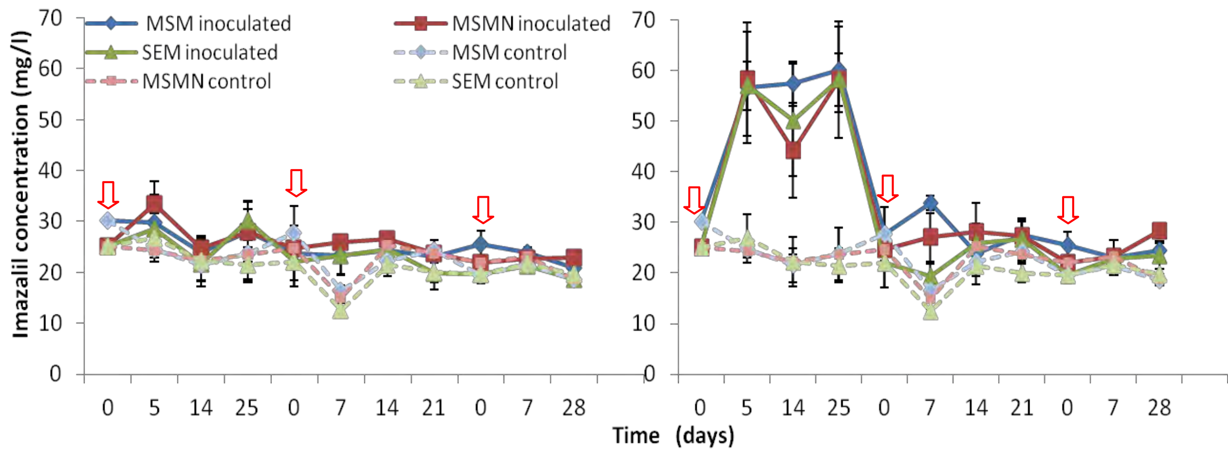

Figure 4.1. The degradation of IMZ in enrichment cultures in MSM, MSMN, and SEM inoculated with soils (first attempt) collected from Cyprus (left) and Agià, Larissa (right). The degradation of IMZ in three successive enrichment cycles is presented. The degradation of IMZ in corresponding non-inoculated controls is also showed (dashed lines). Red arrows indicate the beginning of each enrichment cycle.

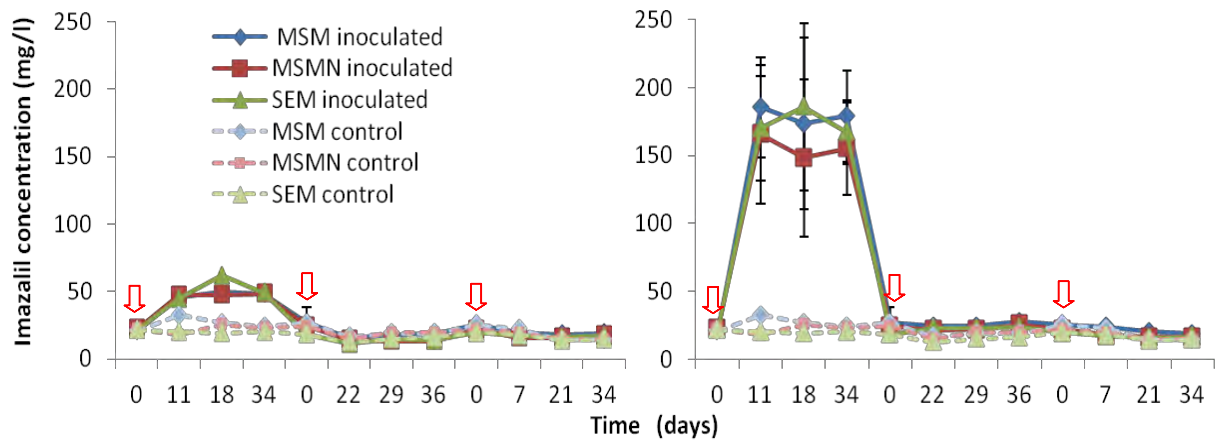

Figure 4.2. The degradation of IMZ in enrichment cultures in MSM, MSMN, and SEM inoculated with fresh soil (second attempt) collected from Cyprus (left) and Agià, Larissa (right). The degradation of IMZ in the following enrichment cycles is shown. The degradation of IMZ in the non-inoculated controls is also presented (dashed lines). Red arrows indicate the beginning of each enrichment cycle.

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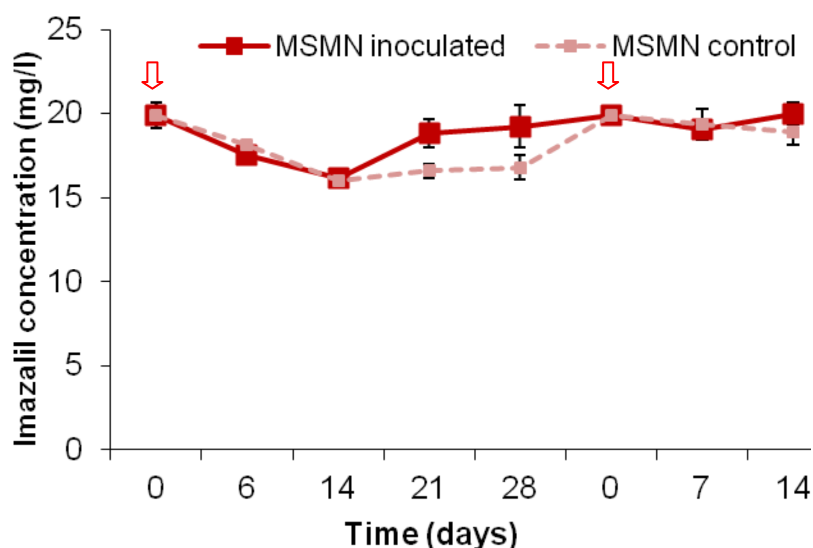

Figure 4.3. The degradation of IMZ in enrichment cultures in MSMN inoculated with aerobic activated sludge obtained from the municipal sewage treatment plant of the city of Larissa. The degradation of IMZ in the two enrichment cycles is shown. The degradation of IMZ in the non-inoculated controls is also presented (dashed lines). Red arrows indicate the beginning of each enrichment cycle.

Interestingly, in the first enrichment cycle inoculated with soil from Agià, Larissa, a substantial increase, instead of a decrease, in the concentration of IMZ was observed upon inoculation (Figures 4.1 and 4.2). This was more evident in the second enrichment experiment (Figure 4.2), where fresh soil from the disposal site from Agia was used. In this case, IMZ concentrations in the inoculated enrichment cultures reached levels of 150-180 mg/l compared to the controls where no change in the concentration of IMZ within the time frame of the experiment was observed. These results indicated that the soil inoculum might have been responsible for the increase in the concentration of IMZ in the enrichment culture. This was verified by extraction and analysis of IMZ residues in the soils used as inoculum which showed that the levels of IMZ in the Agià soil were > 600 mg/kg compared to 150 mg/kg which were found in the soil from the disposal site in Cyprus. For the latter, only a slight increase in IMZ concentration in the enrichment cultures was detected. It is probable that the excess of IMZ residues which were not strongly adsorbed onto soil particles were resuspended from soil into the liquid culture resulting in the observed increase of IMZ concentration in the medium. It is anticipated that this high concentrations of IMZ in the soil from the disposal sites did not allow the establishment and proliferation of soil microorganisms with the capacity to degrade IMZ thus precluding the possibility of isolating bacteria with this phenotype. Those high concentrations of IMZ detected in the soil should have been the result of the continuous disposal of IMZ-containing wastewaters from the fruit-packaging industries and it is in agreement with the high soil persistence of IMZ [EC, 2009; US EPA, 2003]. Those disposal sites constitute a major

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environmental problem which could enable the gradual movement of IMZ residues to adjacent surface and groundwater systems *via* surface runoff or leaching respectively. This risk becomes more acute considering the high toxicity of IMZ to non-target aquatic organisms [EC, 2009].

No degradation of IMZ was observed in MSMN inoculated with activated sewage sludge from the municipal treatment plant of the city of Larissa. Although the microbial biomass found in sewage sludge is characterized by a versatile capacity to degrade organic pollutants, no IMZ-degrading capacity was evident in the enrichment cultures. This could be attributed to the lack of previous exposure of the microflora of the sewage sludge from the municipal wastewater treatment facility of the city of Larissa to IMZ which is a fungicide not expected to be found in municipal wastewaters of the city.

4. Conclusions and Future work

Our attempts to isolate IMZ-degrading bacteria from relevant polluted soils and sewage sludge were not successful in agreement with the limited biodegradability and high environmental persistence of this molecule [EC, 2010]. Our findings however further reinforce the urgent need for the development and implementation of a system that can be used also by small to medium fruit-packaging industries to treat wastewaters containing the fungicide IMZ and other relevant pesticides. Further attempts will focus on the isolation of IMZ-degrading bacteria from other soils showing lower levels of IMZ pollution.

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Chapter 5

Isolation and characterization of bacteria able to degrade *ortho*-phenyl-phenol

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1. Introduction

Ortho-phenyl-phenol (OPP) or 2-phenyl-phenol and its sodium salt are used in the postharvest treatment of fruits (citrus and pears) to control fungal infestations during storage and shipment. OPP is also used as a sanitizer in the fruit packaging plants to kill fungal spores in dump tanks, on bins, and in flume water [Janisiewicz, 1999]. OPP is active against different species of fruit-rotting fungi [EC, 2009]. In the EU, 2-phenyl-phenol has been granted authorization for various postharvest uses and listed in Annex I of the Directive 91/414 [EC, 2010]. OPP is applied in fruit-packaging plants either *via* waxing, dipping, and foam curtain treatments [EC, 2010b] and its concentration in the water used for the treatment of products cannot exceed the 600 mg/l [EFSA, 2008].

OPP is not persistent in the environment and exhibits moderate adsorption onto soil particles and thus it is characterized as moderately mobile. The major degradation route appears to be through biodegradation in aerobic and anaerobic environments [EFSA, 2008], with observed $t_{1/2}$ values ranging from 3 hours to 3 weeks [US EPA, 2006]. Monitoring studies have shown the frequent detection of OPP residues in several environmental compartments including groundwater and drinking water [IARC, 1983], rivers and lagoons [Jonkers *et al.*, 2010], wastewater [Rudel *et. al.*, 1998], and marine sediments [Aguera *et al.*, 2003]. In addition, residues of OPP have been found in canned beverages [Coelhan *et al.*, 2006 and 2009]. So even though OPP is generally considered readily biodegradable under aerobic conditions, its high application rates and continuous introduction into the environment may lead to its gradual accumulation and a "pseudopersistent" situation [Madsen *et al.*, 2001].

Considering the large wastewater volumes produced by the application of OPP, appropriate management practices to handle these wastewaters are required [EC, 2009b]. This need has been laid down by the EC which provided authorization for the use of OPP under the clause *that appropriate measures would be taken to handle the wastewaters produced by the application of OPP before their final environmental discharge* [EC, 2009b]. So far little progress has been done towards the development of such treatment system. In laboratory studies it has been demonstrated that OPP degradation can be photocatalysed by TiO_2 and ZnO [Khodja *at al.*, 2001], but further studies on a prototype or industrial scale system were not produced. Nowadays, direct disposal into creeks, sewage treatment plants, and evaporation ponds, together with land spreading on adjacent field sites, are the most common ways of wastewater management. The use of biobed systems for the depuration of such OPP-containing wastewaters was evaluated with promising results [Omirou *et al.*, 2012]. However, the extreme volumes of wastewaters produced by the citrus fruit packaging plants, where OPP is mostly used, could not

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be handled by a biobed system within a season and a more sophisticated biofiltration system inoculated with tailored made OPP-degrading bacterial inocula is probably a more conducive solution to the given problem.

A few microorganisms able to degrade OPP have been isolated in the past including fungi and bacteria. A fungal strain of *Irpex lacteus* could completely degrade 100 mg/l of OPP within a day [Moon and Song, 2012]. In a similar study, Karas *et al*. [2011] showed that the white rot fungi *Trametes versicolor* and *Pleurotus ostreatus* were able to rapidly degrade OPP through their ligninolytic enzymatic system including laccases and manganese-peroxidases. However, the use of fungi in biological treatment systems is a challenging task due to their limited competitiveness and their limited capacity to degrade other fungicides used in the fruit packaging industry like TBZ [Karas *et al*., 2011]. Regarding bacteria so far two strains have been isolated which were able to degrade OPP, a *Pseudomonas testosterone* strain B-356 [Sondossi *et al.*, 1991] and a *Pseudomonas azelaica* strain HBP1 [Kohler *et al.*, 1988]. The former strain was isolated as a biphenyl degrader and thus the metabolism of OPP was not studied in detail. In contrast, the metabolic pathway of OPP by the latter strain was fully elucidated [Kohler *et al.*, 1993] and the genes involved in the degradation of OPP (the *hbpCAD* genes) and the relative enzymes were detected and isolated [Jaspers *et al.*, 2000 and 2001].

Based on this, our study aimed I) to isolate and identify new bacteria able to rapidly degrade the fungicide OPP and II) to characterize the degrading capacities of the isolated microorganisms in order to get an overview of their application potential for bioremediation.

2. Materials and methods

2.1. Pesticides and other chemicals

OPP (Pestanal®, analytical standard, 99.9% purity) was purchased from Fluka, Sigma-Aldrich. A 1000 mg/l methanol stock solution was initially prepared and used for analytical purposes. A filter sterilized (0.22 μm, MS® PES Syringe Filter, Membrane Solution) aqueous solution of OPP (100 mg/l) was also prepared and used for the preparation of OPP – amended media. Other molecules used in the present study including 2,3-dihydroxybiphenyl (≥98% purity), benzoic acid (A.C.S. reagent, ≥99.5% purity), 1,2-dihydroxybenzene (catechol) (ReagentPlus®, ≥99% purity), and diphenylamine (Pestanal®, analytical standard, 99.9% purity, Fluka) were all purchased from Sigma-Aldrich. For all the above compounds methanol and water stock solutions were prepared as described for OPP.

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2.2. Growth media

The growth media used for isolation of OPP-degrading bacteria *via* enrichment cultures were I) the minimal medium MSMN (OPP does not contain N) and II) the non-selective Soil Extract Medium (SEM), both prepared as described in Chapter 2. Appropriate aliquots of the sterilized water stock solution of OPP (100 mg/l) were added to the media to reach a final pesticide concentration of 20 mg/l, unless otherwise stated. Agar plates were prepared by addition of 15 g of agar per litre of medium before sterilization.

LB was routinely used in liquid or solidified (agar plates) form. Further media were also utilized in the present work in order 1) to isolate and purify the members of the initially isolated OPP-degrading consortia (a, b, c, d), 2) to assess eventual bacterial nutritional deficiencies and to maximize the degradation potential of the isolates (e, f, g, h), and 3) to characterize the degradation capacity of the OPP-degrading bacteria (i). Their composition and preparation is described below:

a) **Modified Sphingomonas-specific L9 minimal salt medium** [Yim *et al.*, 2010]: the components of the medium are listed in Table 3.2, Chapter 3. After 15 min. of autoclaving and cooling, the filter sterilized trace element solution and appropriate amounts of aqueous filter sterilized solutions of the antibiotics streptomycin and piperacillin (100000 mg/l) were added to obtain final concentrations of 100 and 50 μg/ml respectively. The pH of the medium was adjusted to 7;
b) **Pseudomonas basal mineral medium (PB)** [Atlas, 2005]: the components of the medium are shown in Table 5.1. The trace element solution was filter sterilized and added to the autoclaved (15 min.) medium. The pH was adjusted to 7.2;
c) **Medium 465 (Leibniz Institute DSMZ)** [available at http://www.dsmz.de]: the components of the medium are listed in Table 5.2. The trace elements solutions SL-4 and SL-6 were filter sterilized. SL-4 was added to the final medium after 15 min. of autoclaving and cooling. The pH of the medium was adjusted to 7.25;
d) **Medium 660 (Leibniz Institute DSMZ)** [available at http://www.dsmz.de]: the components of the medium are listed in Table 5.3. The trace element solution SL-7 and the $CuSO_4$ 1M solution were filter sterilized and added after heat sterilization of the medium (15 min.) and cooling;
e) **Mandelbaum medium** [Mandelbaum *et al.*, 1993] **plus casamino acids**: the composition (Table 3.1) and the preparation of the medium are described in Chapter 3. The medium was supplemented with 0.15 g/l of casamino acids (casein hydrolysate for microbiology, Fluka, Sigma-Aldrich) added before autoclaving [Onneby *et al.*, 2013];

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f) **MSMN + casamino acids:** 0.15 g/l of casamino acids were added before autoclaving;

g) **MSMN + vitamins**: 20 ml of a filter sterilized vitamin solution [(mg/l) 5 thiamine-HCl; 2 biotin; 2 folic acid; 10 nicotinamide; and 10 pyridoxine-HCl] was added in a liter of medium after autoclaving and cooling;

h) **MSMN + casamino acids + vitamins:** 0.15 g/l of casamino acids were added before autoclaving and 20 ml of the filter sterilized vitamin solution described above were added in a liter of medium after sterilization.

i) **Biobed Extract Medium (BEM):** prepared as described in Chapter 2.

In all the listed media OPP was added as aliquots of a filter sterilized aqueous solution (100 mg/l) after autoclaving and cooling. Corresponding solid media were obtained by adding before autoclaving 15 g of agar per liter of medium as described in Chapter 2.

Table 5.1. Composition of the PB medium.

Component	**Concentration (g/l)**
K_2HPO_4	12.5
KH_2PO_4	3.8
$(NH_4)_2SO_4$	1
$MgSO_4 \cdot 7H_2O$	0.1
Trace element solution	5 ml

Trace element solution:

Component	**Concentration (g/l)**
H_3BO_3	0.232
$ZnSO_4 \cdot 7H_2O$	0.174
$FeSO_4(NH4)_2 \cdot 6H_2O$	0.116
$CoSO_4 \cdot 7H_2O$	0.096
$(NH4)_6Mo_7O_{24} \cdot 4H_2O$	0.022
$CuSO_4 \cdot 5H_2O$	0.0008
$MnSO_4 \cdot 4H_2O$	0.0008

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Table 5.2. Composition of the medium 465.

Component	**Concentration (g/l)**
$Na_2HPO_4 \cdot 2H_2O$	3.5
K_2HPO_4	1
$MgCl_2 \cdot 6H_2O$	0.1
$Ca(NO_3)_2 \cdot 4H_2O$	0.05
Trace element solution SL-4	1 ml

Trace element solution **SL-4**:

Component	**Concentration (g/l)**
EDTA	0.5
$FeSO_4 * 7H_2O$	0.2
Trace element solution SL-6	100 ml

Trace element solution **SL-6**:

Component	**Concentration (g/l)**
$ZnSO_4 \cdot 7H_20$	0.1
$MnCl_2 \cdot 4H_2O$	0.03
H_3BO_3	0.3
$CoCl_2 \cdot 6H_2O$	0.2
$CuCl_2 \cdot 2H_2O$	0.01
$NiCl_2 \cdot 6H_2O$	0.02
$Na_2MoO_4 \cdot 2H_2O$	0.03

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Table 5.3. Composition of the medium 660.

Component	**Concentration (g/l)**
Tris	6.06
NaCl	4.68
KCl	1.49
NH_4Cl	1.07
Na_2SO_4	0.43
$MgCl_2$*6H_2O	0.2
$CaCl_2$*2H_2O	0.03
Na_2HPO_4*12H_2O	0.23
Fe(III)(NH4) citrate	0.005
$CuSO_4$ (1M)	2,5 ml
Trace element solution SL-7	1 ml

Trace element solution **SL-7:**	
Component	**Concentration (mg/l)**
HCl (25%)	1 ml
$ZnCl_2$	70
$MnCl_2$*4H_2O	100
H_3BO_3	60
$CoCl_2$*6H_2O	200
$CuCl_2$*2H_2O	20
$NiCl_2$*6H_2O	20
Na_2MoO_4*2H_2O	40

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2.3. Antibiotic stocks

Stock solutions of the antibiotics used were prepared as described in Chapter 2. The concentrations of antibiotics used in the present study are listed in the Table 5.4. Streptomycin concentration in the medium was 200 μg/ml, but when combined with piperacillin the concentration was adjusted to 100 μg/ml. Antibiotics were added after autoclaving and cooling.

Table 5.4. Working concentration of the antibiotics used in the present work.

Antibiotic	Working concentration (μg/ml)
Amikacin	16
Ampicillin	100
Ciprofloxacin	2
Colistin	2
Doxycycline	25
Lincomycin	10
Nalidixic acid	23
Penicillin	300
Piperacillin	50
Polymyxin B	10
Streptomycin	100/200
Trimethoprim	20
Vancomycin	200

2.4. Pesticide extraction and HPLC analysis

The method used for the extraction of OPP from liquid media was described in Chapter 2. OPP residues were extracted from soil as follows: 10 g of soil were shaken for 1.5 hour at 26°C in 25 ml of acetonitrile (ACN, HPLC Gradient Grade). After 5 min. centrifugation at 11000 rpm, the supernatant was collected in a glass bottle. After filtration (0.45μm PTFE Syringe Filter), the extract was kept at -20°C until HPLC analysis.

OPP residues were detected by HPLC-UV at 254 nm. Separation of OPP was achieved in a Grace Smart RP column (details given in Chapter 2) with a mobile phase composed of 55:44.5:0.5 of ACN:water:25% NH_3 solution (by volume). Under these conditions the retention time (Rt) of OPP was 3.4 min. With the CNW Athena column the liquid phase was modified (70:29.5:0.5 ACN:water:25% NH3 solution) to maintain the same retention time. The HPLC conditions used for the detection of the other molecules tested were as follows:

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a) **Benzoic acid**: mobile phase 60:40 ammonium acetate solution (0.4 g/l ammonium acetate, pH 4.2):ACN and detection at 228 nm (Rt= 2.2 min);
b) **Catechol**: mobile phase 60:40 water:ACN + 0.1% acetic acid glacial and detection at 276 nm (Rt=2.5 min.);
c) **2,3-dihydroxybiphenyl**: mobile phase 60:40 water:ACN + 1% acetic acid glacial and detection at 254 nm (Rt=2.5 min.);
d) **Diphenylamine**: 60:30:10 ACN:water:methanol and detection at 210 nm (Rt=3.5 min.).

2.5. Enrichment culture for the isolation of OPP-degrading bacteria

The enrichment culture technique was used for the isolation of OPP-degrading bacteria, as described in Chapter 2. A soil collected from a wastewater disposal site in Agià, Larissa, Greece, was used as source of pesticide-degrading microorganisms. Three biweekly applications of 20 mg/kg of OPP from a methanol solution of 1000 mg/l were done in the soil as described in Chapter 2. This was done prior to the enrichment culture set up to stimulate the putative OPP-degrading fraction of the soil bacterial community.

Enrichment was initiated in the two media, MSMN and SEM, by addition of 0.5 g of the treated soil. There were three replicates per medium and two corresponding non-amended control flasks to monitor the abiotic degradation of OPP during enrichment. Five enrichment cycles were followed with HPLC measurements at different time intervals after inoculation until complete OPP degradation. At the end of the fifth enrichment cycle a 10-fold dilution was prepared (10^{-1} until 10^{-6}) in sterilized ddH_2O and spread in MSMN + OPP (20 mg/l) agar plates. SEM cultures were not processed since successful degradation was achieved in the selective medium MSMN. Plates were incubated at 26°C for few days and single growing colonies were selected and tested for degrading ability in corresponding liquid cultures. Degrading colonies were spread on LB and MSMN + 20mg/l OPP to check purity. When not pure, the different colonies were picked up, sub-cultured, and checked again for degradation, until morphologically pure degraders were obtained. The purity of the latter was further checked *via* DNA extraction and PCR-DGGE analysis as described in Chapter 2. In cases where DGGE analysis suggested that the cultures were not pure, clone libraries were established to identify the members of the consortium and plan further cultivation conditions to purify them.

2.6. Experiments aiming to isolate, purify, and identify OPP-degrading bacteria

Enrichment cultures resulted in the isolation of mixed OPP-degrading cultures composed of different microbial ribotypes as identified *via* PCR-DGGE analysis and clone libraries screening.

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In order to obtain pure OPP-degrading different approaches were followed and are described below.

2.6.1. Purification of OPP-degrading consortia via successive cultivation in group-selective media

Agar plates from the group-selective media L9 (selective for *Sphingomonas* spp.), PB (selective for *Pseudomonas*), 465 (selective for *Pseudomonas / Sphingomonas*), and 660 (selective for *Achromobacter / Alcaligenes*) were prepared as described above and amended with 20 mg/l of OPP. Aliquots of the MSMN OPP-degrading mixed cultures were serially diluted, spread on the selective agar plates, and left to grow at 30°C in the dark. Growing colonies were selected and used to inoculate liquid MSMN + 20 mg/l OPP for assessment of their degradation capacity. Degradation was determined at 7 days and the degraders were spread on LB plates to test purity. Pellet from morphologically pure cultures was collected by centrifugation and DNA was extracted for DGGE analysis.

In a second attempt to isolate pure OPP-degrading bacteria, the three selective media PB, 465, and 660 amended with 20 mg/l of OPP were prepared in liquid and solid form. Serially diluted aliquots of isolated OPP-degrading consortia cultures were spread on the specific agar plates. Growing colonies were selected, inoculated in the corresponding selective liquid medium, and OPP degradation was measured by HPLC at 7 days. Cultures exhibiting good degrading capacity were spread again on the corresponding medium and colonies were again picked up and tested for OPP degradation in liquid cultures. After three cultivation-degradation cycles, pellet from the degrading cultures was collected for DNA-DGGE analysis from the liquid culture and from the total growing pellet on the specific plates and on LB.

2.6.2. Purification of OPP-degrading consortia with the use of antibiotics

In order to achieve effective purification of OPP-degrading consortia, actively degrading mixed cultures were transferred to liquid cultures of MSMN+OPP (20 mg/l) amended with a range of different antibiotics listed in Table 5.4. The effect of antibiotics on the degrading ability and on the composition of the OPP-degrading consortia isolated was evaluated. Duplicate inoculated and non-inoculated flasks were prepared and incubated on an orbital shaker at 26°C/180 rpm. The degradation of OPP was determined by HPLC at 0, 2, and 5 days. Cultures exhibiting OPP degradation were refreshed in MSMN + OPP + antibiotic and, after degradation was completed, the bacterial pellet was collected for DNA-DGGE analysis. Clone libraries were prepared for the identification of the different members of the degrading consortia.

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2.6.3. Assessing the role of the different members of the OPP-degrading consortia isolated

The role of the different members of the OPP-degrading cultures, eventually purified as described above, was determined either individually or in mixtures. Thus the different pure or mixed bacterial cultures obtained during the different purification steps were combined in all possible ways. The isolates used are described in the "Results and Discussion" section of this Chapter.

The degradation capacity of the different artificial bacterial mixtures obtained was tested in MSMN amended with 50 mg/l of OPP. Duplicate flasks per inocula were prepared together with duplicate non-inoculated controls. All samples were incubated in an orbital shaker at 26°C/180 rpm. OPP degradation was measured by HPLC immediately after inoculation and 1, 2, 3, 4, 8, 11, and 15 days later. The density of the bacterial inocula in each case was determined *via* spread plating in LB as described before. The composition of each bacterial culture was verified by DNA extraction and DGGE analysis.

Based on the results of this study a follow up experiment was undertaken to optimize the degrading capacity of the bacterium responsible for the degradation of OPP and verify the supporting role of other members of the bacterial consortium. Overall, five treatments were considered: MSMN, MSMN+casamino acids, MSMN+vitamins, MSMN+casamino acids+vitamins, and Mandelbaum medium+casamino acids, all supplemented with OPP (20 mg/l). Duplicate inoculated and non-inoculated samples were prepared for each treatment. All cultures were incubated in an orbital shaker at 26°C/180 rpm and samples were taken at 0, 1, 2, 3, 7, and 14 days for determination of OPP degradation. The density of the bacterial inoculum was determined as described before. Before inoculation and at the end of the experiment, the bacterial pellet was collected by centrifugation and the DNA extracted was subjected to DGGE analysis to verify the presence of the intended bacteria in the different treatments.

2.7. Phylogenetic analysis of the OPP-degrading bacterium

The identity of the pure OPP-degrading bacterium finally obtained after the different purification steps was determined after phylogenetic analysis using the full sequence of the 16S rRNA gene as described in Chapter 2.

2.8. Experiments for the characterization of the degrading capacity of the isolated strain

2.8.1. Assessment of bacterial growth during OPP degradation

The growth of the OPP-degrading isolate during degradation of 50 mg/l of the molecule in MSMN was measured in order to investigate if the degradation observed was co-metabolic or the

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strain was actively utilizing OPP as a growth substrate. Three inoculated replicates and two non-inoculated controls were prepared and incubated in an orbital shaker at 26°C/180 rpm. Aliquots from the cultures were removed immediately after inoculation and 4, 8, 12, 16, 24, and 28 hours later and were used for measurement of OPP degradation and for determination of bacterial numbers by spread plating.

2.8.2. Assessment of the ability of the isolate to degrade high pesticide concentrations

The degrading capacity of the isolated strain towards OPP concentrations substantially higher than the 20 mg/l commonly used in all experiments was investigated. Different concentration levels of OPP were considered: 50, 75, 100, 150, 200, 250, 300, and 500 mg/l. In order to achieve such concentration levels in MSMN, appropriate volumes from a filter-sterilized solution of OPP in DMSO (45 g/l) were added to the medium. Aliquots of filter sterilized DMSO were properly added to reach in all samples the same DMSO concentration of 1%. Three inoculated replicates and two non-inoculated controls for each concentration level were prepared. The samples were incubated in orbital shaker at 26°C/180 rpm and OPP degradation was measured by HPLC at the day of inoculation and 1, 2, 7, 14, 21, and 28 days later. The density of the bacterial inoculum was determined by spread plating in LB.

2.8.3. Evaluation of the stability of the degrading capacity of the isolate in nutrient rich medium

The ability of the isolated bacterium to metabolize OPP when transferred into a nutrient rich medium like LB, where OPP was not the sole C source, was evaluated. LB was supplemented with 50 mg/l of OPP. Inoculated triplicates and non-inoculated duplicate controls were prepared and incubated in an orbital shaker at 26°C/180 rpm. OPP degradation was determined *via* HPLC immediately after inoculation and 12, 24, 36, 48, 120, 192, 312, and 504 hours later. The level of the starting inoculum was determined *via* spread plating and the growth of the bacterium during degradation of OPP was determined *via* optical density at 600 nm with a spectrophotometer.

2.8.4. Evaluation of the ability of the isolate to degrade different compounds of similar chemical structure

The ability of the OPP-degrading bacterium to degrade compounds that have been reported to be possible intermediate metabolites of OPP like 2,3-dihydroxybiphenyl, benzoic acid, and catechol, or other pesticides that have similar chemical structures like DPA was determined in MSMN supplemented with 20 mg/l of each of those compounds. Because of the photosensitivity of catechol all cultures containing this compound were covered with aluminium foil to minimize

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exposure to light. OPP was also included for comparative purposes. Three inoculated replicates and two not-inoculated controls were prepared for each compound. All samples were incubated in an orbital shaker at 26°C/180 rpm. Degradation was measured at 0, 2, 7, and 15 days.

In a follow up study, the formation of the above putative metabolites (2,3-dihydroxybiphenyl, benzoic acid, and catechol) during degradation of OPP by the isolated bacterium was also evaluated in MSMN supplemented with OPP (50 mg/l). The experimental set up consisted of three inoculated replicates and two non-inoculated controls. The starting bacterial inoculum was determined *via* spread plating as described before. All samples were placed in an orbital shaker and incubated at 26°C/180 rpm. OPP degradation and the formation of the different putative metabolites were determined by HPLC on 12-hour intervals until complete degradation of OPP was achieved.

2.8.5. Testing the presence of the hbpCAD and hbpR genes in the isolated OPP-degrading strain

A PCR-based analysis was undertaken to detect possible genes that might be involved in the degradation of OPP by the isolated strain. Primers were designed based on the sequences of the genes *hbpA, hbpC, hbpD,* and *hbpR* previously found in the other well-studied OPP-degrading strain *Pseudomonas azelaica* HBP1 [Jaspers *et al.*, 2000 and 2001]. The gene sequences were downloaded from the GenBank® database (http://www.ncbi.nlm.nih.gov/genbank/). The sequence of each gene was elaborated with Primer3 (http://primer3.ut.ee) and the designed primers were further analysed with PrimerSelect™ (Lasergene®, DNASTAR) for secondary structure formations including hairpins, self-dimers, and cross-dimers in primer pairs. The sets of primers used are listed in Table 5.5. The PCR reagent composition and the thermocycling program followed are shown in Tables 5.6 and 5.7 respectively. The PCR products were analysed by agarose gel electrophoresis. DNA extracted from *P. azelaica* strain HBP1 was used as a positive control in all PCR amplifications. The HBP1 strain was regularly grown on agar medium 465 (as suggested by DSMZ) supplemented with 50 mg/l of OPP. The strain was able to degrade OPP and upon completion of its degradation the bacterial pellet was collected and used for DNA extraction.

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Table 5.5. Sequences of the primers designed to amplify the *hbpA*, *hbpC*, *hbpD*, and *hbpR* genes from total DNA of the two OPP-degrading strains. Melting temperatures (Tm) were calculated using the formula described in Chapter 3, Section 2.7. The expected amplicon size is also indicated.

Gene	Primer name	Primer sequence (5'-3')	Tm primer (°C)	Amplicon size (bp)
HbpA	HbpA_f	TCT TTC CAC ATT TCC TCC TGT T	54.0	452
	HbpA_r	AGA CTT TCA GCT CAA TCC CAA G	55.6	
HbpC	HbpC_f	CTT CTG CAG TCG AGT AAT GTC G	57.7	491
	HbpC_r	TGA TGG ATG ATC TTG GAA ACA G	54.0	
HbpD	HbpD_f	CGA GTG ATC TTG CTT GAT TGT C	55.6	568
	HbpD_r	CGT CAG GCA TGA AGT TGA TAA G	55.6	
HbpR	HbpR_f	TAT AGC GGC ACA GTT CAC TTC	55.8	573
	HbpR_r	CAG ATT TAT GCA GGT GGT GTC	55.8	

Table 5.6. Concentrations of the different reagents used for PCR amplifications of the *hbpA*, *hbpC*, *hbpD*, and *hbpR* genes. The reaction volume was 25 μl.

Component	Final concentration
10X Buffer with Mg^{2+}	1X
dNTPs (10mM)	200 μM
Forward Primer (20μM)	0.4 μM
Reverse Primer (20μM)	0.4 μM
Template DNA	Variable
KapaTaq (5U/μl)	1U/50 μl
PCR grade water	Up to final volume

Table 5.7. The PCR programme used for the amplification of the *hbp* genes.

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 min.	1
Denaturation	95	30 sec.	30
Annealing	52	30 sec.	
Extension	72	1 min.	
Final extension	72	10 min.	1

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2.8.6. The effect of pH and temperature on the degrading ability of the OPP-degrading bacterium

The ability of the bacterial isolate to degrade OPP in a range of pH and temperatures was tested as described in Chapter 2.

2.8.7. The effect of the co-presence of other pesticide-degrading bacteria on the degrading capacity of the OPP-degrading isolate

The effect of the co-presence of other pesticides used also in fruit packaging industry and their corresponding degrading bacteria on the degradation of OPP by the isolated strain was determined in MSMN. The molecules considered were TBZ and its corresponding isolated degrading consortium (Chapter 3) and/or DPA and its corresponding degrading isolate (Chapter 6). As described in Chapter 2, all treatments contained the same molar concentration of the three pesticides (0.17 mM).

2.8.8. Assessment of the soil bioaugmentation potential of the OPP-degrading isolate

The bioaugmentation potential of the isolated bacterium was evaluated in an artificially contaminated soil as described in Chapter 2. A soil collected from a field of the National Agricultural Research Foundation of Greece in Larissa was used. Three soil samples of 500 g were treated with appropriate amounts of three different filter-sterilized aqueous solutions of OPP (0.25, 12.5, and 2.5 g/l) resulting in concentrations of OPP in soil of 10, 100, and 500 mg/kg. After pesticide application, soils were kept at 4°C in the dark for four weeks to allow the pesticide residues to age in soil. At the end of this period, the samples were split into two subsamples. The first set of subsamples was inoculated with a fresh culture of the OPP-degrading isolate resulting in a final inoculum level of 2x10^{6} cells/g soil. The second set of subsamples received the same amount of water without bacteria to serve as non-inoculated controls. The moisture content of all samples was adjusted to 40% of the soil water holding capacity by addition of extra water when needed. All soils were subsequently divided into 10 g subsamples which were placed in aerated plastic bags and incubated in the dark at 25°C. Immediately after inoculation and at 5, 10, 20, 30, and 60 days triplicate subsamples from each treatment were removed from the incubator and analysed for OPP residues by HPLC.

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2.8.9. Assessment of the degradation potential of the OPP-degrading bacterium in Biobed Extract Medium and maintenance of its degrading capacity upon exposure to composting-like temperatures

The ability of the isolated OPP-degrading bacterium to metabolize OPP in BEM, that resembles the nutritional status of a biobed substrate, was evaluated. Moreover, the degrading ability of the OPP-degrading bacterium was tested in MSMN and BEM during a 28-day incubation under temperature conditions simulating composting (for more details see Chapter 2).

3. Results and discussion

3.1. Enrichment cultures for the isolation of OPP-degrading bacteria

A rather rapid degradation of OPP was observed in both media tested (MSMN and SEM) with almost complete OPP disappearance observed in 6-7 days in the first two cycles (Figure 5.1). Degradation of OPP was accelerated in the subsequent cycles in both media with complete degradation observed in 2-4 days. Since MSMN enrichments showed the presence of an OPP-degrading community which was able to use OPP as a carbon source, isolation procedures focused on the minimal medium only.

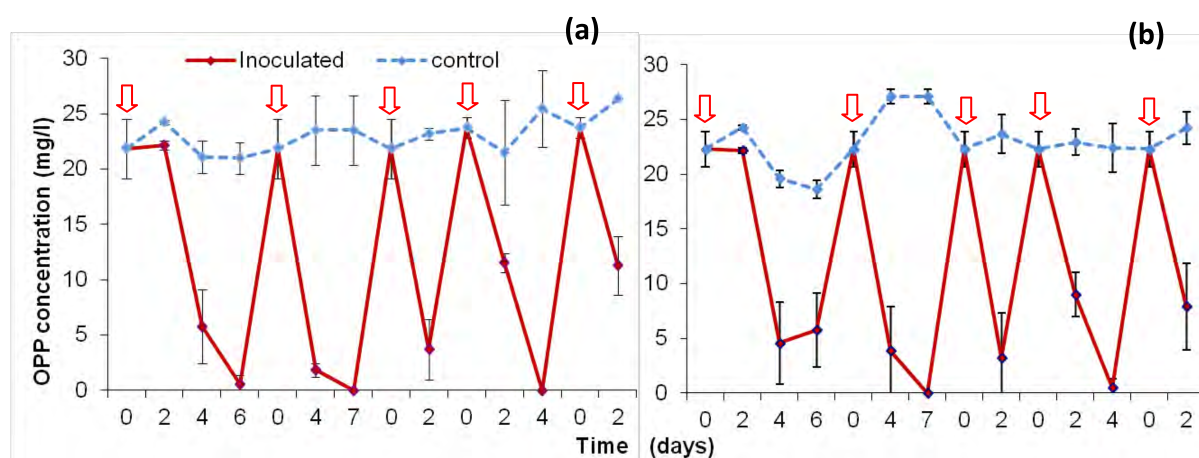

Figure 5.1. The degradation of OPP in MSMN (a) and SEM (b) in successive enrichment cycles. Degradation in the non-inoculated controls is also presented. Each value is the mean of three replicates ± standard deviation. The red arrows indicate the beginning of each cycle.

Aliquots of the fifth enrichment cycle culture were serially diluted and spread on MSMN+OPP agar plates. Approximately 90 colonies were selected and tested for OPP degradation in liquid cultures. Overall, only six displayed high degradation capacity, being able to metabolize >90% of OPP within 7 days (Figure 5.2).

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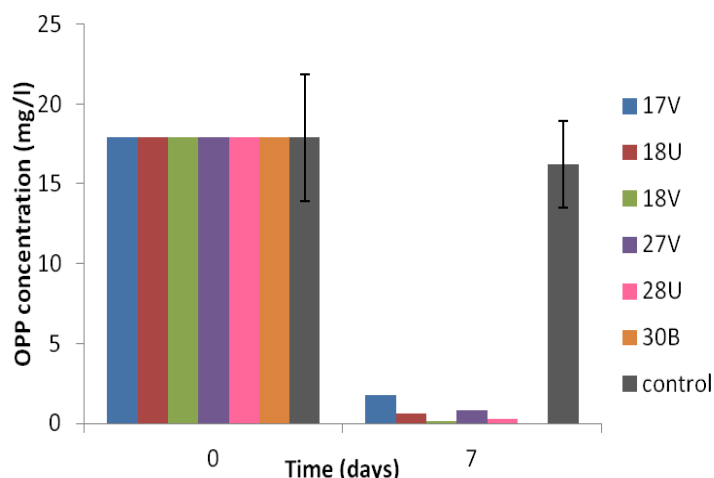

Figure 5.2. OPP degradation by the six positive OPP-degrading consortia initially obtained. Degradation of OPP in non-inoculated cultures (control) is also reported.

Spread plating on MSMN+OPP and LB plates showed that the cultures were not pure and this was verified through DGGE analysis (Figure 5.3). The six OPP-degrading cultures were composed of nine bacterial ribotypes in total which as determined *via* clone libraries belonged to the genera *Pseudomonas*, *Sphingomonas, Achromobacter*, and *Alcaligenes* (Table 5.8). Despite screening of more than 200 clones, no clones for band P2 were obtained, so it was concluded that this band could be an artifact or ghost band. Sequence mismatches, heteroduplexes, chimeric molecules, and single-stranded DNA that arise during PCR amplification can generate visible DGGE-bands and lead to erroneous identification or interpretation of the community diversity [Suzuki and Giovannoni, 1996; von Wintzingerode *et al*., 1997; Wang and Wang, 1997; Qiu *et al*., 2001; Speksnijder *et al*., 2001; Zhang *et al*., 2005; Kušar and Avguštin, 2012].

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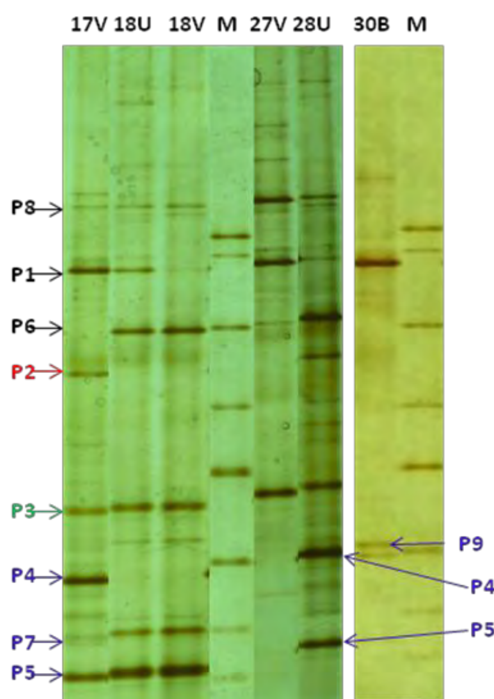

Figure 5.3. DGGE fingerprint of the six OPP-degrading consortia initially obtained. The code names of the major bands are shown and their identity is given in Table 5.8. Lane M: bacterial gel marker.

Table 5.8. Identity of the major members of the OPP-degrading consortia initially isolated. The highest homologies obtained *via* BLAST are presented together with the % of sequence homology and the NCBI accession number of the best matches.

Band	Highest match	Homology (bp)	Homology (%)	NCBI accession number
P1	*Pseudomonas stutzeri* strain S1	1455/1457	99	AY485995
P3	*Sphingomonas haloaromaticamans*	1397/1415	99	X94101
P4	*Achromobacter* sp. strain Ko	1450/1456	99	HQ200411
P5	*Achromobacter* sp. strain MT-E3	1450/1455	99	EU727196
P6	*Pseudomonas putida*	1459/1459	100	AM411059
P7	*Achromobacter* sp. MT-E3	1452/1455	99	EU727196
P8	*Pseudomonas* sp. strain W15Feb29	1446/1450	99	EU681012
P9	*Alcaligenes faecalis*	1450/1455	99	FN433031

3.2. Purification of the OPP-degrading cultures *via* cultivation in group-selective media

Based on the results of the clone libraries which showed that all major members of the consortia belonged to four bacteria genera, selective media for the isolation of those bacteria in pure cultures were used. Thus, the degrading consortia were cultivated in the following media: L9 (selective for *Sphingomonas* spp.), 465 (selective for *Pseudomonas/Sphingomonas* spp), 660 (selective for *Achromobacter/Alcaligenes* spp), and PB (selective for *Pseudomonas* spp).

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Depending on the composition of the initial consortia, each culture was spread on the appropriate agar medium. Growing colonies were selected and checked for OPP degrading ability. DGGE analysis of all the colonies selected showed that none was in pure state. However, it was noted that the ribotype represented by band P3 was present in the DGGE profile of all cultures actively degrading OPP and absent from the fingerprints of all the non-degrading or slowly-degrading cultures (Figure 5.4). As reported in Table 5.8, band P3 showed highest homology (99%) to the 16S rRNA gene of a *Sphingomonas haloaromaticamans* strain*,* known to be involved in the degradation of benzene, catechol, chlorobenzene, 1,3-dichlorobenzene, and 1,4-dichlorobenzene [Wittich *et al*., 2007; Schraa *et al*., 1986].

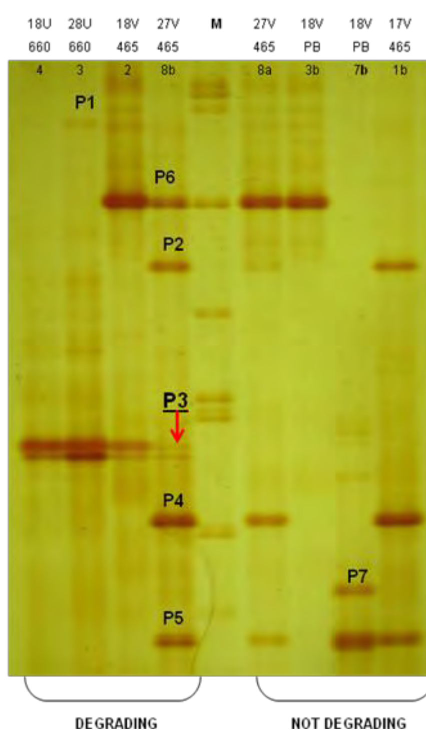

Figure 5.4. DGGE profile of degrading (18U-4, 28U-3, 18V-2, 27V-8b) and non-degrading (27V-8a, 18V3b, 18V-7b, 17V-1b) colonies selected from selective agar plates (660, 465, or PB medium) and tested for degradation of OPP in liquid cultures. Band P3, indicated by a red arrow, was the only band always present in the degrading consortia and missing in the not-degrading ones. Lane M: bacterial gel marker. Major bands are labeled and their identity is reported in Table 5.8.

The cultivation of the initially selected OPP-degrading cultures in group-selective media resulted in less complex OPP-degrading consortia. Based on this, it was hypothesized that prolonged sub-culturing of the given simplified consortia under the same selective conditions will result in further elimination of the members of the OPP-degrading cultures that are not directly involved in the degradation of the molecule and eventually pure OPP-degrading bacterial cultures could be obtained. The OPP-degrading consortia were subsequently spread on

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selective agar plates and growing colonies were again selected and tested for OPP degradation in the corresponding liquid medium. Subsequently, cultures showing active degradation were spread again on the corresponding selective plates. After three such cycles, the composition of the degrading cultures obtained was determined *via* DNA-DGGE analysis. As shown in Figure 5.5 no pure degrading cultures were obtained. The simpler OPP-degrading consortium was composed of two bands, P1 (*P. stutzeri*) and P3 (*S. haloaromaticamans*) and was able to degrade 20 mg/l of OPP within 2 days. DGGE fingerprinting of the total bacterial community growing on MSMN and LB agar plates showed that both bacterial were actively growing on the two media (Figure 5.5).

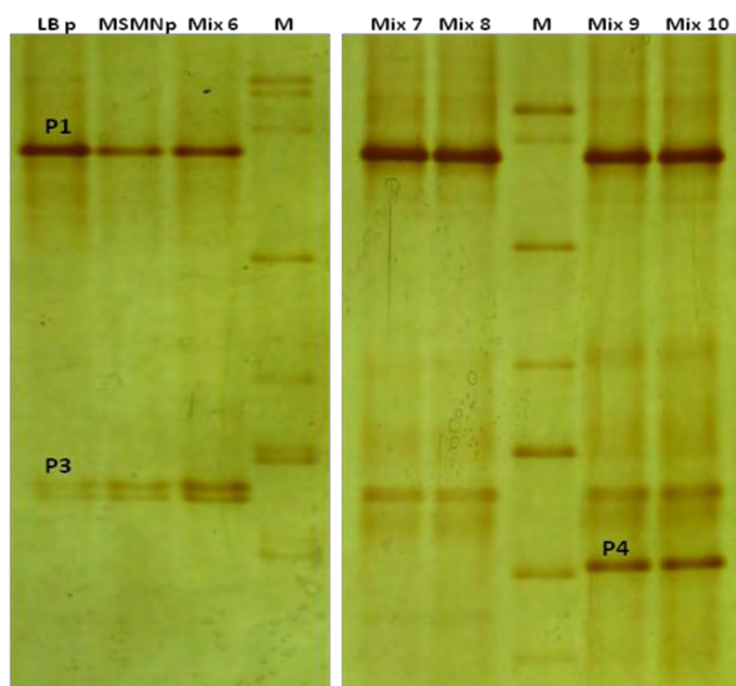

Figure 5.5. Right: DGGE fingerprint of OPP-degrading consortia (named Mix) obtained after three cycles in selective media. **Left:** Growing profile of the Mix6 on LB (LBp) and MSMN+OPP (MSMNp) agar plates and in liquid culture (Mix6). Lane M: DGGE bacterial marker. The major bands are labeled and their identity is showed in Table 5.8.

3.3. Purification of OPP-degrading consortia *via* exposure to a range of antibiotics

One of the fast degrading two-member consortium (Mix6) was treated with different antibiotics at concentration levels listed in Table 5.3 [Vaz-Moreira *et al*., 2011; Lalucat *et al*., 2006; Tattawasart *et al*., 1999], aiming to identify the member of the consortium responsible for the degradation of OPP and to obtain it in pure culture. The degrading ability of the consortium was maintained only in the presence of nalidixic acid, trimethoprim, streptomycin, colistin,

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ciprofloxacin, polymyxin B, and lincomycin, in which 20 mg/l of OPP were completely degraded within 2 days (Figure 5.6).

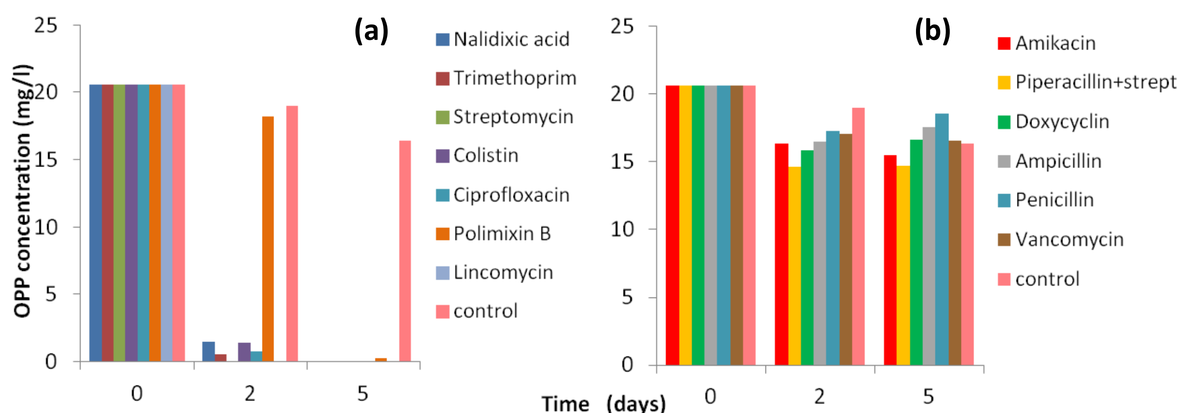

Figure 5.6. The effect of different antibiotics on the degrading ability of the two-member OPP-degrading consortium Mix6 composed of *P. stutzeri* P1 and *S. haloaromaticamans* P3. The degradation of OPP in the presence of antibiotics that did not affect degradation (a) and that halted degradation (b) is presented. Degradation of OPP in non-inoculated cultures (control) is also reported.

After subculturing in fresh media, in order to eliminate the possible presence of DNA derived from dead cells, DNA was extracted for DGGE analysis. The results are shown in Figure 5.7. A clone library was prepared to confirm bands identification and to identify a new band named P10 appearing in the fingerprints (Table 5.9). The bacterial 16S rRNA sequences obtained were registered in the GenBank database and are available at http://www.ncbi.nlm.nih.gov/ providing the accession numbers reported in Annex I, Section 3.2. The new band P10, representing a *Starkeya novella* ribotype, corresponded to a minor member of the isolated consortia, previously not detectable by DGGE possibly because its population levels were below the resolution limit of the technique. After elimination of other major members of the consortium, this bacterium found favorable conditions to grow up and eventually becoming detectable. The bacterium represented by the band P1 (*P. stutzeri*) was tolerant only to trimethoprim, polymyxin B, and lincomycin and was never detected in a pure state in cultures exhibiting high degradation capacity (Figure 5.7). Thus, the presence of this bacterium did not seem to be critical for the degradation of OPP. On the contrary, band P3 was present in all degrading treatments and appeared to be in pure state in the culture treated with streptomycin (Figure 5.7). In all the other treatments, the bacterium was in co-culture either with the *P. stutzeri* ribotype (band P1), or the *S. novella* ribotype (band P10), or with both. Despite the initial rapid rates of degradation of OPP observed in the streptomycin-treated culture which was composed only from *S. haloaromaticamans* subsequent refreshing resulted in a substantial retardation in the degradation rates.

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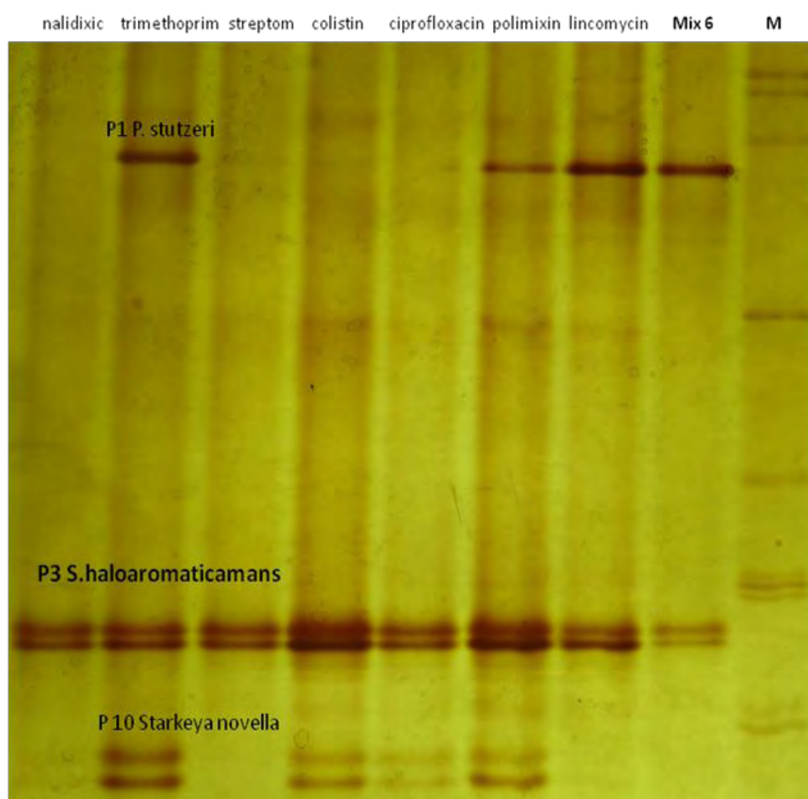

Figure 5.7. DGGE fingerprint of the two-member OPP-degrading consortium Mix6 after treatment with different antibiotics. The profile of the treatments that maintained the degrading ability is shown. The new band P10 was identified *via* cloning and sequencing (Table 5.9). Lane M: DGGE bacterial marker.

Table 5.9. Highest homologies obtained *via* BLAST of the 16S rRNA gene sequences of the three main bacteria present in the DGGE fingerprinting of the OPP-degrading consortium after antibiotic treatments. Percentage of sequences homology and NCBI accession number of the best match are shown.

Band	Highest match	Homology (bp)	Homology (%)	NCBI accession number
P1	*Pseudomonas stutzeri* strain S1	1455/1457	99	AY485995
P3	*Sphingomonas haloaromaticamans* strain A175	1402/1415	99	NR_044902
P10	*Starkeya novella* strain R8-541	1401/1407	99	JQ659944

Overall these data provide strong evidence that the bacterium represented by the band P3 identified as *S. haloaromaticamans* could be mainly responsible for the degradation of OPP. However, the gradual reduction of degradation rates upon sub-culturing in pure culture might suggest a supporting role for the other two bacteria present in the original OPP-degrading mixed cultures which were characterized by a stable degrading phenotype.

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3.4. Assessing the role of other bacterial members of the OPP-degrading consortium

Trying to understand the possible role of the bacterial partners of the strain P3 on OPP metabolism, the degradation capacity of the strains P1, P3, and P10 in pure culture or in different combinations was assessed. A pure culture of the *P. stutzeri* strain P1 which was not degrading OPP was obtained from MSMN+OPP agar plates and a pure culture of the *S. haloaromaticamans* strain P3 was also available from the streptomycin treatment. In addition, bacterial cultures composed of P1, P3, and P10 or of P3 and P10 were obtained after exposure of the initial OPP-degrading culture (Mix6) to trimethoprim and colistin respectively. For comparison purposes the initial OPP-degrading culture Mix6 was also included in the study. Finally, a two member consortium composed of *P. stutzeri* P1 and *S. haloaromaticamans* P3 was prepared by combining the two pure bacteria artificially. All tests were performed in MSMN + OPP.

All natural consortia considered (Mix6, trimethoprim- and colistin-treated Mix6) were able to degrade 50 mg/l OPP in 2 days (Figure. 5.8). A slightly slower degradation of OPP was observed in the artificially composed mixture of *P. stutzeri* P1 and *S. haloaromaticamans* P3 which degraded the fungicide in 3 days. The slight delay in the degradation of OPP by the artificially composed mixture of P1 and P3 could be attributed to the lower initial densities of the two bacteria in this culture ($8.8x10^4$ cells/ml) compared to the three natural bacterial mixed cultures considered, where bacterial densities were $4x10^6$ cells/ml. Indeed, the effect of the inoculum cell density on pesticide degradation rates have been demonstrated in many studies [e.g. Nwanyanwu and Abu, 2011; Wang *et al*., 2007; Lakshmi and Velan, 2011]. On the contrary, the pure culture of strain P1 did not degrade OPP during the course of the study, while P3 in pure culture degraded only 50% of the initial amount of OPP in 15 days. Thus, effective degradation of OPP was observed only when P3 was combined with another bacterium at least.

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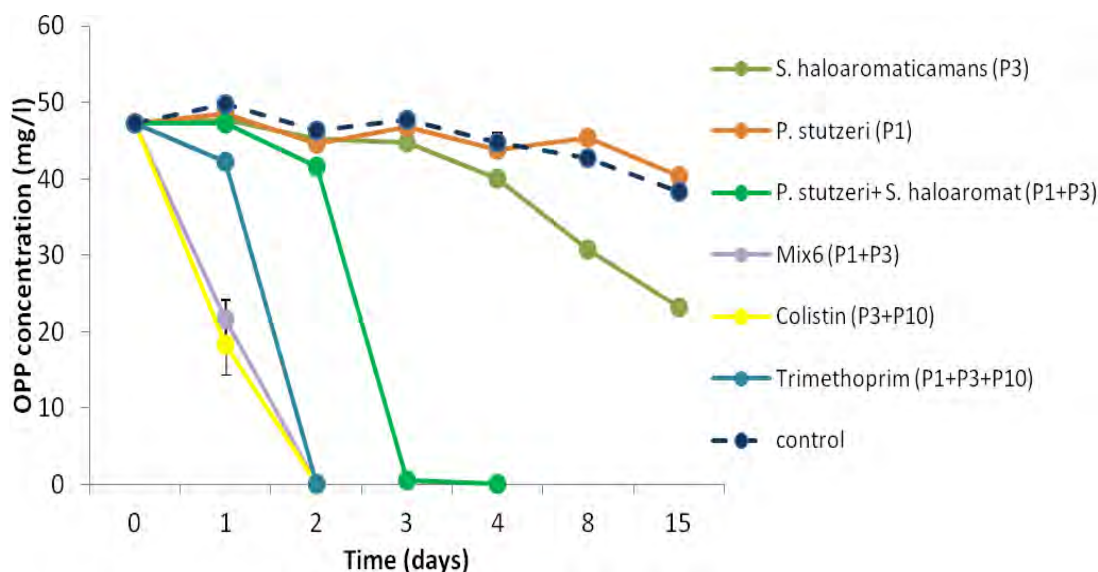

Figure 5.8. The degradation of OPP by the bacteria *S. haloaromaticamans* P1, *P. stutzeri* P3, and *S. novella* P10, present in the fingerprinting of the OPP-degrading consortium, when cultured individually or in different natural or artificial combinations. Degradation of OPP in non-inoculated cultures (control) is also shown.

These results suggest that *S. haloaromaticamans* strain P3 requires the presence of other bacteria of the community to effectively degrade OPP. It is possible that *P. stutzeri* P1 and *S. novella* P10 could play a role in the degradation of toxic metabolites produced during the metabolism of OPP thus enabling its complete degradation. A toxic role of metabolites on pesticide-degrading bacteria has been demonstrated for example in the case of chlorpyrifos whose main metabolite 3,5,6-trichloro-2-pyridinol (TCP) if accumulates can reduce the degradation rate of the parent compound [Racke *et al*., 1990; Singh *et al*., 2003]. Similarly, the formation of the antibiotic protoanemonin during microbial degradation of 4-chlorocatechol, a central intermediate of the catabolism of chloroaromatic compounds, has been shown to induce cell death and consequent inhibition of degradation [Blasco *et al.,* 1995]. Interestingly, Pelz *et al*. [1999] isolated a bacterial consortium able to metabolize 4-chlorosalicylate with strain MT1 being the dominant degrading strain of the parent compound. However, other members of the consortium named MT3 and MT4 exhibited a protective effect on MT1 by actively metabolizing two toxic intermediates, 4-chlorocatechol and protoanemonin, produced during degradation of the parent compound by MT1.

When a toxic compound is produced during degradation of an organic pollutant an initial rapid degradation phase followed by a slowing down of the degradation rates due to the progressive accumulation of the toxic metabolite is expected. However, the degradation kinetic of OPP by the bacterium P3 deviated from this pattern (Figure 5.8). Moreover, if that was the

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case then the substitution of strain P1 or P10 with other randomly selected bacteria not belonging to the isolated consortia would result in inhibition of OPP degradation. In order to test this hypothesis the effect of co-cultivation of P3 with bacteria not belonging to the OPP-degrading consortium, a *P. monteilii* strain isolated as DPA degrader (Chapter 6) and a TBZ-degrading consortium composed of different bacterial members (Chapter 3) was evaluated. Results showed that *S. haloaromaticamans* strain P3 was able to rapidly degrade OPP in the presence of the DPA- and TBZ-degrading bacteria whereas no degradation was observed when P3 was inoculated alone in MSMN (Figure 5.9). These results did not support the formulated hypothesis of a detoxification role of strains P1/P10 during OPP degradation by bacterium P3. Alternatively, the other bacteria could provide to P3 essential nutrients which the latter is not able to attain from the liquid medium or is not able to synthesize.

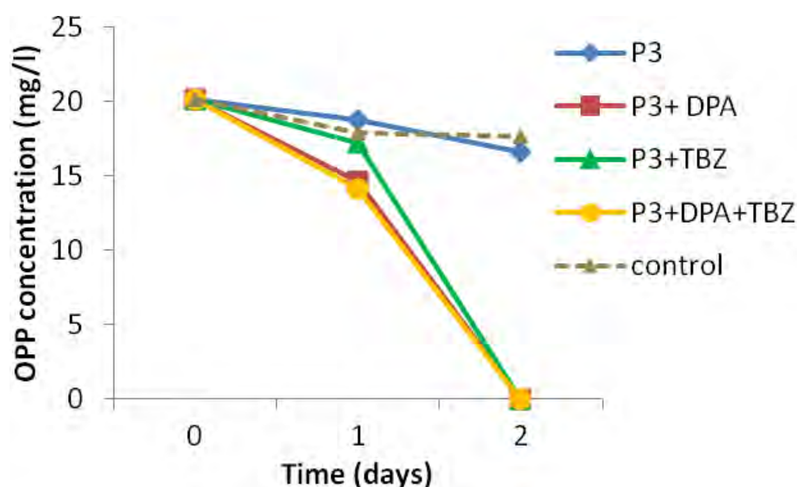

Figure 5.9. The degradation of OPP in MSMN by *S. haloaromaticamans* strain P3 in the co-presence of other pesticides used in the fruit packaging industry and their corresponding degrading bacteria. P3+DPA: co-inoculation with *P. monteilii* strain DPA; P3+TBZ: co-inoculation with a TBZ-degrading consortium; P3+DPA+TBZ: co-inoculation with both DPA- and TBZ-degrading bacteria. Degradation of OPP in the non-inoculated control is also presented.

3.5. Effect of different essential nutrients on the degrading capacity of the isolated strain

In order to test the second hypothesis expressed above a new experiment was undertaken where the MSMN medium was supplied with different additives including casamino acids (as a source of amino acids), vitamins, or both and the degradation of OPP by the pure strains P3 and P1 was evaluated. Purity was checked with DNA-DGGE analysis prior to inoculation and confirmed at the end of the experiment (Figure 5.10). The inoculum density was similar for both bacteria and corresponded to $3x10^4$ cells/ml. Degradation of OPP by the two bacteria was also determined in

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the Mandelbaum medium supplemented with casamino acids. The OPP-degrading consortium Mix6 composed of P1 and P3 was also included in the study for comparative purposes.

No degradation of OPP was observed in the cultures inoculated only with *P. stutzeri* strain P1 (Figure 5.11) confirming its inability to actively metabolize OPP. Similarly no appreciable degradation of OPP was evident in the MSMN cultures inoculated only with *S. haloaromaticamans* P3. In contrast, rapid degradation of OPP by strain P3 was observed in MSMN and Mandelbaum medium supplemented with casamino acids. Degradation in those treatments was comparable to the degradation observed in MSMN inoculated with the initial OPP-degrading consortium Mix6 (Figure 5.11). Vitamins when added alone did not appear to enhance the degradation of OPP.

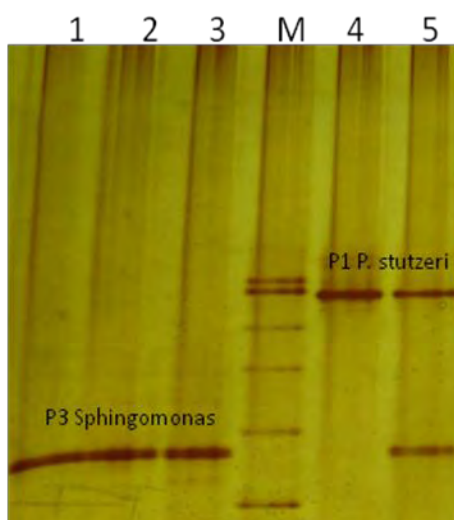

Figure 5.10. DGGE fingerprint of the bacterial cultures to verify that only the intended strains were growing during the experiment. Lanes 1 – 3: cultures inoculated with *S. haloaromaticamans* P3; Lane 4: culture inoculated with *P. stutzeri* P1; Lane 5: culture inoculated with original mixed consortium Mix6. Lane 1: MSMN + casamino acids; lane 2: MSMN + casamino acids + vitamins; lane 3: Mandelbaum medium + casamino acids; Lane M: bacterial gel marker.

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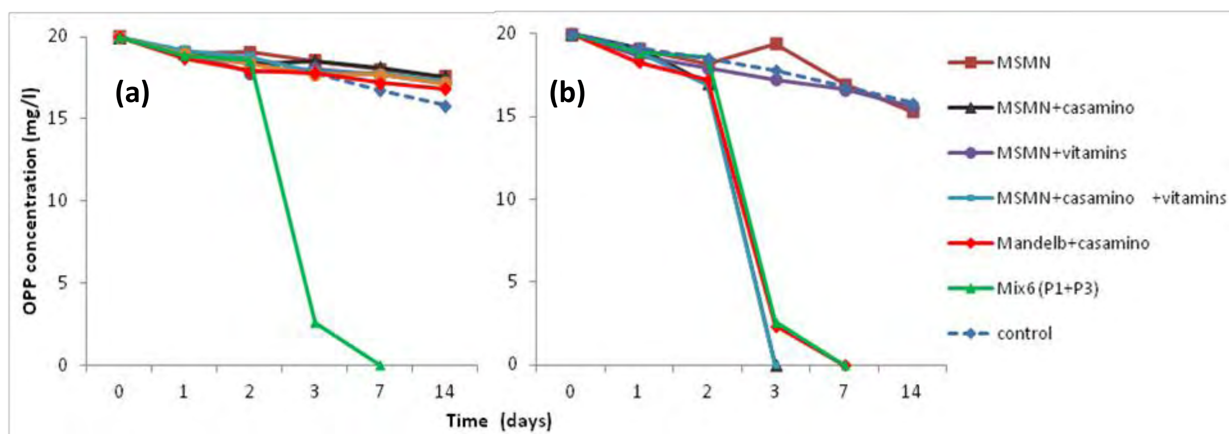

Figure 5.11. The degradation of OPP by *P. stutzeri* strain P1 (a) and *S. haloaromaticamans* strain P3 (b) in different selective media supplemented or not with casamino acids and/or vitamins. The degradation of OPP by the initially obtained mixed consortium Mix6 and in non-inoculated controls is also shown.

These last experiments confirmed the catalytic role of *S. haloaromaticamans* strain P3 in the degradation of OPP. However its ability to metabolize the fungicide resulted dependent on the presence of casamino acids. Alternatively, the presence of *P. stutzeri* strain P1 or *S. novella* strain P10 in co-culture with the strain P3 could alleviate the need for amino acids and reestablish the degradation of OPP in a similar manner. These results suggest that the strains P1/P10 have probably a supporting role in providing amino acids essential for the growth and the degradation activity of the strain P3. This kind of cooperative metabolic activities in bacterial consortia during degradation of organic pollutants in which the degrading bacteria depend on secondary strains which provide various growth factors has been reported before [Hay *et al*., 2001; Maymò-Gatell *et al*., 1997; Senior *et al*., 1976]. Thus in the presence of extra amino acids in the form of casamino acids, P3 was able to carry out normal cellular functions, to degrade OPP, and to proliferate. When *P. stutzeri* strain P1 and the other members of the consortium were eliminated (after treatment with antibiotics) the strain P3 could keep its degradation capacity for a few refreshing cycles, probably using amino acids derived from lysed cells or previously liberated in the medium and brought in the new culture with the inoculum or accumulated in the cell. However the progressive reduction in amino acids availability could not support new cell growth, and the degrading population was progressively reducing as well as the degradation rates. It cannot be excluded that, after prolonged lack of amino acids, cells entered in a dormant state which has been previously described for bacteria exposed to nutrient deficiency and that allows them to survive through a general reduction in their metabolism [Schwarz and Forchhammer, 2005].

Auxotrophy, which is defined as the inability of an organism to synthesize a particular organic compound required for its growth, has been described in the case of other isolated strains

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belonging to the genus *Sphingomonas*. Sørensen *et al.* [2002] isolated a *Sphingomonas* sp. strain SRS2 that could efficiently degrade the herbicide isoproturon only when L-methionine was delivered to SRS2 by a second bacterium. In the same way, a *Sphingobium* sp. T51 was found to need a combination of L-arginine and L-methionine to efficiently metabolize 4-cloro-2-methylphenoxyacetic acid [Onneby, 2013]. Hay *et al*. [2001] isolated a *Sphingomonas* sp. from a triclosan-degrading consortium, but its degradation capacity depended on the presence of other bacteria or a complex medium, thus suggesting a requirement for growth factors or nutrients from its surrounding environment. Our findings strongly indicate that the isolated *Sphingomonas* sp. strain P3 has metabolic deficiencies that should be fulfilled by other bacteria or provided externaly in order to actively degrade OPP. The essential amino acid/s required for growth have not been identified. The bacterium morphology on LB plate is shown in Figure 5.12.

Figure 5.12. Colony morphology of *S. haloaromaticamans* strain P3 on LB plate.

Phylogenetic analysis confirmed that the isolated strain P3 belonged to the genus *Sphingomonas* and most probably to the species of *S. haloaromaticamans* (Figure 5.13). Thus the OPP degrading strain was named as *S. haloaromaticamans* strain P3. This is the first report of isolation of a S*phingomonas* strain able to degrade OPP. Indeed, previous studies have reported the isolation of *Pseudomonas* OPP-degrading strains [Kohler *et al*., 1988; Sondossi *et al*., 1991]. Bacteria belonging to the genus *Sphingomonas* are particularly versatile in the degradation of organic pollutants [Aylward *et al*., 2013]. Karpouzas *et al.* [2005] reported the isolation of a *Sphingomonas paucimobilis* strain able to rapidly degrade the organophosphates cadusafos and ethoprophos. In addition, a *Sphingomonas* strain, as depicted from studies presented in Chapter 3, was responsible for the degradation of TBZ.

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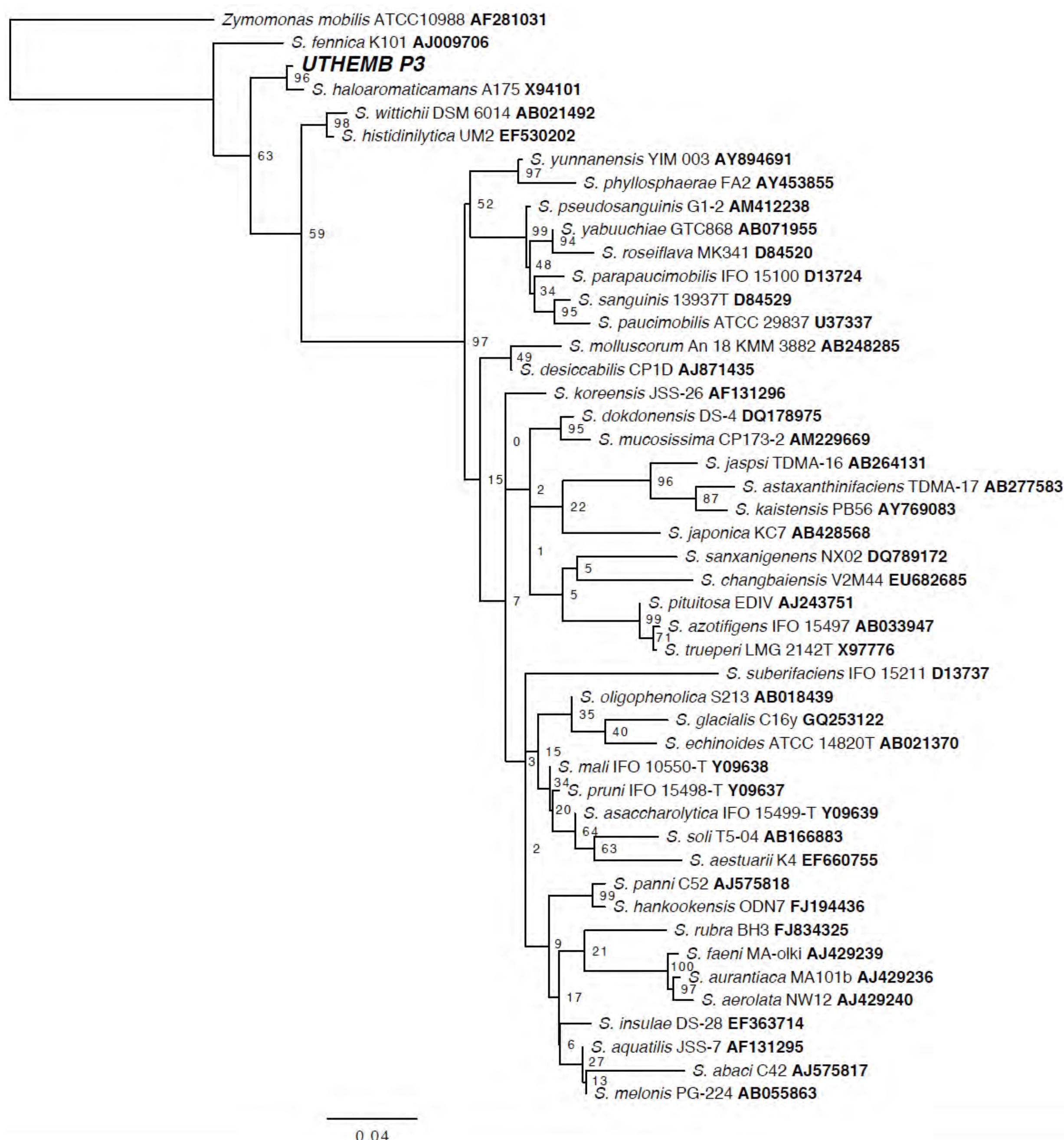

Figure 5.13. Phylogenetic analysis of the isolated OPP degrader P3-*Sphingomonas* (indicated as UTHEMB P3: **U**niversity of **Th**essaly, laboratory of **E**nvironmental **M**icrobiology and **B**iotechnology **P3)** based on the complete 16S rRNA gene sequence. 1000 bootstrap replicates were run with RAxML following the GTRGAMMAI (**G**eneral **T**ime **R**eversible with **GAM**ma rate heterogeneity and considering **I**nvariable sites) model. The bootstrap support is expressed in scale from 0 to 100. The NCBI accession numbers of each bacterium is indicated.

3.6. Characterization of the degradation capacity of the OPP-degrading strain

3.6.1. Assessment of the bacterial growth during degradation of OPP

The ability of *S. haloaromaticamans* strain P3 to degrade OPP and its concurrent growth on this substrate were studied in a liquid culture experiment. As shown in Figure 5.14, the OPP-degrading strain was able to rapidly degrade the fungicide within 24 hours with a concurrent

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stoichiometric increase in the bacterial population in the medium which reached a peak of $3.9x10^8$ cells/ml at 28 hours. At that point, no OPP was available in the culture, but the bacteria showed to be still in the logarithmic phase, probably multiplying at the expense of byproducts of the degradation. This degradation profile suggested that the *S. haloaromaticamans* strain P3 can effective degrade OPP and use it as an energy source for its proliferation.

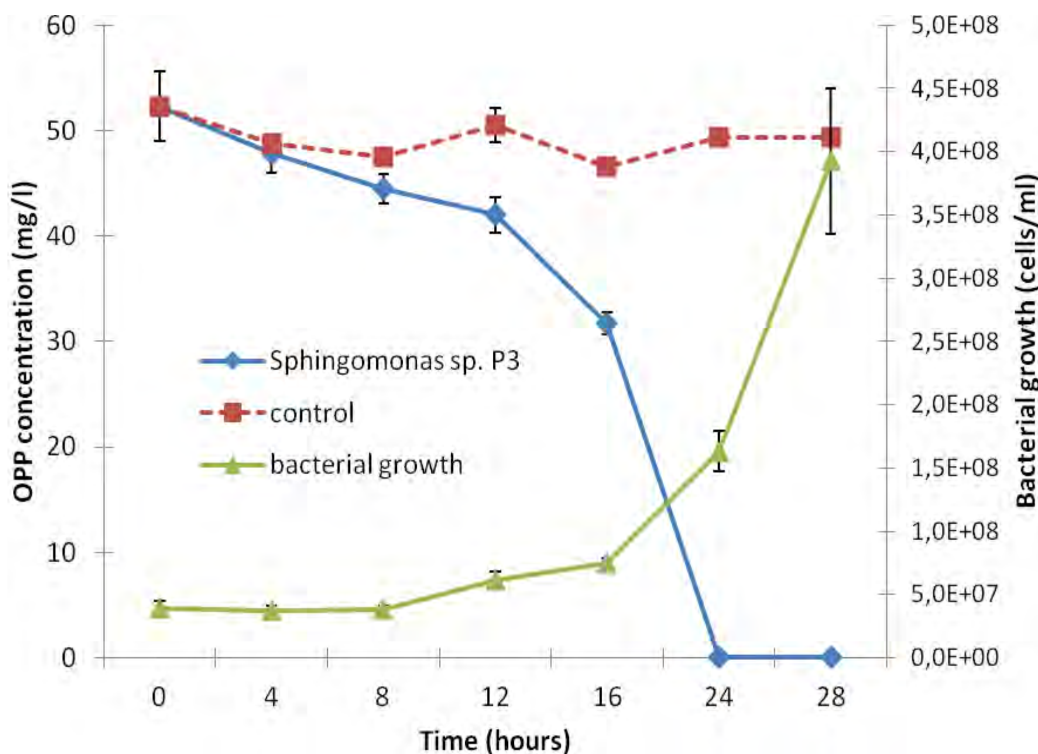

Figure 5.14. Degradation of OPP by *S. haloaromaticamans* strain P3 and the corresponding growth of the bacterium in MSMN+casamino acids. Degradation in non-inoculated cultures (control) is also presented. Each value is the mean of the triplicates ± the standard deviation.

3.6.2. Assessment of the ability of the isolate to degrade high OPP concentrations

The ability of the isolated bacterium to degrade high concentrations of OPP in MSMN+casamino acids was evaluated. The starting bacterial inoculum was $2.2x10^6$ cells/ml. The isolated strain was able to completely degrade 50 and 75 mg/l of OPP in one day, while 2 days were required for complete degradation of 100 mg/l of OPP (Figure 5.15). Degradation rates were slower when the isolate was exposed to 150 mg/l of OPP where degradation was completed in 7 days. No degradation of OPP was observed at higher concentration levels. The lack of degradation at concentrations higher than 150 mg/l could be due to a toxic effect of OPP on the bacterial isolate [Rani *et al.*, 2008; Felsot and Dzantor, 2009]. The re-use of pesticide-containing water solution for more than one application is a common practice in the fruit packing-houses. This recycling process commonly results in a decrease in the concentration of OPP in the wastewater to levels

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of 10% of the starting dose (600 mg/l maximum [EFSA, 2008]). Therefore the OPP-degrader isolate is expected to be effective in the treatment of OPP-contaminated wastewaters.

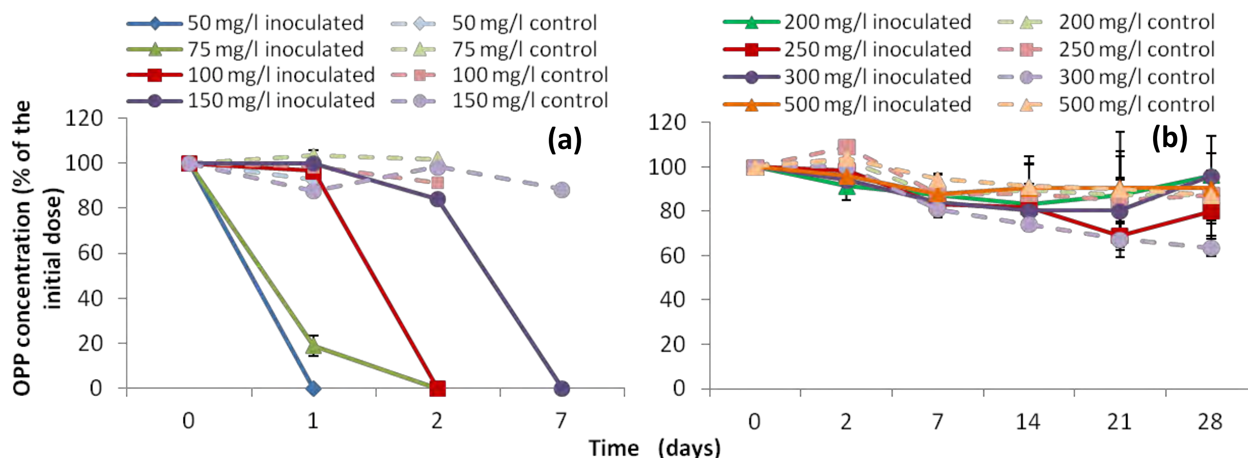

Figure 5.15. The degradation of different concentration levels of OPP by *S. haloaromaticamans* strain P3. **(a)** OPP concentration levels that the strain P3 was able to degrade and **(b)** OPP concentration levels that the strain P3 failed to degrade during the course of the study. Degradation of OPP in non-inoculated cultures (control) is also presented. Error bars represent the standard deviation of the mean.

3.6.3. Evaluating the degrading ability of the isolate in rich growth media

No OPP degradation was observed within 21 days in the nutrient rich medium LB. These results are in agreement with previous studies which have demonstrated that additional C sources may inhibit aromatic compound biodegradation. In a study conducted by Ampe *et al.* [1998] a *Ralstonia eutropha* strain degraded phenol less effectively in the presence of acetate. In turn, phenol degradation by a *Pseudomonas putida* strain was inhibited by the presence of extra low molecural weight organic acids (e.g., succinate, lactate, and acetate) or carbohydrates (e.g., glucose and gluconate) [Mueller *et al*., 1996]. Moreover, glucose enrichment repressed catechol degradation by *Pseudomonas* sp. CF600 [Mrozik *et al*., 2006]. Thus bacteria possess regulatory mechanisms that allow them to preferentially utilize certain C sources when a mixture of several other substances is supplied [Mrozik *et al*., 2007]. Indeed, it has been demonstrated that promoters of biodegradative operons are down-regulated in response to exponential growth in rich media irrespective of the presence of an effector [Cases and de Lorenzo, 2001]. This phenomenon is referred to as catabolite repression or exponential silencing. The nutrient rich LB medium could exert such a repression effect on the catabolic mechanism of strain P3. This study provides preliminary evidence that the enzymes involved in OPP degradation are inducible. These mechanisms that allow cells not to waste energy on the unnecessary formation of enzymes for degradation are not well clarified. In fact, other cases are described in which degradation was

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strictly connected with the presence in the medium of extra C and N sources, suggesting a co-metabolic degradation process [Ellegaard-Jensen *et al*., 2013; Yun *et al*., 2009].

3.6.4. Cross-feeding studies: assessment of the ability of the isolate to degrade compounds of similar chemical structure

The capacity of the OPP-degrading isolate to metabolize 2,3-dihydroxybiphenyl, benzoic acid, and catechol that constitute possible metabolites of OPP was evaluated. In addition its capacity to degrade DPA was also checked, based on the similarity of its chemical structure and its practical use along with OPP in the fruit-packaging plants. The chemical structures of the molecules tested are shown in Figure 5.16.

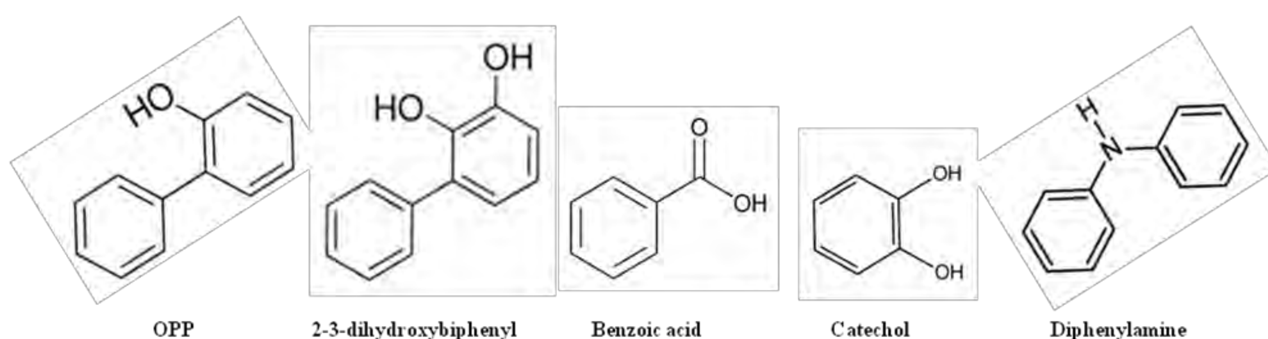

Figure 5.16. The chemical structure of the tested molecules.

As shown in Figure 5.17, 2,3-dihydroxybiphenyl was unstable in the medium and was completely transformed even in the non inoculated cultures within 2 days. Benzoic acid and catechol were both completely degraded by the strain P3 within 2 days. However, substantial catechol degradation (80%) was also evident in the non-inoculated controls. This general instability of some of the phenolic molecules tested is not surprising since phenolic compounds exist in a partially dissociated state in water and could become particularly reactive when exposed to light and O_2 [Thomson, 1964]. The *S. haloaromaticamans* strain P3 showed no capacity to degrade DPA despite its structural similarity with OPP (both bi-phenolic compounds but with different substitutes in the rings). Previous studies have demonstrated that the overall size and orientational flexibility of the two aromatic rings is important for metabolic pathway activation [Jaspers *et al*., 2000]. Thus, the strain P3 was able to degrade benzoic acid and catechol, whereas its capacity to degrade 2,3-dihydroxybiphenyl was not confirmed experimentaly due to the instability of this molecule under the experimental conditions used.

The formation of these putative metabolites during degradation of OPP by strain P3 was further evaluated. No residues of catechol, benzoic acid, and 2,3-dihydroxybiphenyl were detected throughout the study, despite the rapid degradation of OPP (50 mg/l were completely

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degraded in 2 days). These results might indicate that those compounds are effectively not produced in the degradation pathway followed by the isolated bacterium. Alternatively, the production of those metabolites could be a very rapid process which does not allow its transient accumulation in the medium. The latter hypothesis is in agreement with previous findings by Jaspers *et al*. [2000] which showed that the OPP-degrading strain *P. azelaica* HBP1 had developed mechanisms to ensure the rapid elimination of 2,3-dihydroxybiphenyl that exerts a toxic effect on the bacterial cells.

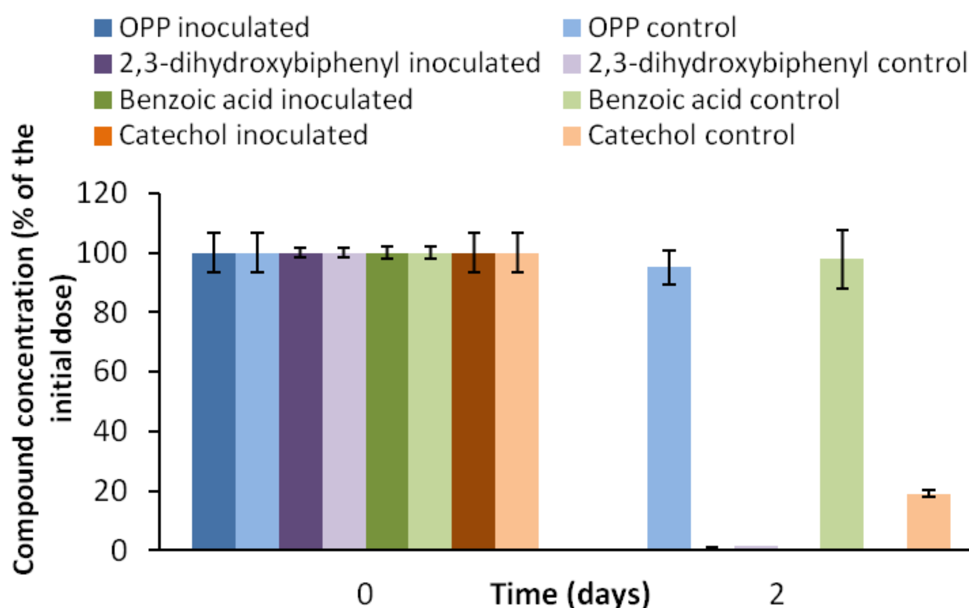

Figure 5.17. Degradation of OPP and its possible metabolites by the OPP-degrading strain P3. Degradation of OPP in non-inoculated cultures (control) is also reported. Error bars represent the standard deviation of the mean.

So far there are two pathways that have been reported for the microbial degradation of OPP. In the first and better studied, *P. azelaica* HBP1 metabolizes OPP through a *meta* cleavage pathway (Figure 5.18) [Kohler *et al.*, 1988]. The first step in the degradation is catalyzed by HbpA, a flavin adenine dinucleotide-containing 2-hydroxybiphenyl-3-monooxygenase, that catalyzes the NADH-dependent *ortho* hydroxylation of OPP to 2,3-dihydroxybiphenyl. Next, HbpC, a 2,3-dihydroxybiphenyl-1,2-dioxygenase, catalyzes the *meta* cleavage, resulting in 2-hydroxy-6-oxo-6-phenyl-2,4-hexadienoic acid [Kohler *et al.*, 1993]. The last compound is then hydrolyzed by the *meta*-cleavage product hydrolase HbpD to 2-hydroxy-2,4-pentadienoic acid (2-oxopent-4-enoate) and benzoic acid [Kohler *et al.*, 1993]. 2-hydroxy-2,4-pentadienoic acid is easily transformed into pyruvate that enter in the glycolysis, and in acetaldehyde that enter in the pyruvate metabolism [Denef *et al.*, 2005; Shingler *et al.*, 1992]. Benzoic acid is further converted by benzoate 1,2-dioxygenase to catechol, which is the substrate for the lower *meta*-cleavage

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pathway [Jaspers *et al*., 2000] that involves ring fusion and eventual production of pyruvate and acetaldehyde [Denef *et al*., 2005; Shingler *et al*., 1992]. The upper pathway resembles the major catabolic pathway of biphenyl, where a dioxygenase catalyze the first-step instead of the monooxygenase which is needed for the first step in the microbial degradation of OPP. This common pathway has been found in different bacteria, such as *Pseudomonas* sp. [Kikuchi *et al*., 1994], *Sphingomonas yanoikuyae* [Kim and Zylstra, 1999], *Bulkhoderia xenovorans* [Denef *et al*., 2005], *Micrococcus* sp. [Bevinakatti and Ninnekar, 1992], and *Dyella ginsengisoli* [Li *et al*., 2009].

A second degradation pathway for OPP was reported in a biphenyl-degrading *Pseudomonas testosterone* strain B-356 [Sondossi *et al*., 1991]. Based on this, OPP degradation proceeds *via* dioxygenation of the non-hydroxylated aromatic ring of OPP, subsequent rearomatization, and *meta* cleavage. Considering that this pathway was reported in a bacterium which was primarily a biphenyl degrader and its capacity to degrade OPP was attributed to the relaxed specificity of dioxygenases, it was hypothesized that the metabolism of OPP by the isolated strain P3 followed the same pathway as the one reported in the only other OPP-degrading bacterium, *P. azelaica* HPB1.

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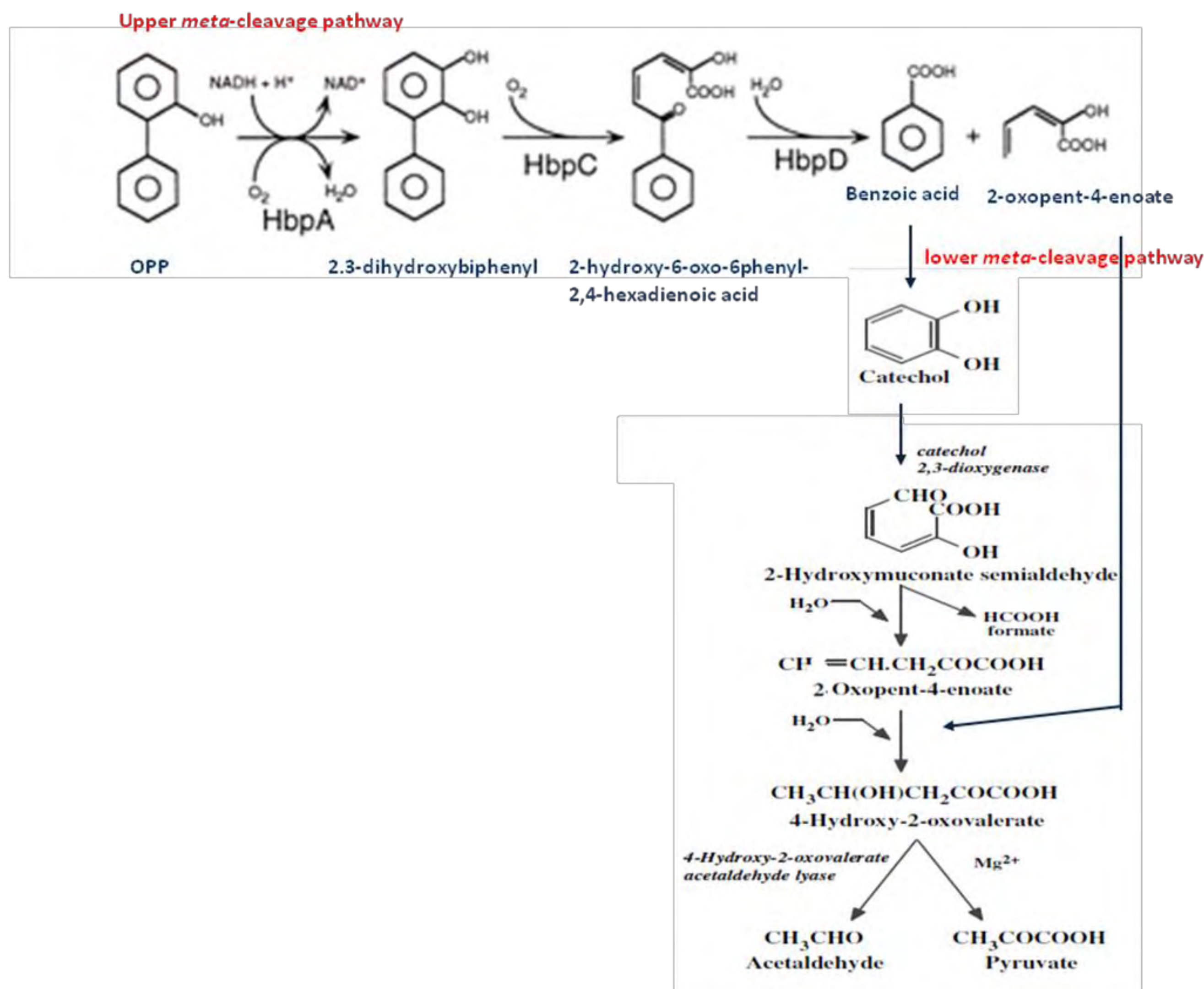

Figure 5.18. The metabolic pathway of OPP by *P. azelaica* HBP1 (sources: upper pathway Jaspers *et al*. [2000]; lower pathway Zeyaullah *et al*. [2009]).

3.6.5. Testing the presence of hbp genes in the OPP-degrading strain

The previous experiments did not elucidate the metabolic pathway of OPP in strain P3. Therefore, the presence in the strain P3 of the *hbp* genes known to be involved in the upper pathway of the metabolism of OPP in *P. azelaica* strain HBP1 was investigated *via* PCR. The *hbp* operon consists of three structural genes, *hbpA* (encoding the 2-hydroxybophenyl-3-monooxygenase-step) 1), *hbpC* (encoding the 2,3-dihydroxybiphenyl-1,2-dioxygenase, step 2), and *hbpD* (encoding for the meta-cleavage product hydrolase, step 3), and a regulatory gene, *HbpR*. The *hbpCAD* genes are organized in a small noncontiguous gene cluster and *HbpR* is located directly upstream, as shown in Figure 5.19 [Jaspers *et al*., 2000 and 2001].

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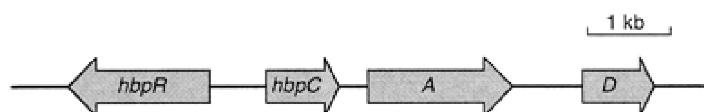

Figure 5.19. Genetic organization of the *hbp* genes in *P. azelaica.* The orientation and size of the genes are indicated by arrows; solid lines represent non-coding DNA (adapted by Jaspers *et al*. [2000]).

PCR on total DNA from the strain P3 and from the *P. azelaica* strain HBP1 (used as positive control) showed positive amplification only from *P. azelaica* HBP1 DNA, while no PCR amplicons were detected for any of the four genes tested when DNA from the strain P3 was used as template (Figure 5.20). These results indicated that the isolated *S. haloaromaticamans* strain P3 does not carry the genes contained in the *hbp* operon of *P. azelaica*. However, the fact that no identical genes were detected does not preclude the possibility that a similar degradation pathway is followed, considering also the capacity of the strain P3 to degrade key-metabolites such as benzoic acid and catechol. Furthermore, even in the case of biphenyl degradation, the *bph* gene clusters identified to date in different bacteria demonstrate that, while maintaining the same degradation pathway, some are very similar but some are very different in terms of gene organization and the structure of each gene [reviewed by Furukawa *et al*., 2004].

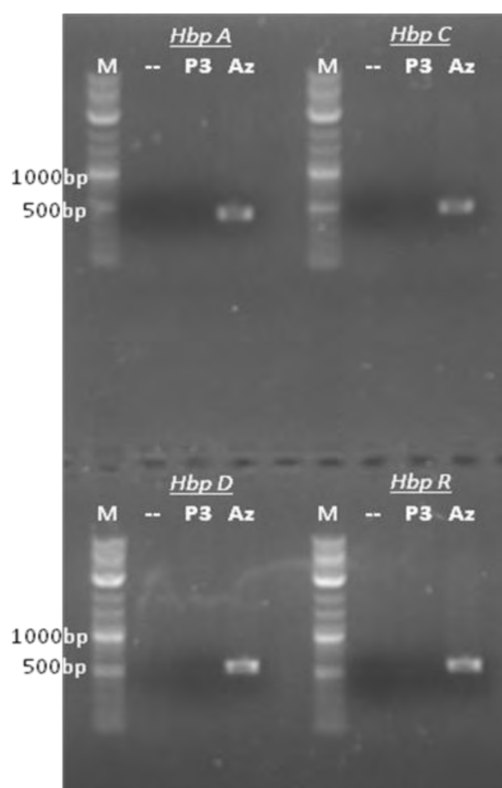

Figure 5.20. 1.5% agarose gel electrophoresis showing the PCR products amplified from the total DNA of *S. haloaromaticamans* strain P3 (lanes P3) and *P. azelaica* strain HBP1 (lanes Az). The four genes amplified are indicated. “--“ (minus) indicates the blank for each PCR reaction. Lane M: molecular size marker.

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3.6.6. The effect of pH and temperature on the degrading ability of the OPP-degrading bacterium

Strain P3 was able to rapidly degrade OPP in the wide range of pH tested (Figure 5.21). The more rapid degradation of OPP was observed at slightly acidic to neutral pH (5.5 and 6.5), where degradation of OPP was completed in a day. A slight retardation in the degradation was observed at more acidic (4.5) and at more alkaline pH levels (7.5-9) where complete degradation occurred in 2 days. OPP was stable under abiotic conditions in the range of pH tested, as expected [EFSA, 2008]. The bacterial starting inoculum for all the treatments was $3x10^5$ cells/ml.

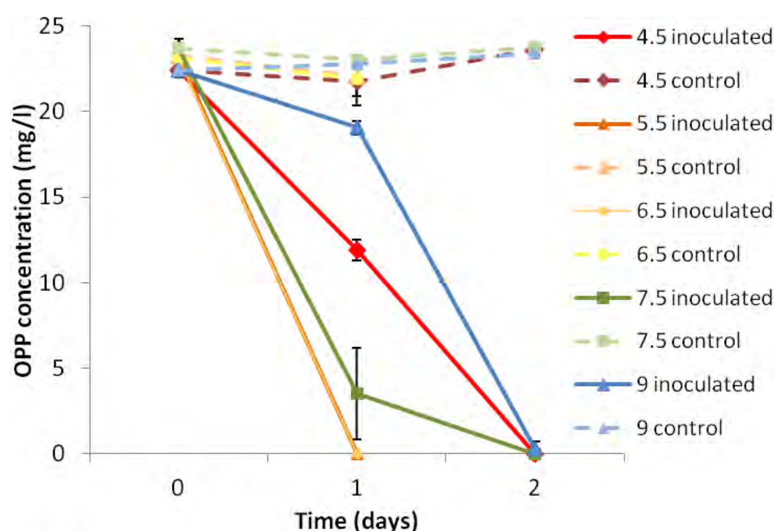

Figure 5.21. The effect of pH on the capacity of *S. haloaromaticamans* strain P3 to degrade OPP. Degradation of OPP in non-inoculated cultures (control) is also reported. Bars represent the standard deviation of the mean.

Regarding the effect of temperature on OPP degradation by strain P3, differences were observed between the treatments. OPP was completely degraded by strain P3 in 2, 5, 8 and 30 days at 37, 26, 15 and 4°C respectively (Figure 5.22). The bacterial inoculum was $3x10^5$ cells/ml. Those results indicate that strain P3 was able to degrade OPP at slower rates even a 4°C which might be encountered during its application in biofilter systems. Overall the data from the pH and temperature tests suggest that the strain P3 is a good candidate for bioremediation applications since it maintains its degrading capacity even at extreme pH and temperature conditions that might encounter during bioaugmentation and industrial applications.

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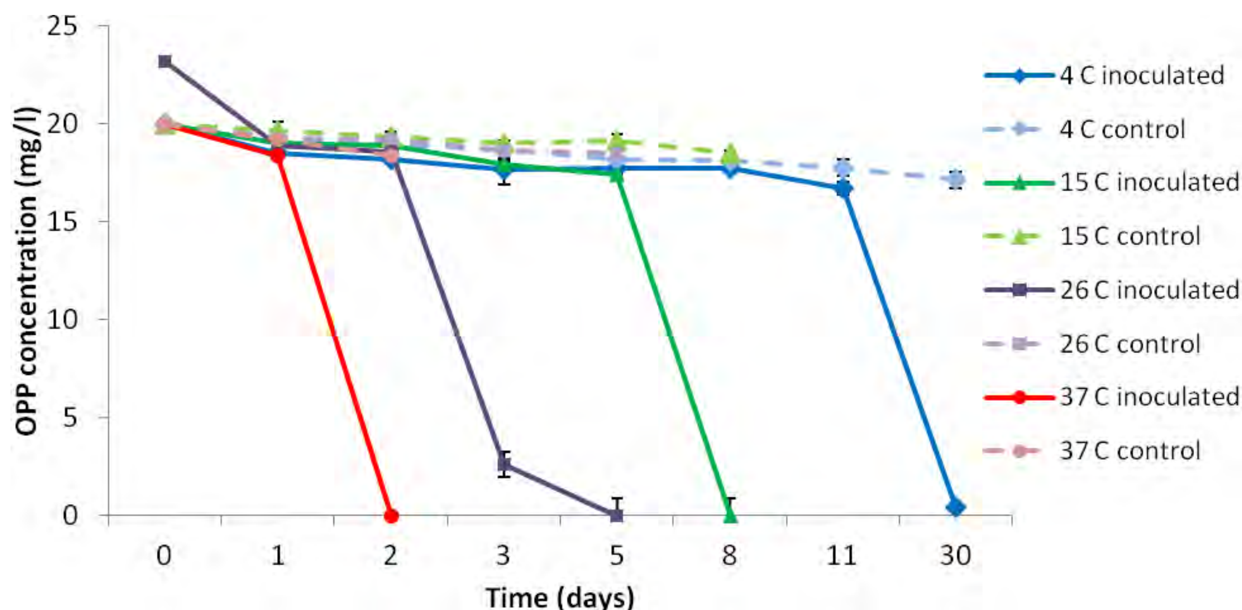

Figure 5.22. The effect of different temperatures on the degrading ability of the *S. haloaromaticamans* strain P3. Degradation of OPP in non-inoculated cultures (control) is also reported. Bars represent the standard deviation of the mean.

3.6.7. The effect of the co-presence of other pesticides and of their respective degrading bacteria on the degrading capacity of the OPP-degrading isolate

The degradation of OPP by the strain P3 was evaluated in the presence of TBZ and DPA and of their respective degrading bacteria (Chapter 3 and 6). The starting bacterial inoculum for strain P3 was $2x10^5$ cells/ml. The co-presence of other pesticides and their respective degraders did not affect the degrading capacity of strain P3 which degraded OPP in 2 days in all possible combinations tested (Figure 5.23). These results are important for a practical point of view since this bacterium is expected to be used as an inoculum along with other tailored-made inocula in biofilter systems for the treatment of wastewater from the fruit packaging industry. In such an application approach the strain P3 will be co-inoculated with other pesticide-degrading inocula in the biofilter systems and it will be exposed to wastewaters which might contain mixtures of OPP, TBZ, and DPA.

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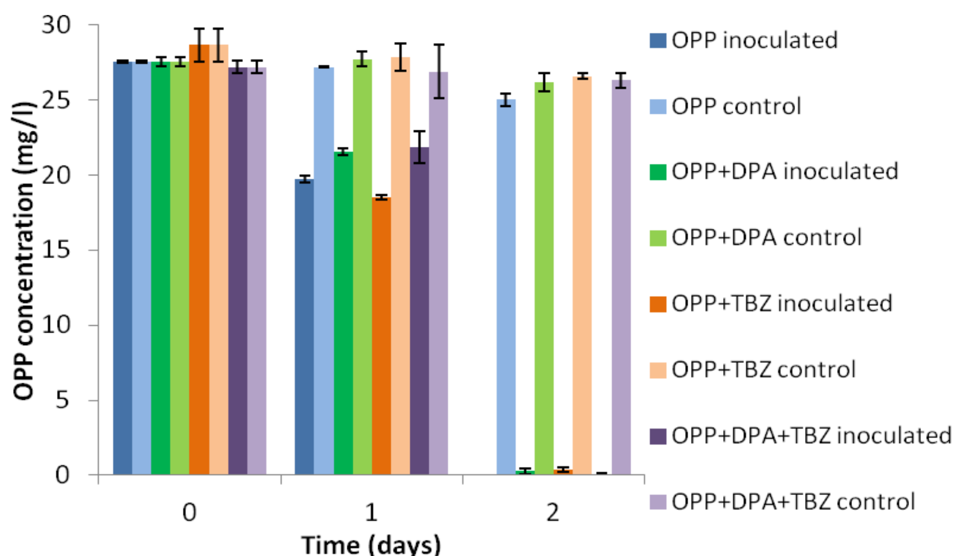

Figure 5.23. The degradation of OPP by *S. haloaromaticamans* P3 in the co-presence of TBZ (OPP+TBZ), DPA (OPP+DPA), TBZ and DPA (OPP+TBZ+DPA), and their respective degrading bacteria. Degradation of OPP in non-inoculated cultures (control) is also reported. Bars represent the standard deviation of the mean.

3.6.8. Assessment of the soil bioaugmentation potential of the OPP-degrading isolate

The capacity of the *S. haloaromaticamans* strain P3 to degrade OPP in an artificial contaminated soil was evaluated. For the three concentration levels considered, 10, 100, and 500 mg/kg, no differences in the dissipation rates between the inoculated and the non-inoculated samples were observed. Thus complete dissipation of OPP was evident in both inoculated and control samples within 5 days from inoculation in the two lower concentration levels, whereas nearly 20 days were needed for the complete dissipation in the higher concentration level (Figure 5.24). The data obtained did not clarify the bioaugmentation potential of the OPP-degrading isolate since it appears that OPP was not particularly persistent in soil and it rapidly dissipated either by abiotic processes or due to the contribution of the indigenous soil microflora. The limited persistence of OPP in soil observed in our study is in agreement with previous regulatory studies that showed $t_{1/2}$ values from three hours to three weeks [US EPA, 2006; EFSA, 2008].

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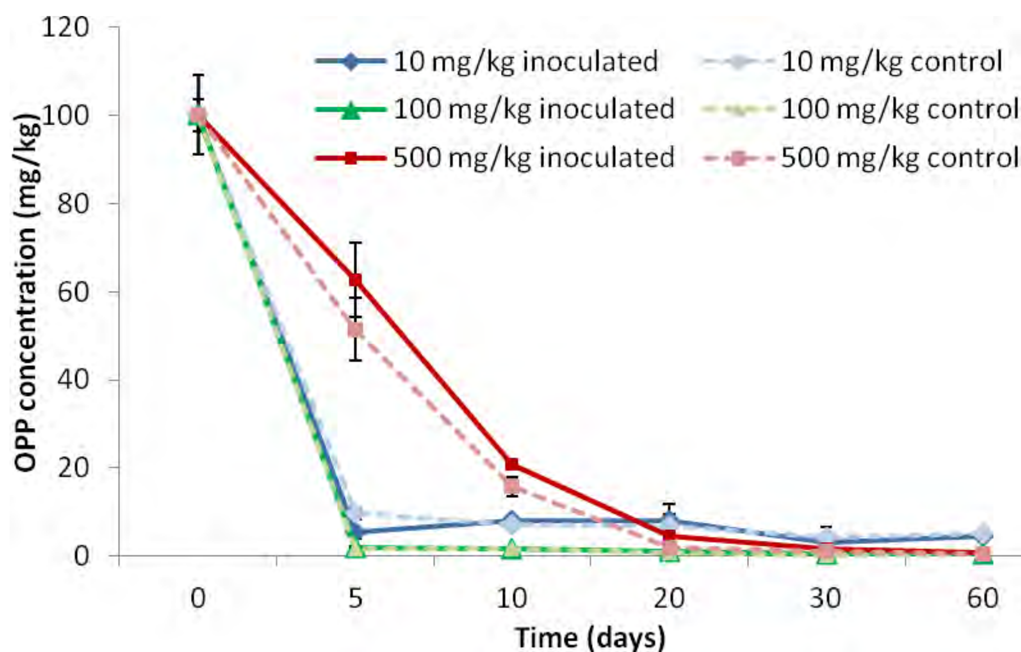

Figure 5.24. The degradation of OPP by *S. haloaromaticamans* strain P3 inoculated in a soil previously treated with 10, 100, and 500 mg/kg of the fungicide. The degradation of OPP in non-inoculated controls is also presented. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

3.6.9. Assessment of the degradation potential of the OPP-degrading strain in Biobed Extract Medium and maintenance of its degrading capacity upon exposure to composting-like temperatures

BEM is a liquid medium derived from biobed substrates, thus imitating its nutritional conditions. It is supposed to be richer than MSMN, containing extra C and energy sources alternative to OPP. As shown in Figure 5.25, the rapid degradation of OPP was maintained in BEM and it was comparable to the rates observed in MSMN, with almost complete degradation within 2 days. The bacterial starting inoculum in both media was of $5x10^5$ cells/ml. The maintenance of the degrading ability of the OPP-degrading bacterium in BEM, where no casamino acids were added, suggests that there were alternative elements in this medium which satisfied the nutritional needs of the strain P3 allowing it to actively degrade OPP. These results further stresses the adaptability of the *S. haloaromaticamans* strain P3 as inoculum in a biobed system, in which the bacterium is supposed to maintain the degradation ability, and its potential for future implementation in the bioaugmentation of biobeds receiving OPP-containing wastewaters.

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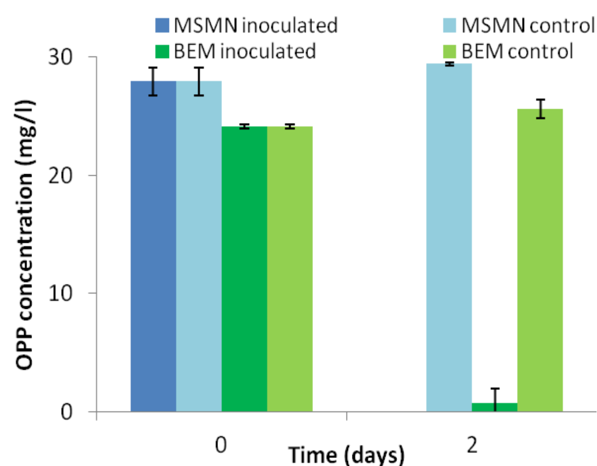

Figure 5.25. The degradation of OPP in MSMN and BEM inoculated with the *S. haloaromaticamans* strain P3. Degradation of OPP in non-inoculated cultures (control) is also reported. Each value is the mean of three replicates ± standard deviation.

The use of biobeds for the depuration of pesticide-containing wastewaters results, at the end of the life cycle of the system, in the release of a spent biobed matrix which could contain high pesticide loads. Thus, decontamination of biobed materials before release in the environment is needed. The use of composting combined with bioaugmentation with specific pesticide-degraders has been proposed as an environmental friendly and cheap way to decontaminate those materials [Karanasios *et al*., 2012]. During composting, the temperature levels reached (up to 40-60°C) could be critical for bacterial survival. Thus, the tolerance and degrading activity of strain P3 at such composting temperatures were evaluated.

Liquid cultures of the strain P3 in MSMN+ and BEM + OPP were exposed to a temperature profile measured during a 28-days composting experiments by P. Karas (PhD student, Group of Plant and Environmental Biotechnology, University of Thessaly, Greece). As shown in Figure 5.25, temperatures fluctuated between 25 to 50°C, with the thermophilic phase (50°C) lasted for 4 days. OPP was completely degraded in both media within 7 days, before the thermophilic phase was reached (Figure 5.26). A substantial abiotic degradation of OPP was also observed in the non-inoculated controls which could be attributed to the prolongation of the experiment, since OPP is thermostable at 50°C [EFSA, 2008].

At the end of the experiment, when the bacterial cultures were transfered in fresh corresponding media and incubated at 26°C no degradation was observed within 28 days (data not shown) suggesting that the bacterium did not eventually survived the simulated thermophilic phase. These results suggest that if bioaugmentation of OPP-contaminated spent biobed substrates is applied in conjunction with composting, the bacterium should be inoculated in advance to allow OPP degradation before the thermophilic phase is reached.

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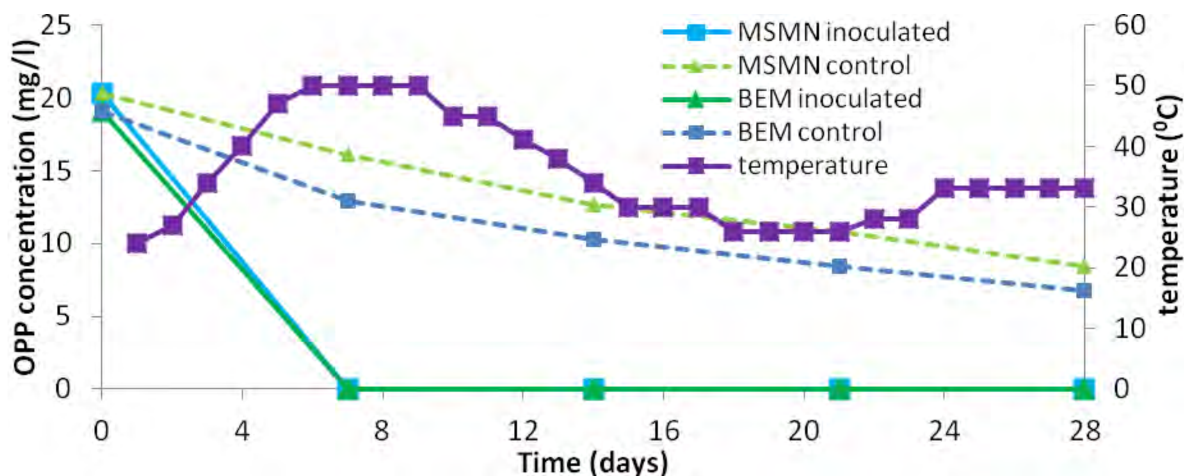

Figure 5.26. OPP degradation in MSMN and BEM inoculated with *S. haloaromaticamans* strain P3 and exposed for a 28-day period to a composting-temperature scheme. The degradation of OPP in non-inoculated cultures of the same media is also shown.

4. Conclusions and Future work

OPP is a fungicide widely used in the fruit packaging industry for the control of fungal infestations during storage. Its application results in the production of large wastewater volumes containing high levels of OPP (up to 600 mg/l) which, according to EU legislation, should be treated on site since their direct environmental release constitute an unacceptable risk to aquatic ecosystems. Thus an efficient and economic system for the treatment of OPP-contaminated wastewaters and in a wider sense for the detoxification of wastewaters from the fruit packaging industry is needed. In this context the development and implementation of biofiltration systems inoculated with tailored-made OPP-degrading bacterial inocula could be the way forward. Thus, the isolation of microorganisms able to rapidly degrade OPP constitutes the first step towards this goal. In addition, the currently applied practice of direct disposal of OPP-contaminated wastewaters in abandoned field sites have resulted in the creation of heavily polluted hotspots which need direct decontamination. For such situations, bioaugmentation with OPP-degrading microorganisms appears as a possible method.

This study resulted in the isolation of a *Sphingomonas haloaromaticamans* bacterium (strain P3), from a soil collected from a wastewater disposal site, which was able to degrade OPP and use it as energy source. A series of tests demonstrated that the ability of strain P3 to degrade OPP was dependent on the external supply of amino acids, provided in the form of casamino acids in the medium. Alternatively the strain P3 was able to degrade OPP in the presence of other bacteria which did not appear to contribute to the degradation of OPP or to the transformation of its intermediate metabolites but they could probably provide essential elements

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that strain P3 was not able to synthesize. Although the metabolic pathway of OPP was not elucidated, the isolated strain P3 was able to degrade molecules which have been reported as key metabolites during degradation of OPP by the only other OPP-degrading bacterial strain known so far, *P. azelaica* HBP1. In addition, the *hbp* genes known to be involved in the upper metabolic pathway of OPP in *P. azelaica* were not detected suggesting the presence of an alternative genetic armoury for the degradation of OPP by the isolated *S. haloaromaticamans* strain P3. On-going studies aim to elucidate the metabolic pathway of OPP in the isolated strain P3 using a combination of high resolution analytical methods (LC-MS/MS or GC/MS) and genomic / proteomic analysis. The latter will also allow us to identify the genes and enzymes involved in OPP degradation.

The *S. haloaromaticamans* strain P3 maintained its degrading capacity in a range of temperature and pH conditions, in the co-presence of different pesticides and competing pesticide-degrading microorganisms, and also in liquid media resembling the nutritional status of biobed substrates suggesting that it could be a good candidate for future applications in the biological treatment of wastewaters from the fruit packaging industry. Practical future aspects of the project will focus on the development, evaluation, and implementation of a biological treatment unit inoculated with the isolated strain for the depuration of real-life agro-industrial effluents from citrus fruits packaging plants, mostly situated in Peloponnese, Greece where the fungicide OPP is heavily used.

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Chapter 6

Isolation and characterization of bacteria able to degrade diphenylamine

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1. Introduction

Diphenylamine (*N-phenylbenzenamine*) is an antioxidant used at post-harvest level to control the physiological disorder called scald on apples and pears. DPA is generally applied by drenching at concentrations of 400-2000 g/l [Rudell *et al*., 2005; EC, 2012]. DPA is authorized for use in several countries including USA, Canada, Australia, Chile, Argentina, Uruguay, South Africa, and countries adopting Codex standards. In contrast, in 2009 the EC did not grant authorization for inclusion of DPA into Annex I of the Directive 91/414/EEC, which lists all the authorized substances in the EU. The non-approval of the active substance DPA, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council, was based on the identification of unacceptable risks for the consumers. In particular it was noted that "*it was not possible to perform a reliable consumer exposure assessment, as data were missing on the presence and toxicity of unidentified metabolites of the substance, as well as on the possible formation of nitrosamines (carcinogens) during storage of the active substance and during processing of treated apples. Moreover, no data was available on the potential breakdown or reaction product of diphenylamine residues in processed commodities*" [EC, 2012]. Despite that and based on the proven lack of alternative pesticides which could replace DPA on the market, in 2012 major fruit-producing countries like France, Ireland, UK, Portugal, Spain, Italy, and Greece have granted exemption authorization for DPA for a period of 120 days.

The drenching application of DPA results in the formation of large wastewater volumes with high concentration levels of DPA. Based on EFSA [2012], *management measures tailored to local practice and legislation need to be put in place to control the waste disposal of spent application solution and prevent accidental spillage entering sewers or surface water drains*. Considering the high toxicity of DPA on aquatic organisms [Drzyzga, 2003; EC, 2008], the direct disposal of DPA-containing wastewaters without any prior treatment entails a serious risk for the aquatic organisms inhabiting receiving water resources and should be mitigated. Several attempts have been made for the development of treatment systems for the detoxification of DPA-contaminated wastewaters. Flaim and Toller [1989] proposed the use of filtering system based on a mixture of peat moss, manure, clay, and dolomite sand that was able to remove 2 g/l of DPA from wastewater with an efficiency of 98.9%. However the specific filter was not able to handle the high wastewater volumes produced by the fruit-packaging industries and was abandoned. The potential of using enzymatic treatment to remove DPA from buffered synthetic wastewater was also tested. This treatment method includes oxidative polymerization of DPA using laccase from the fungus *Trametes villosa*, followed by removal of those polymers *via* adsorptive micellar flocculation. In batch reactors 95% substrate conversion was achieved in

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three hours [Saha *et al*., 2008]. However this method is considered laborious for handling high wastewater volumes, it has relatively high cost and results in the production of DPA-containing solid waste which will need further handling.

The lack of a reliable method for the treatment of wastewaters from the fruit-packaging industry has forced most fruit-packaging industries to discharge their DPA-contaminated wastewaters into nearby abandoned fields or into creeks, streams, evaporation ponds or directly into the municipal sewage treatment system increasing the likelihood for environment contamination and degradation of ecosystems quality. Thus there is an urgent need for the development and implementation of methods for the treatment of DPA-contaminated wastewaters. Biological treatment systems based on specialized DPA-degrading inocula could be an efficient and economically feasible solution for the treatment of those wastewaters. Alternatively, modified biobed systems augmented or not with DPA-degrading microorganisms could be also a viable alternative.

According to the Technical Guidance Document on Risk Assessment, DPA is regarded as not readily biodegradable. In fact, using non adapted inocula, only 25% mineralisation was achieved. Christodoulatos *et al*. [1997] reported the ability of three *Pseudomonas* strains and of a mixed activated sludge culture to rapidly degrade DPA ($t_{1/2}$ = 1.40 days in a bioreactor) at concentration levels of 15 to 60 mg/l. More recently a *Burkholderia* sp. and a *Ralstonia* sp. strain were isolated as DPA-degraders and the degradation pathway and the genes involved were determined [Shin and Spain, 2009]. It was shown that DPA was metabolized to catechol and aniline which were further transformed *via* relevant pathways which are ubiquitous in soil bacteria. DPA microbial degradation was also observed in a sewage sludge experiment [Gardner *et al*., 1982], in anoxic sediment-water batch enrichments and in cultures of newly isolated sulfate-reducing bacteria [Drzyzga and Blotevogel, 1997; Drzyzga *et al*., 1996]. Apart from bacterial degradation, white rot fungi like *Trametes versicolor* and *Pleurotus ostreatus* were able to degrade 10 mg/l of DPA within 2 hours with parallel stimulation of their lignolytic enzymatic system suggesting its involvement in the transformation of DPA [Karas *et al*., 2011].

Based on this, our study aimed to isolate and identify bacteria able to rapidly degrade the antioxidant DPA. Subsequent focus was given to the characterization of the degrading capacities of the isolated microorganisms in order to get an overview of their application potential.

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2. Materials and methods

2.1. Pesticides and other chemicals

Diphenylamine (Pestanal®, analytical standard, 99.9% purity) was purchased from Fluka, Sigma-Aldrich. Methanol stock solutions (1000 mg/l) were prepared and serial dilutions were used for analytical purposes, while a filter sterilized (0.22 μm, MS® PES Syringe Filter, Membrane Solition) 100 mg/l water stock solution of DPA, was also prepared and used for the preparation of DPA-amended media.

A formulation (No Scald DPA 31.8 EC, Decco Iberica, Post-Cosecha s. a. u., Paterna, Valencia, Spain) containing 318 g/l of DPA was available and used in certain cases as described below. A 20 g/l stock solution of DPA in dimethyl sulfoxide (DMSO, 99.9%, ChemLab) was also prepared, filter sterilized (0.22 μm, PTFE Syringe Filter, sterilized) and used in certain cases as described below.

Other molecules used in the present work were catechol (ReagentPlus®, ≥99% purity, Sigma-Aldrich), aniline (≥99% purity, ChemLab Supply), and *ortho*-phenyl phenol (OPP) (Pestanal®, analytical standard, 99.9% purity, Fluka, Sigma-Aldrich). For those compounds stock solutions in methanol (1000 mg/l) and water (100 mg/l) were prepared as described for DPA.

2.2. Growth media

For the isolation of DPA-degrading bacteria *via* enrichment the two minimal salts media MSM and MSMN were used. The two media were amended with DPA using a 100 mg/l filter-sterilized aqueous stock solution aiming to a final concentration of 20 mg/l unless otherwise stated. Agar plates of the corresponding media + DPA were prepared in the same way but with the addition of 15 g/l of agar before sterilization (Chapter 2). Other media used were LB and Biobed Extract Medium (BEM) which were prepared as described in Chapter 2.

2.3. Pesticide extraction and HPLC analysis

The residues of DPA and of other molecules studied were extracted from liquid media as described in Chapter 2. Regarding DPA extraction from soil, 10 g of soil were mixed with 25 ml of acetonitrile (ACN). The mixture was agitated for 1.5 hours in an orbital shaker at 200 rpm and then centrifuged at 11000 rpm for 5 minutes. The supernatant was collected in glass bottles and filtered (45μm, PTFE Syringe Filter) before analysis in an HPLC system described in Chapter 2.

DPA residues were determined at 210 nm using a mobile phase of 60:30:10 ACN:water:methanol (by volume). Under these conditions DPA showed a retention time of 3.5

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min. Apart from DPA, compounds which were hypothesized to be main metabolites of DPA were analysed *via* HPLC. Thus, catechol was eluted using a mobile phase of 60:40 water:ACN (by volume) + 0.1% acetic acid and its residues were determined at 276 nm (retention time 2.5 min). Aniline was eluted using a mobile phase of 60:40 ammonium acetate solution (0.4 g/l at pH 4.2):ACN (by volume) and detection was achieved at 254 nm (retention time 3.4 min). Finally OPP residues were determined as described in Chapter 5.

2.5. Enrichment culture for the isolation of DPA-degrading bacteria

The enrichment culture technique was followed for the isolation of DPA-degrading bacteria. A soil from a wastewater disposal site in the area of Agià, Larissa, Greece, was used as a source of DPA-degrading bacteria. The soil was treated with a fresh addition of DPA (10 mg/kg), as described in Chapter 2, and was incubated at 26°C for a week. After this period, 0.5 g of the soil was used to inoculate fresh cultures of MSM and MSMN + DPA which constituted the only C and N or the sole C source respectively. The degradation of DPA in the enrichment cultures was monitored regularly by HPLC analysis and when > 50% degradation occurred the next enrichment cycle was initiated. In total, four enrichment cycles were employed and when > 50% of DPA was evident in the fourth cycle, aliquots of the media were used for the preparation of 10-fold serial dilutions which were plated in the corresponding MSM or MSMN+ 20 mg/l DPA agar media. Upon incubation at 26°C for 4-5 days, growing colonies were selected and used to inoculate fresh MSMN and MSM + DPA (20 mg/l) liquid media and DPA degradation was checked. Cultures exhibiting >50% in 7 days were considered as positive and were plated on LB and MSM or MSMN+ 20 mg/l DPA agar plates to verify purity. The bacteria that appeared as pure in plates were harvested from the corresponding liquid cultures and the pellet obtained was used for DNA extraction as described before.

2.6. Molecular analysis of isolated bacteria

Upon isolation of DPA-degrading bacteria, DNA extraction and amplification of their full length 16S rRNA gene were done as described in Chapter 2. DGGE analysis and clone libraries were employed (as described in Chapter 2) in order to verify purity and identify the bacteria.

2.7. PCR amplification of the *rpoD* and *gyrB* genes

In order to obtain a more robust phylogenetic analysis of our Pseudomonas isolated bacteria the genes *gyrB*, encoding the beta-subunit of the enzyme gyrase, and *rpoD*, encoding the sigma 70 subunit of the RNA polymerase, were amplified using the primers UP1E/APrU [Yamamoto *et*

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al., 2000] and PsEG30F/PsEG790R [Mulet *et al*., 2009] respectively. The sequences of the primers are listed in Table 6.1. The PCR components are listed in Table 2.2, Chapter 2. The PCR thermocycling conditions were as described by Mulet *et al*. [2009] (Tables 6.2 and 6.3). The amplification products for *gyrB* and *rpoD* were 1000 and 700 bp respectively and their size was verified *via* agarose electrophoresis in 1% agarose gel. The PCR products were subsequently purified, ligated into pGEM vector, and transformed into competent *E. coli* cells. White colonies of the transformed cells were selected for plasmid extraction and sequencing.

Table 6.1. Sequences of the primers used to amplify the genes *gyrB* [Yamamoto *et al*., 2000] and *rpoD* [Mulet *et al*., 2009] from the DPA-degrading strain.

Gene target	Primer Name	Primer sequence (5' – 3')
gyrB	UP1E	CAG GAA ACA GCT ATG ACC AYG SNG GNG GNA RTT YRA
	APrU	TGT AAA CGA CGG CCA GTG CNG GRT CYT TYT CYT GRC A
rpoD	PsEG30F	ATY GAA ATC GCC AAR CG
	PsEG790R	CGG TTG ATK TCC TTG A

Table 6.2. The PCR thermocycling program used for the amplification of the *gyrB* gene.

Steps	Temperature (^oC)	Time (min.)	Cycles
Initial denaturation	94	2	1
Denaturation	94	1	
Annealing	50	1	35
Extension	72	1	
Final extension	72	10	1

Table 6.3. The PCR thermocycling program used for the amplification of the *rpoD* gene.

Steps	Temperature (^oC)	Time	Cycles
Initial denaturation	94	2 min.	1
Denaturation	94	1 min.	
Annealing	53	1 min.	30
Extension	72	40 sec.	
Final extension	72	10 min.	1

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2.8. Sequences alignment and construction of phylogenetic tree

Sequenced bacterial genes were aligned with the program MegAlign™ (Lasergene®, DNASTAR). The phylogenetic tree of the isolated degrader was constructed as described in Chapter 2. To obtain a better classification at species level, the sequences of the three housekeeping genes *16S rRNA*, *gyrB*, and *rpoD* were considered together for phylogenetic analysis. Every sequenced gene was processed as elucidated in Chapter 2; then, before the Topali step, the three resulting blocks of every sequence were concatenated in a single one (concatenated alignment) with the GBlocks software (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The concatenated alignment was then processed in the next steps.

2.9. Experiments for the characterization of the degrading ability of the isolate

2.9.1. Assessment of the bacterial growth during DPA degradation

The growth kinetics of the isolated bacterium during DPA degradation was evaluated in both MSM and MSMN. Three inoculated replicates and two not-inoculated controls were prepared. The cultures were inoculated from a fresh culture of the DPA-degrading bacterium and the inoculation was determined by spread plating. Samples were shaken at 26°C/180 rpm and degradation was measured by HPLC at the time of inoculation (T0) and 4, 8, 12, 16, and 24 hours later. At the same sampling times, aliquots of appropriate of the degrading cultures were serialy diluted and spread on LB agar plates for bacterial counting.

2.9.2. Ability of the DPA-degrading bacterium to degrade high DPA concentration

The ability of the isolated bacterium to degrade a wide range of DPA concentrations in liquid cultures was evaluated. Thus MSM was prepared and amended with appropriate volumes of filter sterilized aqueous solutions of DPA (3.1, 16, and 52 g/l) prepared by the commercial formulation of DPA in order to get final DPA concentrations in MSM of 20, 120, 600, 1000, and 2000 mg/l. For every concentration level, three inoculated cultures and two not-inoculated controls were considered. The cultures were inoculated from a fresh culture of the DPA-degrading bacterium and the inoculation was determined by spread plating. All cultures were placed in an orbital shaker and incubated at 26°C/180 rpm. DPA degradation was measured by HPLC at 0, 2, and 4 days after inoculation as described before.

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2.9.3. Assessment of the ability of the isolate to degrade other compounds including possible metabolites of DPA

The ability of the DPA-degrading isolate to degrade possible metabolites (catechol and aniline) and compounds of similar chemical structure (*ortho*-phenyl-phenol, OPP) was evaluated. Because of the photosensitivity of catechol, methanol and water stocks and media containing catechol were covered with aluminium foil in order to minimize abiotic losses. OPP and catechol degradation was evaluated in MSMN (because of the lack of N in their molecules), while DPA and aniline degradation was evaluated in MSM. For each compound, three inoculated replicates and two non-inoculated controls were prepared. All cultures were inoculated by a fresh culture of the DPA-degrading isolated in MSM. The inoculum density was determined *via* spread plating on LB. All samples were incubated in an orbital shaker at 26ºC/180 rpm and degradation of the different compounds was measured by HPLC at 0, 12, and 24 hours after inoculation. In the case of OPP, degradation measurements were also taken at 6, 13, 20, 30, and 37 days.

A parallel experiment to determine the formation of aniline and catechol during degradation of DPA by the degrading isolate was set up. The appearance of the two possible metabolites was followed in MSM and MSMN amended with DPA (20 mg/l). Three inoculated and two not-inoculated cultures were prepared and incubated in an orbital shaker at 26°C/180 rpm. Inoculation of the cultures was done as described above. The degradation of DPA and the formation of the two metabolites were followed by HPLC at 0, 4, 8, 12, 16, and 24 hours.

2.9.4. Assessment of the effects of pH and temperature on DPA degradation by the isolated bacterium

The capacity of the DPA-degrading isolate to degrade DPA in a range of pH and temperatures was evaluated in MSM as described in Chapter 2.

2.9.5. The effect of the co-presence of other pesticide-degrading bacteria on the degrading capacity of the DPA-degrading isolate

The degrading ability of the DPA-degrading strain was tested in the presence of *ortho*-phenyl-phenol (OPP) and the corresponding OPP-degrading bacterium (Chapter 5), or thiabendazole (TBZ) and the corresponding TBZ-degrading consortium (Chapter 3). Triple mixtures containing DPA, TBZ, OPP, and their corresponding degrading bacteria were also tested. As described in Chapter 2, all media contained the same molar concentration of the three pesticides (0.17 mM). DPA was added in the medium using a filter sterilized (0.22 μm, PTFE Syringe Filter, sterilized)

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DMSO stock solution of 20000 mg/l. The DMSO amount in the medium did not exceed 0.3% in order not to induce deleterious effects on the degrading bacteria.

2.9.6. Assessment of the bioaugmentation potential of the DPA-degrading isolate

The ability of the isolate to degrade a range of concentrations of DPA in soil was evaluated. A soil with no previous history of pesticide contamination was collected from a field of the National Agricultural Research Foundation of Greece, Larissa. Upon its collection, it was handled as described in Chapter 2. Three 500 g soil-samples were treated with appropriate amounts of filter sterilized water stock solutions of DPA (0.2, 2, and 9 g/l) prepared by the commercial formulation of DPA resulting in concentrations of 20, 200, and 1000 mg/kg of DPA in soil. The soils were kept at 4^oC for 30 days in order to facilitate aging of DPA residues simulating a realistic spillage situation. After this period, each soil sample was split into two portions. The first subsample was inoculated with appropriate amounts of a fresh MSM culture of the DPA-degrading bacterium to reach an inoculum level in soil of $2x10^6$ cells/g of soil. The concentration of the inoculum was verified by spread plating on LB. The second set of subsamples received the same amount of water without inoculum to serve as non-inoculated controls. All soils were subsequently separated into subsamples of 20 g, placed into plastic bags, and incubated at 25^oC. Immediately after inoculation and 2, 5, 10, 20, and 30 days later three inoculated and two non-inoculated subsamples were taken for determination of DPA residues in HPLC. Moisture content in the soil was adjusted to 40% of the soil water holding capacity and maintained at this level by regular addition of water if needed.

2.9.7. Assessment of the degrading ability of the DPA-degrading isolate in BEM and maintenance of its degrading capacity upon exposure to composting-like temperatures

The ability of the DPA-degrading isolate to degrade DPA in BEM, which resembles the composition of a biobed substrate, was tested as described in Chapter 2. Similarly, the degrading capacity and survival of the DPA-degrading isolate when exposed to a temperature profile for a period of 28 days simulating the composting process was also tested as described in Chapter 2.

3. Results and discussion

3.1. Enrichment culture for the isolation of DPA-degrading bacteria

In both enrichment media a rapid degradation of DPA was observed right from the first cycle, with complete disappearance of DPA within 2 days (Figure 6.1).

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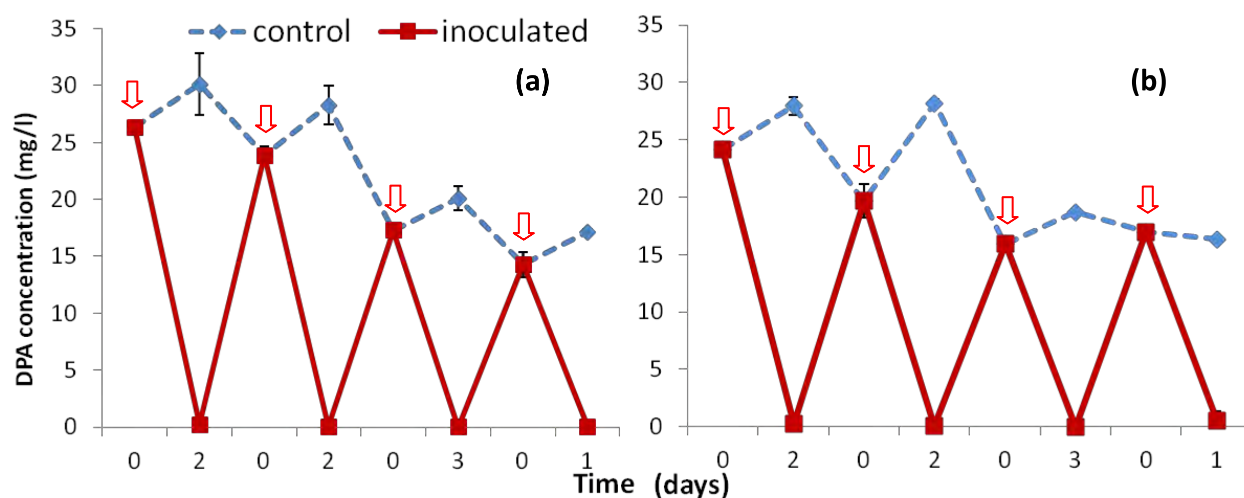

Figure 6.1. The degradation of DPA in the enrichment cultures in MSM (a) and MSMN (b). Degradation in the non inoculated controls is also presented. Each value is the mean of three replicates + standard deviation. The red arrows indicate the beginning of each enrichment cycle.

The final enrichment cultures in both media were spread on the respective agar plates. In total 33 colonies were selected and assayed for DPA degradation in the corresponding liquid media. 14 of them were able to completely degrade DPA within 5 days (Figure 6.2). After streaking on LB agar plates, all isolates appeared to be composed of the same single morphotype. Two cultures from each medium, MSM 1 and 3 (M1/M3) and MSMN 4 and 6 (N4/N6) were selected and were grown on the respective agar medium. The total bacterial growth from the plate was collected and was used to inoculate fresh liquid cultures of the corresponding media in order to check degradation of DPA and the composition of the degrading cultures *via* DGGE analysis. Complete DPA degradation occurred within 2 days. DGGE profiles of the degrading cultures were identical and comprised of four main bands (Figure 6.3). These results suggested that all four DPA-degrading bacteria tested were able to grow on the agar media used. However, the appearance of more than one bands in the DGGE profiles challenged the initial observation about the purity of the isolates obtained.

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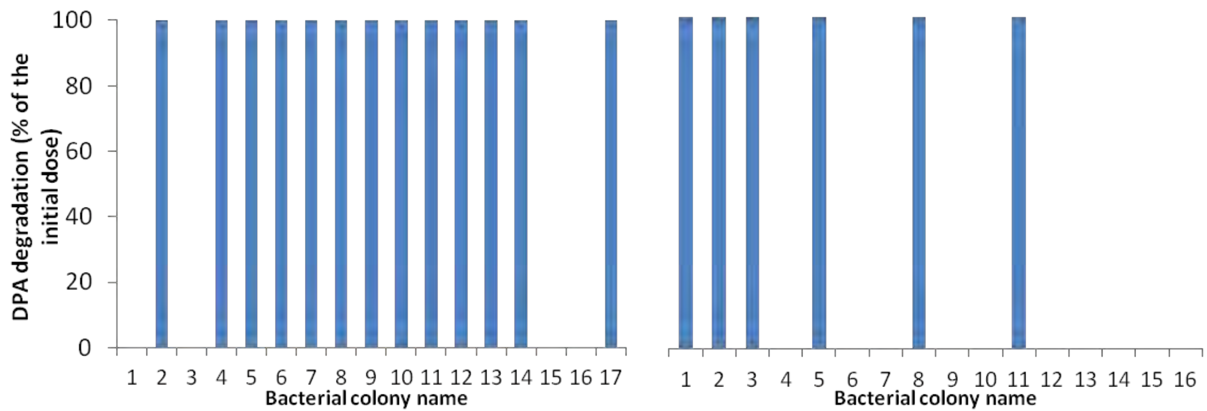

Figure 6.2. DPA degradation in liquid cultures by the 33 colonies selected from MSM (left) and MSMN (right) + DPA plates. DPA degradation was determined 5 days after inoculation.

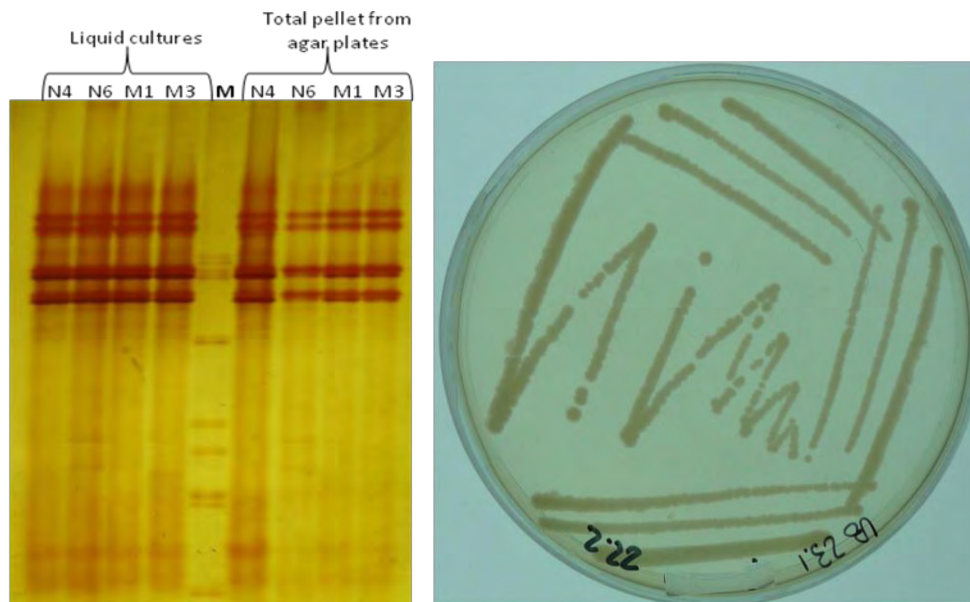

Figure 6.3. Left: DGGE profile of the four selected DPA-degrading cultures N4, N6, M1, M3 obtained after growth in liquid cultures and LB agar plates. **Right:** DPA-degrading cultures growing on LB plates.

In order to check the hypothesis that the different bands belong to morphologically indistinguishable bacteria present in the degrading cultures, 21 colonies from an actively growing DPA-degrading culture were randomly selected from M1 and N4 and were subjected to colony PCR and DGGE analysis. As shown in Figure 6.4, all 21 single colonies selected gave a DGGE profile identical to the initial DPA-degrading cultures composed of the same four dominant bands.

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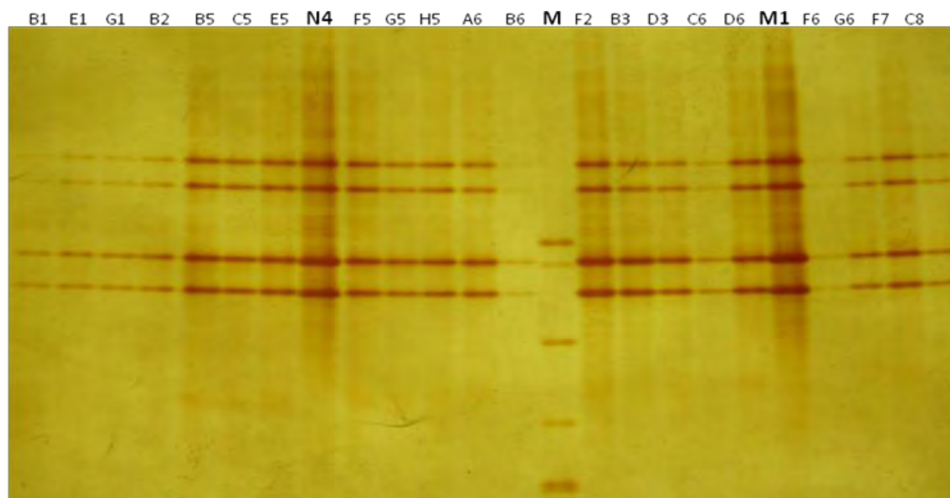

Figure 6.4. Colony PCR - DGGE fingerprints of the single colonies selected from DPA-amended agar plates. The colonies derived from the DPA-degrading culture N4 are to the left of the marker M and the colonies derived from the DPA-degrading culture N1 are to the right.

These results suggested that the isolated DPA-degrading bacterium was in pure state but possessed an inherent polymorphism in the different copies of the 16S rRNA gene. Genes encoding the 5S, 16S, and 23S rRNAs are typically organized into an operon in members of the domain Bacteria and the copy number of rRNA operons per bacterial genome varies from 1 to as many as 15 [Andersson *et al*., 1995; Bercovier *et al*., 1986; Rainey *et al*., 1996]. Due to gene conversion [Liao, 2000], the multiple copies of 16S rRNA genes of a given bacterial strain are generally identical, with the vast majority of 16S rRNA sequences showing less than 1% of different nucleotides [Acinas *et al*., 2004; Pei *et al*., 2010]. On the contrary, for other strains, the 16S rRNA sequences can differ substantially between operons [e.g. Pei *et al*., 2010; Wang *et al*., 1997; Yap *et al*., 1999]. This sequence heterogeneity within 16S rRNA genes has an effect on community analysis techniques based on 16S rRNA genes, such as DGGE, where multiple signals can be generated from a single organism [Crosby and Criddle, 2003; Nubel *et al*., 1996]. Thus, following the formulated hypothesis, the isolated DPA degrader would possess at least four different 16S rRNA gene sequences that give four different DGGE bands. In fact, even if it has been demonstrated that the estimation of genetic diversity can be affected by sequence errors introduced by polymerase and formation of chimeric DNA molecules during the PCR reaction [Speksnijder *et al*., 2001; Wang and Wang, 1997], this is considered a random process, thus it is not expected to result into the same base anomalies and sequence infidelity in independent samples, giving the same DGGE profile, as happening in our case.

In order to identify the sequence polymorphism in the V3 region of the 16S rRNA gene which was used for fingerprinting, the full sequence of N4 was analysed. All the different selected clones showed high homology (>99%) to *Pseudomonas putida* strains. Sequencing

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alignment showed a very high similarity in the entire gene sequence (data not shown). The use of an error-proof polymerase and of a lower number of amplification cycles minimized the possibility of PCR-driven errors in the sequences obtained. Analysis of the sequences of the V3 region of the clones obtained (200 bp) showed high homology with the exception of six bases where polymorphism was noted (Table 6.4) resulting in acquisition of four different sequences that are in agreement with the four DGGE bands observed. Of the four different type-sequences, three differed by one nucleotide and the fourth showed polymorphism in four bases. It appears that in samples of low complexity DGGE is able to distinguish between short sequences which differ by only one base [Fischer and Lerman, 1979; Fodde and Losekoot, 1994]. These results seemed to support the hypothesis of a polymorphism in the 16S rRNA gene of the isolated DPA-degrading strain. The four complete 16S rRNA genes sequences are reported in Annex 1, Section 3.3. The sequences were registered in the GenBank database and are available at http://www.ncbi.nlm.nih.gov/ providing the accession numbers reported in Annex I.

Table 6.4. Analysis of the 200 bp fragments of the V3 region of the 16S rRNA gene of DPA-degrading culture N4. The different bases are highlighted in red. The position of the forward (Muyzer F) and reverse (Muyzer R) primers in each clone sequence is also indicated. The different colors (violet, yellow, green, and blue) indicate the four fragment-combinations.

Clones	Position (bp) in the sequence						
N4	Primer Muyzer F	383	431	481	482	484	Primer Muyzer R
A	309-325	A	T	C	T	T	486-502
B	309-325	G	T	C	T	T	486-502
C	309-325	A	C	A	A	C	486-502
D	309-325	A	T	C	T	T	486-502
E	309-325	A	C	C	T	T	486-502

3.2. Analysis of the *rpoD* and *gyrB* genes

Despite of the general use of 16S rRNA gene for the phylogenetic characterization of bacteria strains to the genus level, its resolution at the species level is low for certain bacterial genera like *Pseudomonas* that is one of the more diverse bacterial genera [Moore *et al.*, 1996; Anzai *et al.*, 2000; Yamamoto *et al.*, 2000]. Thus, the sequences of other housekeeping genes provide better resolution and some of those have been studied for *Pseudomonas* [Mulet *et al.*, 2010; Yamamoto *et al.*, 2000]. Such genes have been proposed including *gyrB* encoding the beta-subunit of gyrase, which is responsible for negative supercoiling of DNA during replication, and *rpoD* that

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encodes the sigma 70 subunit of RNA polymerase. These genes evolve more slowly than typical protein-coding genes but more rapidly than rRNA genes [Tayeb *et al*., 2005].

It has been demonstrated that the co-analysis of three concatenated genes (16S rRNA, *gyrB,* and *rpoD*) is sufficient for a reliable phylogenetic analysis of bacteria belonging to the genus *Pseudomonas* [Mulet *et al*., 2010]. Thus, to verify the initial hypothesis that the DPA-degrading bacterium isolated is in pure state and carries sequence polymorphisms in the different copies of its 16S rRNA gene, the sequences of *gyrB* and *rpoD* were also obtained *via* PCR and analyzed. Clones containing PCR fragments from each of the two genes were sequenced and in both cases all clones showed high homology (>99%) to *Pseudomonas putida* strains (Table 6.5) in agreement with the identification provided when the sequence of the 16S rRNA gene was utilized. Alignment of the sequences obtained from all the clones carrying the same gene showed no nucleotide differences verifying further our hypothesis that the DPA-degrading culture is composed of a single bacterium showing polymorphism in the different copies of the 16S rRNA gene. The *gyrB* and *rpoD* sequences obtained were submitted in the GenBank database and are available at http://www.ncbi.nlm.nih.gov/ providing the accession numbers reported in Annex I, Section 3.3. From the concatenated alignment of the three genes 16S rRNA, *gyrB,* and *rpoD* (3096 bp) a phylogenetic tree was constructed (Figure 6.5). The concatenation resulted in the assignment of the DPA-degrading bacterium within the *P. putida* group as defined by Mulet *et al.* [2010] and it was most closely related to a *Pseudomonas monteilii* strain.

Table 6.5. Highest homologies of the sequences of *gyrB* and *rpoD* obtained by the DPA-degrading isolates M1 and N4 *via* BLAST. The percentage of sequences homology and the NCBI accession number of the best match are shown.

Isolates	**Highest match**	**Homology (bp)**	**Homology (%)**	**NCBI accession number**
		gyrB		
M1	*Pseudomonas putida* KT2440	904/913	99	AE015451
N4	*Pseudomonas putida* KT2440	904/913	99	AE015451
		rpoD		
M1	*Pseudomonas putida* F1	708/710	99	CP000712
N4	*Pseudomonas putida* F1	708/709	99	CP000712

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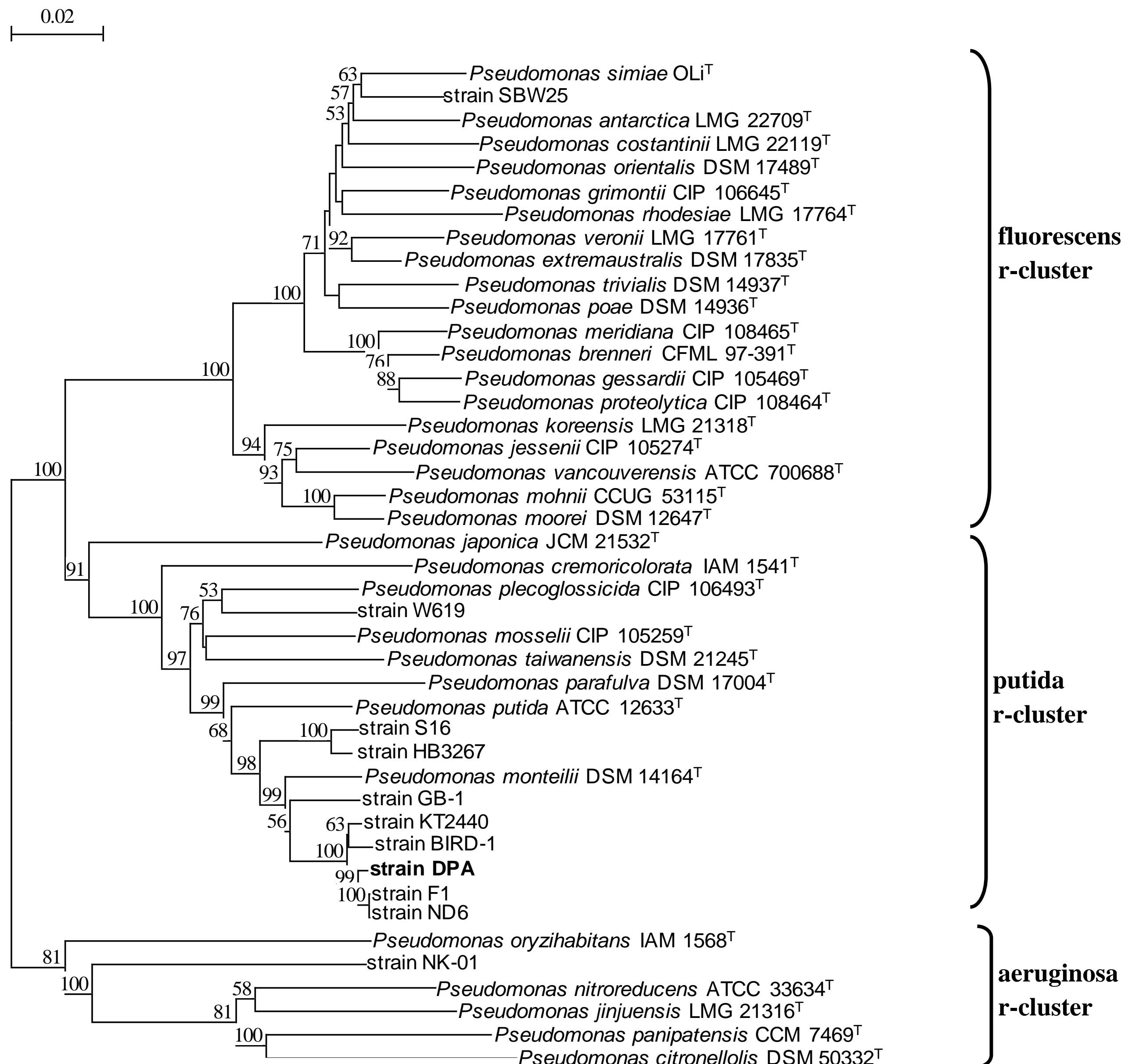

Figure 6.5. Phylogenetic analysis of the DPA-degrading bacterium based on concatenated alignment of the three genes 16S rRNA, *gyrB*, and *rpoD* (total 3096 bp). 1000 bootstrap replicates were run with PhyML (maximum likelyhood method). The bootstrap support is expressed in scale from 0 to 100. The three well-supported clusters in the 16S rRNA phylogeny (fluorescens, putida, and aeruginosa) as described by Bodilis *et al*. [2012] are indicated.

Overall the phylogenetic analysis confirmed the isolation of a pure DPA-degrading strain which belongs to the *P. putida* subgroup and whose closest relative is *P. monteilii*. Therefore, the isolate will be called *P. monteilii* strain DPA. So far there is only one other report regarding the involvement of a *P. monteilii* strain N-502 in the rapid degradation of organic pollutants such as bisphenol A, a molecule with a biphenyl structure that resembles DPA [Masuda *et al*., 2007]. However, total genome sequencing of a *P. monteilii* strain QM showed the presence of gene

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clusters involved in the transformation of benzoate (*benABCD*), catechol (*catABC*), phenol (*dmpK, dmpLMNOP*), salicylate (*nahG*), and *p*-hydroxybenzoate (*pobA*), indicating its verstatility in degradation of aromatic compounds, similar to other *Pseudomonas* strains [Ma *et al*., 2012]. In addition, the DPA-degrading isolate clustered together with *Pseudomonas* strains which are known to be involved in the degradation of organic pollutants. Thus the *Pseudomonas* ND6 strain was isolated from industrial wastewater in Tianjin, China by selection for its ability to degrade naphthalene [Zhang *et al*., 2000], while strain S16 was identified as nicotine degrader [Wang *et al*., 2007]. Similarly *Pseudomonas* strain F1 is one of the most well studied toluene-degrading bacterium [Zylstra and Gibson, 1989] which was also able to degrade other substituted phenols and trichloroethylene [Wackett and Gibson, 1988]. Moreover, *Pseudomonas putida* KT2440 is characterized from a wide metabolic versatility, which enables the strain to degrade a large variety of natural and man-made aromatic compounds through different characterized catabolic pathways [Nelson *et al*., 2002; Jimenez *et al*., 2002].

Regarding the polymorphism of the 16S rRNA gene, effectively it has been demonstrated that this is an intrinsic characteristic of the genus *Pseudomonas*, where copy numbers of the rRNA operons per bacterial genome varies from 4 (*P. aeruginosa*, PAO 1 strain [Stover *et al.*, 2000]) to 7 (*P. putida*, KT2440 strain [Nelson *et al*., 2002]). In particular, all strains belonging to the *P. putida* cluster, where our isolate was assigned, showed to possess 6 or 7 copies of the 16S rRNA gene [Bodilis *et al*., 2012]. Moreover, most of the *Pseudomonas* have at least two different 16S rRNA alleles. For example, genome analysis of the *P. putida* KT2440 strain (closely related to the DPA degrader, Figure 6.5) showed the presence of two alleles differing by only one nucleotide in the V3 hypervariable region and two heteroduplexes, resulting in a DGGE profile composed of two dominant bands (the two alleles) and two extra faint bands (the two heteroduplexes) [Bodilis *et al*., 2012]. These data further support the hypothesis of the polymorphisms in the 16S rRNA gene of our isolate. The fact that all four DGGE bands produced by our bacterium appear as dominant and that sequencing revealed 4 different sequences, the presence of four different alleles in the genome of our isolate is possible. Bodilis *et al.* [2012] showed that there is a correspondence between DGGE band intensity and the number of allele copies in a bacterial genome. Thus, the equal intensity of the DGGE bands in the profile of our DPA-degrading *Pseudomonas* strain could be a first indication for the presence of four alleles in the genome of the DPA-degrading isolate. Multiple rRNA operons may provide a growth advantage. Indeed, multiple rRNA operons allow transcriptional initiation from multiple loci, permitting a rapid increase in the intracellular concentration of rRNA and a rapid response to favorable changes in growth conditions [Klappenbach *et al*., 2000; Stevenson and

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Schmidt, 1997; Condon *et al*., 1995]. Therefore, the very rapid and efficient degradation of DPA in the enrichment cycles but also by our isolate in pure cultures could be attributed to the polymorphism in the rRNA operons of the isolated DPA degrader.

This potential adaptive significance of rRNA operon multiplicity was demonstrated in soil microcosms by the reproductive success of diverse 2,4-dichlorophenoxy-acetic acid-(2,4-D)-degrading bacteria containing a significantly greater number of rRNA operons per genome during competition for a pulse of the molecule [Klappenbach *et al*., 2000]. In fact, the majority of 2,4-D-degrading bacteria possessed between one and four rRNA operons in unamended microcosms, while, among the species detected in microcosms amended with 10 or 100 ppm of 2,4-D, 7 of 13 and 11 of 14 species, respectively, possessed between five and seven rRNA operon copies. Thus, the effect of selection for 2,4-D-degrading species with higher rRNA operon copy number in amended microcosms was significant. A possible distinct pattern of use of 16S rRNA operon sequences for the assembly of ribosomes and their specific regulation has been suggested [Nubel *et al*., 1996].

3.3. Characterization of the bacterial degrading ability

3.3.1. Assessment of the growth of the bacterium during degradation of DPA

The growth kinetics of *P. monteilli* during degradation of DPA were determined to verify the capacity of the isolated bacterium to actively grow on DPA and use it as a C or as a C and N source. As shown in Figure 6.6, DPA was completely degraded within 24 hours in both media. The two media were inoculated with a similar starting inoculum of *ca.* $3x10^6$ cells/ml. Degradation of DPA proceeded with stoichiometric growth of the DPA-degrading bacterium in both media which peaked (5.5 to $6.5x10^7$ cells/ml) at 24 hours when DPA degradation was completed. Overall the results obtained suggest that the isolated strain was able grow on DPA and use it as both C and N source.

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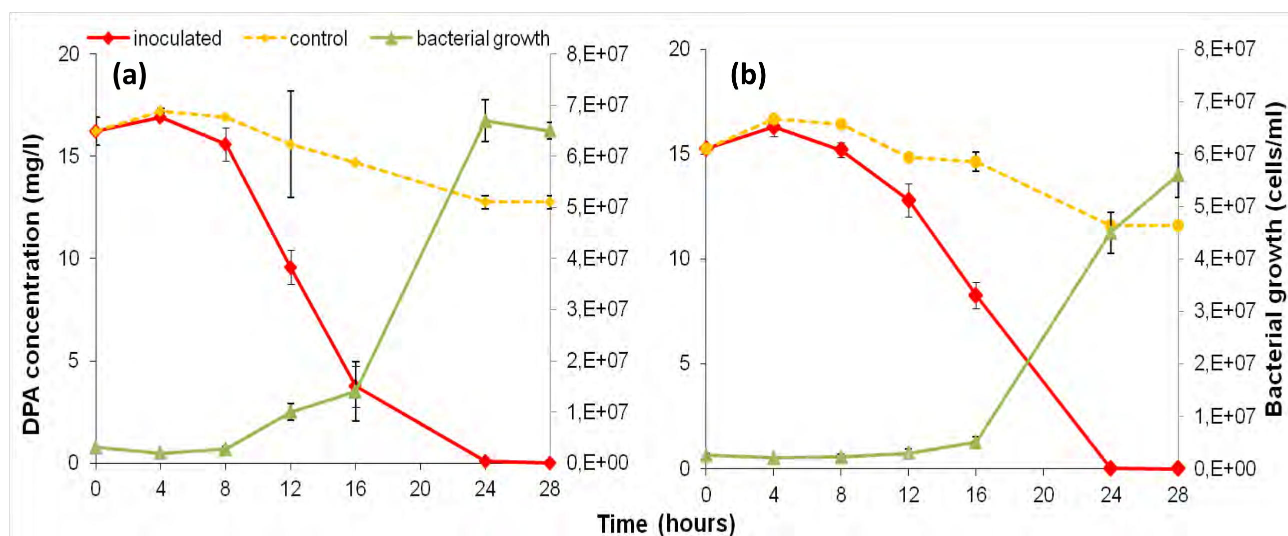

Figure 6.6. The growth kinetics of the isolated bacterium during DPA degradation in MSMN (a) and MSM (b). The degradation of DPA in non-inoculated controls is also shown. Error bars represent the standard deviation of the mean.

3.3.2. Assessment of the degradation capacity of the isolate at high DPA concentration levels

The isolate *P. monteilii* (starting inoculum: $2.5x10^6$ cell/ml) was able to rapidly degrade particularly high concentrations of DPA in MSM. As shown in Figure 6.7, the DPA-degrading isolate was able to degrade 25, 120, and 600 mg/l of DPA within 2 days and achieved 85% degradation of the 1000 mg/l concentration in the same time. In addition, the DPA-degrading isolate was able to degrade the highest DPA concentration tested, 2000 mg/l within 4 days. The degradation of DPA in the non-inoculated controls was negligible verifying the high degrading capacity of our isolate against particularly high DPA concentrations (up to 2000 mg/l) which might encounters if applied for biodepuration of DPA-containing wastewaters. This is the first report of a bacterium able to metabolize rapidly such high levels of DPA. Previously reported DPA-degrading bacteria were able to degrade up to 60 mg/l of DPA [Christodoulatos *et al.*, 1997; Shin and Spain, 2009].

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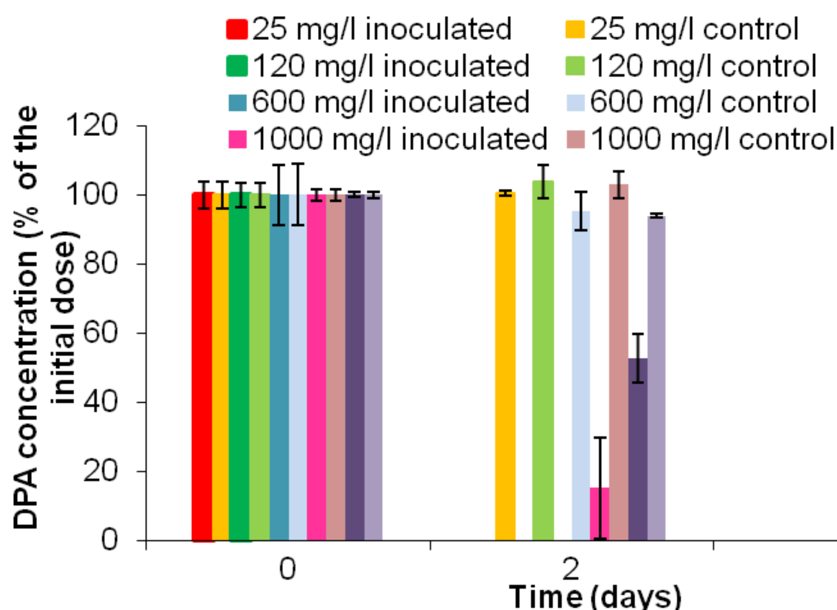

Figure 6.7. Degradation of a range of concentrations of DPA by the isolate *P. monteilii*. The degradation in non inoculated controls is also shown. Error bars represent the standard deviation of the mean.

3.3.3. Assessment of the degradation capacity of the isolate against other molecules

The ability of *P. monteilii* to degrade other molecules including possible metabolites (aniline and catechol) and pesticides with similar chemical structure and having practical relevance like *ortho*-phenyl-phenol (OPP) was evaluated. The chemical structure of the three molecules tested is shown in Figure 6.8.

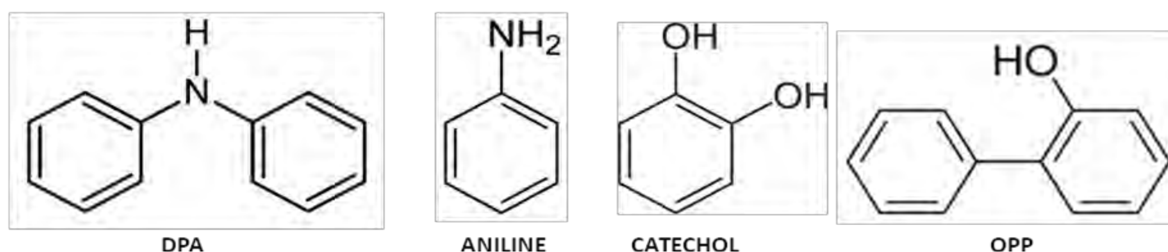

Figure 6.8. The chemical structure of the molecules tested for degradation by the isolated *P. monteilii*.

P. monteilli was able to slowly degrade OPP with 80% degradation observed in 37 days compared to negligible degradation in the non-inoculated control (Figure 6.9). The low degradation capacity of the DPA-degrading bacterium against OPP could be of cometabolic nature and could be linked with the activity of monooxygenases or dioxygenases with relaxed substrate-specificity that could attack one of the two aromatic rings adding one or two atoms of oxygen then converted by dehydrogenases in hydroxylated groups [Suenaga *et al.*, 2009]. A

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subsequent ring cleavage following mechanisms described for OPP [Kohler *et al*., 1988] or biphenyl [e.g. Kikuchi *et al*., 1994; Kim and Zylstra, 1999; Denef *et al*., 2005] degradation, allowing feeding of the isolated *P. monteilii* on derived molecules, cannot be excluded.

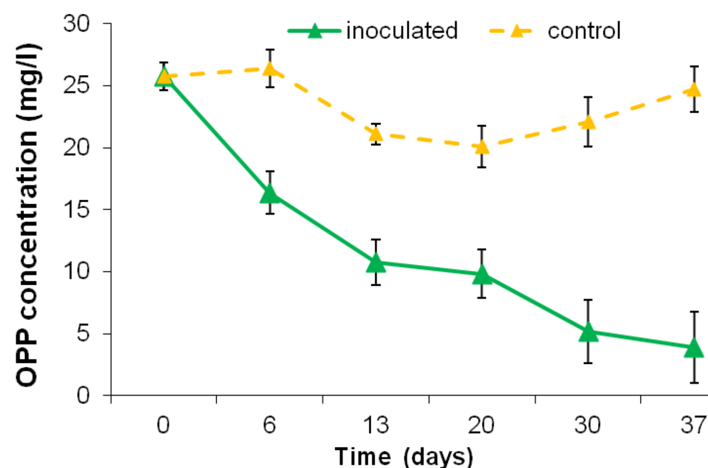

Figure 6.9. The degradation of OPP by the DPA-degrading strain *P. monteilii*. Degradation in non-inoculated cultures (control) is also shown.

Regarding degradation of aniline and catechol, they were both completely degraded within 24 hours showing a similar degradation pattern as DPA (Figure 6.10). The starting bacterial inoculum was 2.8×10^6 cells/ml. Aniline and DPA showed limited degradation in the non-inoculated samples. In contrast considerable abiotic degradation was observed in the case of catechol, although microbial degradation was still faster. Previous studies have demonstrated the instability of catechol which reacts with light and O_2 resulting in a rapid degradation [Thomson, 1964].

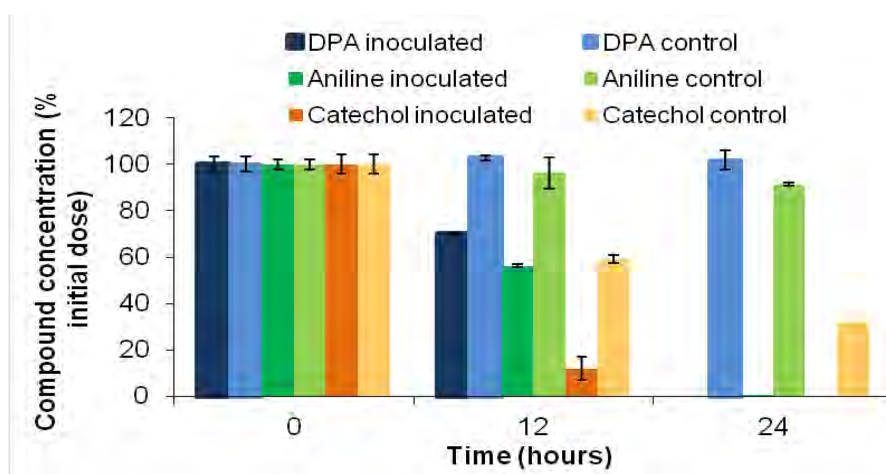

Figure 6.10. Degradation of DPA, aniline, and catechol by *P. monteilii.* Degradation in non-inoculated controls is also presented. Error bars represent the standard deviation.

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Aniline and catechol have been identified as the main intermediates during degradation of DPA [Shin and Spain, 2009]. Previous studies have shown that aniline constitutes the central metabolic product during degradation of DPA under both aerobic and anaerobic conditions [Shin and Spain, 2009; Gardner *et al.*, 1982; Drzyzga and Blotevogel, 1997]. The aniline produced is then transformed to catechol by aniline dioxygenase (Figure 6.11) [Zissi and Lyberatos, 1999; Kahng *et al.*, 2000; Liu *et al.*, 2002; Liang *et al.*, 2005]. Catechol, in turn, is subject to *ortho* or *meta* cleavage, depending on the microbial strain and/or the nature of the growth substrate. However, both pathways bring to the production of final metabolites which are readily usable by the cell (Figure 6.11) [Shingler *et al.*, 1992; Denef *et al.*, 2005]. These two metabolic pathways of catechol are ubiquitous among environmental bacteria and are known as the lower metabolic pathway of different aromatic compounds such as biphenyl, substituted biphenyls, naphthalene [e.g. Shingler *et al.*, 1992; Denef *et al.*, 2005; Kohler *et al.*, 1988; Kikuchi *et al.*, 1994; Goyal and Zylstra, 1997].

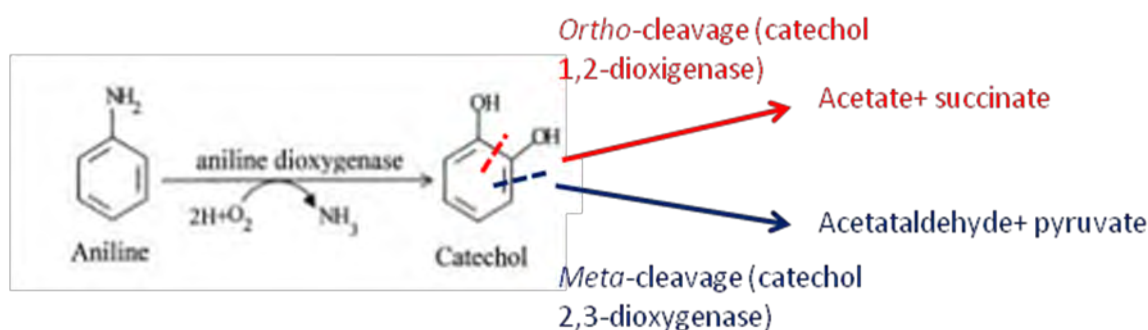

Figure 6.11. The *ortho* and *meta* cleavage pathways of catechol by microorganisms (from aniline to catechol: source Liu *et al.* [2002]).

The *P. monteilii* strain DPA was able to rapidly transform aniline and catechol which could be a first indication for the complete mineralization of DPA. In a recent study by Shin and Spain [2009] the full metabolic pathway of DPA to aniline was elucidated. The DPA-degrading strain *Burkholderia* sp. JS667 was shown to convert DPA to aniline and catechol *via* attack of the 1,2 position of the benzene ring by a dioxygenase (Figure 6.12a). This intermediate converts spontaneously to aniline and catechol. The aniline that is formed is converted to catechol which is then transformed to Krebs cycle intermediates by the *meta*-cleavage pathway (Figure 6.11). The genes that encode the DPA degradation to aniline and catechol (*dpaAaAdAc*) and the aniline degradation to acetaldehyde and pyruvate were identified [Shin and Spain, 2009]. Genomic analysis of the degrading operons suggested a recent recruitment of the genes encoding the DPA dioxygenase by an original most common aniline degrading bacterium. Therefore, the key

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enzyme for DPA degradation is a DPA dioxygenase that catalyzes the attack of the benzene in the first step of the pathway. Such a dioxygenase able to attack the benzene ring and initiate DPA degradation is expected to be also present in the isolated *P. monteilii* strain DPA.

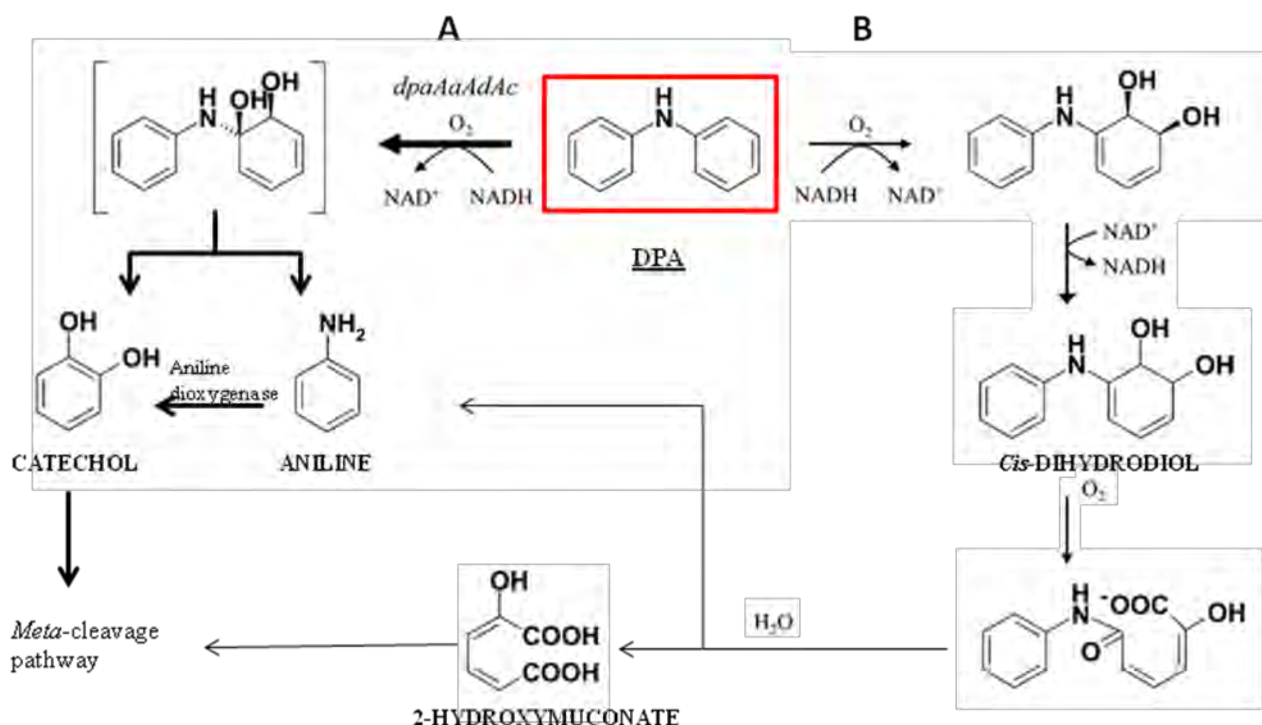

Figure 6.12. Possible DPA degradation pathways. (A) Pathway proposed for DPA metabolism by *Burkholderia* sp. JS667 (B) Alternative route proposed (source: Shin and Spain [2009]). Both pathways converge in the degradation route of aniline *via meta*-cleavage.

Based on our previous results and the results of Shin and Spain [2009], the formation of aniline and catechol during degradation of DPA by our isolate was investigated. None of the two molecules was detected during the degradation of DPA which was completed in 24 hours. A very fast degradation of the intermediates could be an explanation for the absence of accumulation in the medium. Indeed our data are in agreement with previous observations by Shin and Spain [2009] who found no formation of DPA metabolites in actively growing cultures of *Burkholderia* sp. JS667. Substantial amounts of aniline were detected only when succinate-grown cells of JS667 were transferred to medium containing DPA. Moreover, the lack of an induction period before DPA disappearance and aniline accumulation in the first phase indicated that the initial enzymes of the pathway are at least partially constitutive, whereas enzymes that catalyze aniline degradation are inducible causing the transient accumulation of aniline observed.

It was not possible to establish the DPA degradation pathway followed by the *P. monteilii* strain DPA. However, based on previous studies [Shin and Spain, 2009] and on the ability of the

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DPA-degrading bacterium to rapidly degrade aniline and catechol, it is hypothesized that DPA degradation proceeds *via* the intermediate formation of aniline and catechol which are further transformed. Genomic analysis of the DPA-degrading bacterium is on-going and is expected to shed light in the metabolic pathway of DPA by the isolated bacterium.

3.3.4. The effect of pH and temperature on the degrading ability of the DPA-degrading isolate

P. monteilii (starting *inoculum* $1.2x10^6$ cells/ml) was able to rapidly degrade DPA independently of the pH of the medium with almost complete degradation of the molecule occurring within 1 day in all pH tested (4.5 to 9) (Figure 6.13). No DPA degradation was observed in the non-inoculated samples at all pH levels which is in agreement with the generally high hydrolytic stability of DPA at pH 5, 7, and 9 [US EPA, 1998].

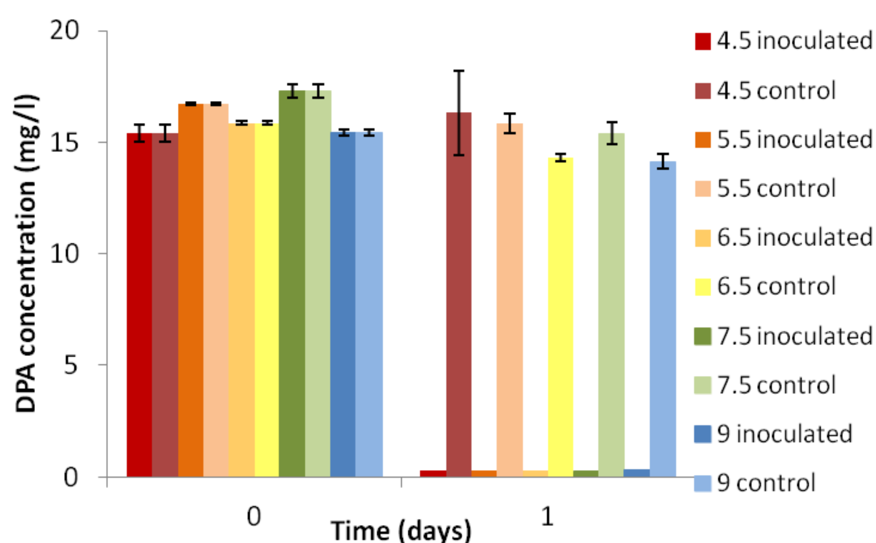

Figure 6.13. DPA degradation by *P. monteilii* at different pH. The degradation in non-inoculated controls is also presented.

When the degrading ability of the bacterium was tested at different temperatures (pH of the medium adjusted to 6.5) differences in the degradation rates were observed (Figure 6.14). The starting bacterial inoculum was $3x10^6$ cells/ml in all treatments. The maximum degradation rates were observed at 26ºC where complete degradation of DPA was evident in 1 day. In contrast relatively slower degradation rates were observed at 15 and 37ºC where complete or nearly complete degradation of DPA was measured at 3 days. At 4°C the same degradation rates as in the non-inoculated controls were observed suggesting ceasation of the biotic degradation of DPA at such temperature levels.

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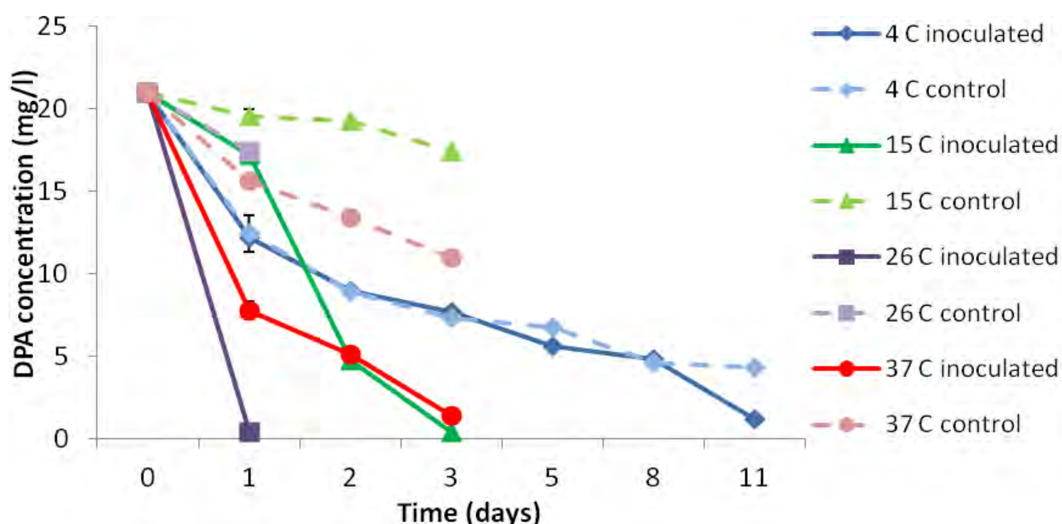

Figure 6.14. The degradation of DPA by *P. monteilii* at different temperatures. Degradation in corresponding non-inoculated control cultures is also presented.

The isolated strain *P. monteilii* was able to rapidly degrade DPA in a wide range of pH (4 to 9) and at different temperatures, making it suitable for bioremediation applications. Previous studies have demonstrated that members of the *Pseudomonas* genus are high tolerant to wide ranges of pH (4-8) and temperature (4–37°C) [Moore *et al*., 2006].

3.3.5. The effect of the co-presence of other pesticide-degrading bacteria on the degrading capacity of the DPA-degrading isolate

The ability of the DPA-degrading isolate to degrade DPA in the co-presence of TBZ and / or OPP and their respective degrading bacteria was assessed. The starting bacterial inoculum was of 10^6 cells/ml. The results showed that the degradation capacity of *P. monteilii* strain DPA was not affected by the co-presence of one or of both TBZ and OPP and their respective degrading bacteria. Indeed in all cases complete degradation of DPA was registered in a day (Figure 6.15).

The lack of any antagonistic or inhibitory effect on the degrading capacity of the DPA-degrading isolate is considered a desirable characteristic regarding the biotechnological potential of this strain if we consider that in many fruit packaging industries DPA is often used together with fungicides such as TBZ or OPP, thus producing wastewaters containing a mixture of substances. These results indicate that *P. monteilii* degrading activity will be not influenced in a biopurification system by the co-presence of other pesticides in the treated wastewaters.

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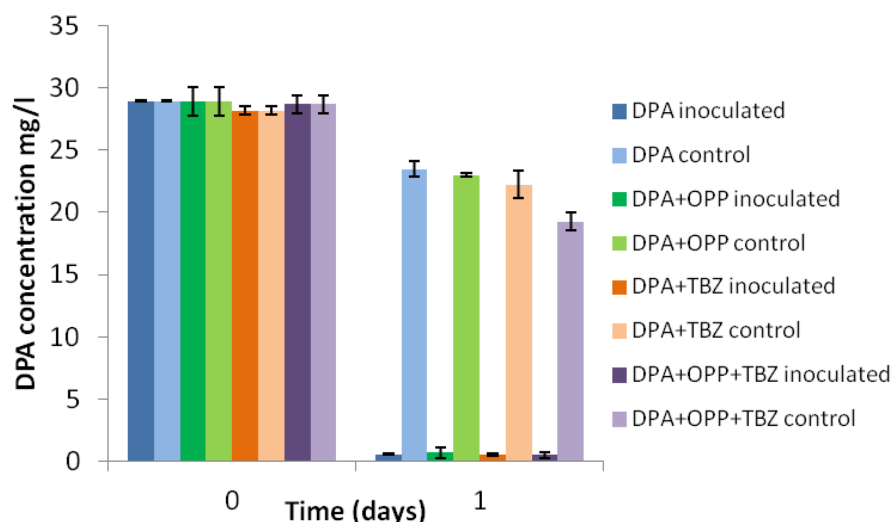

Figure 6.15. DPA degradation by *P. monteilii* in the co-presence of thiabendazole (TBZ), *ortho*-phenyl-phenol (OPP), TBZ and OPP (DPA+TBZ+OPP), and of their respective degrading bacteria. Degradation of DPA in the non-inoculated controls is also presented.

3.3.6. Assessment of the bioaugmentation potential of the DPA-degrading isolate

The bioaugmentation capacity of *P. monteilii* strain DPA was assessed in a soil amended with a range of DPA concentrations (25, 150, and 1000 mg/kg). The artificially contaminated soils were inoculated with $2.5x10^6$ cells/gr of bacterium. In the soils inoculated with the *P. monteilii* strain a substantial acceleration in the dissipation of DPA relatively to the corresponding non inoculated samples was observed (Figure 6.16). In particular, for all concentration levels tested, DPA was almost completely dissipated within 5 days after inoculation compared to the corresponding controls where only 9, 41, and 43% dissipation was measured in the samples amended with 25, 150, and 1000 mg/kg level respectively. After this rapid phase, dissipation rates declined, probably due to the limited availability of the pesticide adsorbed onto the soil particles. A similar dissipation pattern for DPA in soil with a rapid dissipation phase during the first 7 days and a slower phase thereafter was obsereved by Liu [1993]. This dissipation trend was observed also in other soil dissipation studies, where a similar reduction of the dissipation rates by inoculated bacteria after an initial rapid dissipation phases was observed [Karpouzas and Walker, 2000; Cullington and Walker, 1999]. A gradual dissipation of DPA from the non-inoculated samples was evident with *ca*. 2.4, 16, and 12% of DPA remaining in the soils amended with 25, 150, and 1000 mg/kg after 30 days. These dissipation rates are in agreement with previous studies which have reported $t_{1/2}$ values of DPA in aerobic soils between 7 to 28 days [Howard, 1991].

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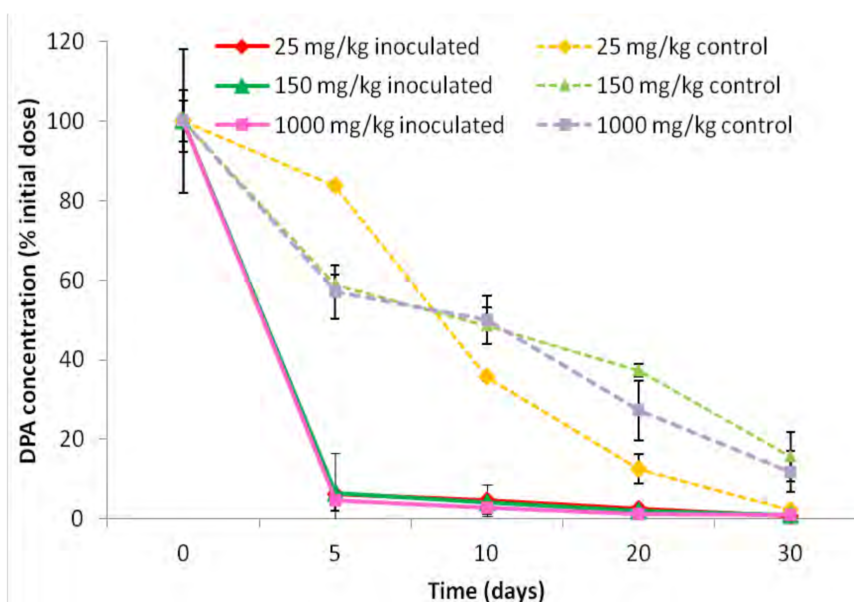

Figure 6.16. The dissipation of 25, 150, and 1000 mg/kg of DPA in soil inoculated or non-inoculated (control) with the DPA-degrading isolate *P. monteilii.*

The bioaugmentation potential of previously isolated pesticide degrading *Pseudomonas* strains was demonstrated also in other studies. Karpouzas and Walker [2000] confirmed that the *P. putida* epI isolated from soil *via* liquid enrichment culture was capable of successfully removing ethoprophos residues when reintroduced into the soil. Similarly, a diazinon-degrading *Pseudomonas* sp. strain was able to rapidly degrade 100 mg/kg in soil [Cycon *et al.*, 2009]. However, soil bioaugmentation with pesticide-degrading bacteria is not always successful. The failure of inoculants to enhance the dissipation of pesticide in natural soil environments have been reported [Goldstein *et al*., 1985; Block *et al*., 1993; Morra, 1996] and was attributed to a combination of factors like non-optimal temperature, pH, moisture and organic matter content, pollutant bioavailability, insufficient inoculum density, or competition with the indigenous microflora for substrates, antagonism, and predation [Karpouzas and Walker, 2000; Awashti *et al*., 2000].

Overall our data suggest that the DPA-degrading *P. monteilii* strain has high bioaugmentation potential and it is able to dissipate particularly high concentration of DPA in soil (1000 mg/kg). Such high concentration levels of DPA are expected to accumulate after long-term disposal of DPA-contaminated wastewaters in soil. Thus the application of this isolate for the decontamination of DPA-contaminated disposal sites or as bioaugmentation agent for enhancement of the degradation capacity of biobed systems should be explored.

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3.3.7. Assessment of the degrading ability of the DPA-degrading isolate in BEM and maintenance of its degrading capacity upon exposure to composting-like temperatures

The degradation capacity of *P. monteilii* against DPA was assessed in BEM, a liquid growth medium that resembled the nutritional composition of a biobed substrate. BEM and MSM were inoculated with 2.5×10^6 cells/ml of the DPA-degrading isolate. Degradation of DPA in both media (BEM and MSM) proceeded equally rapidly with complete degradation of the antioxidant observed within 2 days (Figure 6.17). This result offers a first indication for the adaptability of *P. monteilii* strain DPA to biobed conditions and its potential for future implementation in the bioaugmentation of biobeds receiving DPA-containing wastewaters.

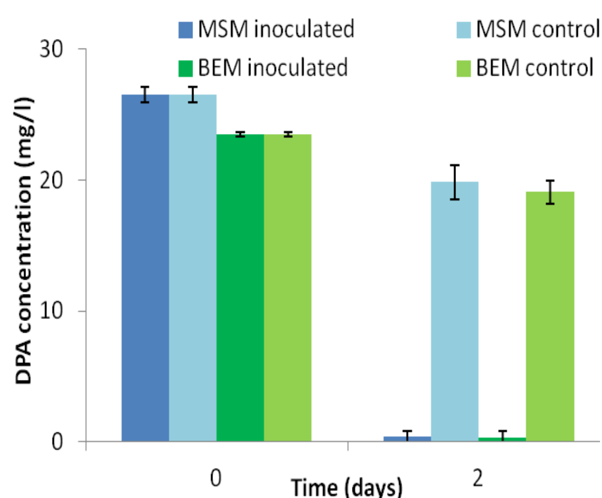

Figure 6.17. The degradation of DPA in MSM and BEM inoculated or not inoculated (control) with *P. monteilii*.

Considering that spent biobed substrates could accumulate and retain considerable amounts of DPA during their use, their decontamination before environmental release is needed. Composting and bioaugmentation with specific pesticide-degraders, or a combination of both, have been proposed as potential methods to decontaminate those substrates [Karanasios *et al*., 2012]. The effect of composting on the activity and mostly on the survival of the pesticide-degrading microorganisms added to the substrate should be known. Thus an experiment was undertaken in order to assess the degrading activity and survival of the DPA-degrading isolate when incubated for a 28-day following a temperature profile simulating composting. The temperature profile used was obtained by a composting study on spent mushroom substrate undertaken by P. Karas (PhD student, Group of Plant and Environmental Biotechnology, University of Thessaly, Greece). As shown in Figure 6.18, during the thermophilic phase the temperature reached levels of 50^oC which were maintained for a short period of 4 days. In both media assessed (BEM and MSM) the degradation of DPA by the degrading isolate was

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completed within 7 days, thus before the thermophilic phase was reached. When refreshed in new medium at 26°C, none of the cultures revived their degradation capacity (data not shown). The loss of DPA degrading capacity could be attributed to cell death after long incubation at temperatures far higher than mesophilic range.

Overall these results demonstrate that the DPA-degrading *P. monteilii* strain could be used as a self-sustained method for the decontamination of DPA-contaminated biobed substrates. Alternatively, if bioaugmentation with *P. monteilii* is used in combination with composting, bacterial application should precede the thermophilic composting phase which will probably result in its elimination.

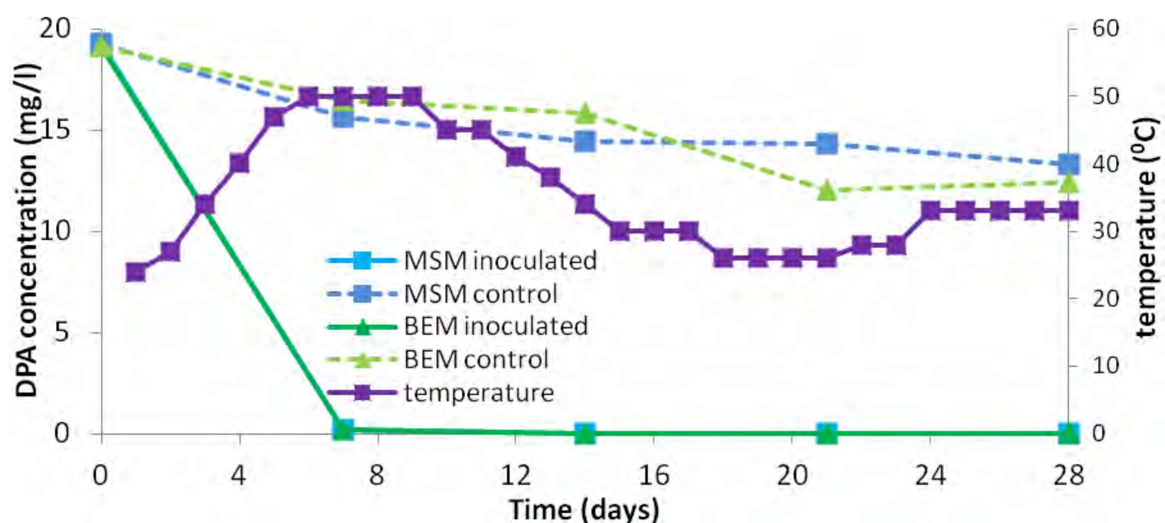

Figure 6.18. DPA degradation by *P. monteilii* in MSM and BEM when incubating according to a composting temperature scheme. Temperature profile is shown in z axis. The degradation of DPA in non-inoculated cultures (control) of the same media is also shown.

4. Conclusions and Future work

DPA is an antioxidant used in fruit packaging industry to protect apples and pears from the development of the physiological disorder called scald. DPA is not currently authorized for use at EU level [EC, 2012]. However several member states have provided exceptional authorization for 120 days to products containing DPA considering that there is no other pesticide in the market that could effectively replace this pesticide. One of the problems identified by its use is the production of large wastewater volumes containing high levels of DPA. Considering the high toxicity of DPA to aquatic organisms the direct environmental disposal of those wastewaters without prior treatment and detoxification is a serious environmental threat. In the absence of effective, sustainable, and economic methods to treat these wastewaters, the development and

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implementation of biofiltration systems based on tailored-made pesticide-degrading bacterial inocula could be the way forward.

Towards this aim, this study isolated a new DPA-degrading bacterium which was particularly effective in the degradation of DPA. The isolated bacterium, identified *via* molecular-based taxonomic analysis as *P. monteilii*, was able to rapidly degrade up to 2000 mg/l of DPA in liquid medium and 1000 mg/kg of DPA when inoculated in soil and to use the pesticide as the sole C and N source. The isolate was able to degrade DPA under a range of pH and temperature conditions (always mesophilic) and in the presence of different pesticides and competing pesticide-degrading microorganisms. Overall the DPA-degrading isolate showed high potential for future biotechnological applications including the *in situ* decontamination of DPA-polluted wastewater disposal sites, bioaugmentation of biobed systems receiving DPA-contaminated wastewaters, and also as tailored made inocula in biofiltration systems devoted to the treatment of wastewaters from the fruit packaging industry.

The metabolic pathway of DPA by the isolated bacterium was not elucidated although our data indicate that catechol and aniline, which have been proposed as intermediates in the degradation of DPA, could be rapidly decomposed by the *P. monteilii* strain DPA. Based on this, future studies will focus on the elucidation of the metabolic pathway of DPA using a combination of high resolution analytical methods (LC-MS/MS or GC/MS depending on the nature of metabolites produced) and genomic and proteomic analysis. The latter will also allow us to identify the genetic background of the isolated DPA-degrading strain and provide information on the evolutionary mechanism behind the degradation capacity of this bacterium.

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Chapter 7
Final Conclusions

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The wastewaters produced by the fruit packaging-industry constitute a serious environmental concern, considering the high load of persistent and toxic pesticides contained. In fact, their direct disposal into the environment without prior treatment is prohibited because of the high risk for deleterious effects on the functioning of aquatic and terrestrial ecosystems. Even so, nowadays no cheap and effective methods to treat such wastewaters are available. Biological decontamination techniques, based on the degradative ability of microorganisms, could offer a possible solution. However little is available in the literature regarding the biodegradation of pesticides used in the fruit-packaging industry. The acquisition of microorganisms able to actively degrade those pesticides is a prerequisite for the future development and implementation of biological treatment facilities for the decontamination of those agro-industrial effluents. **Within this wider goal, the main objectives of this thesis were a) to isolate bacteria able to rapidly degrade the most common pesticides used in the fruit packaging industry, thiabendazole (TBZ), imazalil (IMZ), *ortho*-phenyl-phenol (OPP), and diphenylamine (DPA), and b) to characterize different crucial aspects of their degradative ability.**

Enrichment cultures from soils used as wastewater disposal sites in Greece and Cyprus resulted in the isolation of a *Sphingomonas haloaromaticamans* and a *Pseudomonas monteilii* which were able to actively degrade the pesticides OPP and DPA respectively. In addition, three TBZ-degrading consortia composed of two to more than five bacterial members were isolated from the same wastewater disposal site in Greece. A combination of molecular, microbiological, and biochemical methods resulted in the identification of a *Sphingomonas* strain which was the common member of all three TBZ-degrading consortia responsible for the degradation of TBZ. However it was not possible to obtain the *Sphingomonas* strain in pure culture since it was refractory of growing in the solid media tested. In contrast, all our attempts to isolate bacteria able to degrade the most persistent pesticide of the ones used in the fruit-packaging plants, IMZ, failed. This is the first report for the isolation of microorganisms able to actively degrade TBZ and use it as a C and N source, and only the second report in the literature of the isolation of bacteria able to actively degrade the other two chemicals, OPP and DPA.

Although it was not possible to elucidate the metabolic pathways followed by the three isolated bacteria during the respective pesticide degradation, different evidences seem to indicate a possible complete mineralization of the molecules, which is desirable for bioremediation purposes since it results in the irreversible removal of the chemicals from the environment, although this remains to be verified. Degradation is not always synonymous to detoxification, thus it is essential to identify the metabolites produced and the final products of the pesticide degradation in order to assess the detoxification potential of the isolated degrading bacteria. This

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will be particularly interesting for TBZ, for which our tests indicated the lateral thiazole ring cleavage as a first step in its metabolism suggesting the establishment of a novel pathway.

Characterization of the degrading capacities of the isolated bacteria showed promising results such as their capacity a) to maintain their degrading capacity in a wide range of pH (4-9) and temperatures (4-37ºC), with the only exception of the TBZ-degrading consortium which showed limited degradation capacity at alkaline conditions (pH = 9) and low temperatures (4^oC), b) to actively degrade the three pesticides at concentrations levels which are expected to encounter in the wastewaters produced by the fruit packaging plants, with more striking example being the capacity of the *P. monteilii* strain to degrade up to 2000 mg/l of DPA, the highest capacity ever reported for DPA microbial degradation, c) to effectively degrade spillage level concentrations of the studied pesticides when inoculated in soil, and d) to degrade the target pesticide in the co-presence of other pesticides and their corresponding degrading bacteria. Overall our results suggest that the pesticide-degrading bacteria isolated in this study could be valuable tools for the deployment of future bioremediation and biodepuration regimes including:

a) Decontamination of wastewaters from the fruit packaging industry: biological systems for the treatment of wastewaters from the fruit packaging industry appear as promising solution to the current lack of alternative methods. The pesticide-degrading bacteria isolated in our study could be used as starting-inocula for biological treatment systems enabling their full implementation. Their capacity to maintain active under various environmental conditions and pesticide concentration levels supplied either individually or in mixtures, situations which are expected to encounter in practice, demonstrate their potential for implementation in such biopurification applications.
b) Bioaugmentation of soil from wastewater disposal sites: the current lack of cheap, sustainable, and efficient methods to treat the wastewaters from the fruit packaging industry have forced their owners to dispose those wastewaters to adjacent abandoned field sites resulting in the build up of particularly high pesticide levels in soil, as shown for IMZ in the current thesis (Chapter 4). Those sites constitute a serious environmental problem and their remediation is urgently needed. Our findings indicate that the isolated bacteria could be utilized for the recovery of such soil sites, especially for TBZ which is considered a particularly persistent chemical in the soil.
c) Bioaugmentation of Biobed Systems: these biopurification systems can be used for the decontamination of wastewater produced by the fruit packaging industries as long as appropriate water management schemes are implemented to handle the high wastewater volumes that are produced. Our results in BEM showed that our bacteria could be used in

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bioaugmentation strategies in order to enhance the dissipation potential of biobed systems especially for persistent or mobile chemicals like TBZ and OPP respectively.

d) Decontamination of Spent Biobed Substrates: at the end of the life cycle of a biobed system, the spent biobed substrate is expected to contain high pesticide loads and its decontamination before release in the environment is needed. Our results suggest that bioaugmentation of TBZ, OPP, and DPA contaminated spent biobed substrates with the respective isolated bacteria could be an effective and cheap way to treat such materials and avoid alternative treatment methods which are particularly costly.

Future considerations

The current thesis provided new insights in the microbial degradation of pesticides used in the fruit-packaging industry which have serious practical implications for the future reduction of the environmental footprint of the fruit postharvest industry, a particularly dynamic sector in the Mediteranean sea basin. At the same time new research challenges have arisen which we aim to further undertake in the future including:

a) The elucidation of the metabolic pathway of the studied pesticides is of primary importance in order to further outline the detoxification capacity of our isolates. This will be tackled with the use of advanced analytical tools like LC-MS/MS or GC-MS/MS (for volatile metabolites).

b) Functional analysis of the genes/enzymes involved in the degradation of the studied pesticides by our isolates. On-going genomic and proteomic analysis of the isolated bacteria are expected to shed light on this. The results obtained are expected to further facilitate our effort to elucidate the metabolic pathway of the studied pesticides.

c) Considering the widespread use of IMZ in fruit-packaging plants and its high environmental toxicity and persistence further attempts will be made in order to isolate bacteria able to degrade IMZ, thus complementing the inventory of degrading-bacteria which could be used as starting inocula in biological wastewater treatment systems.

d) Not disregarding the practical significance of our findings, a future focus will be to explore the full-scale application of our pesticide-degrading isolates as tailored-made inocula in a biological treatment system specifically used for the treatment of wastewaters from the fruit packaging industry.

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Annex I

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1. Chapter 3

3.6. Q-PCR experiment

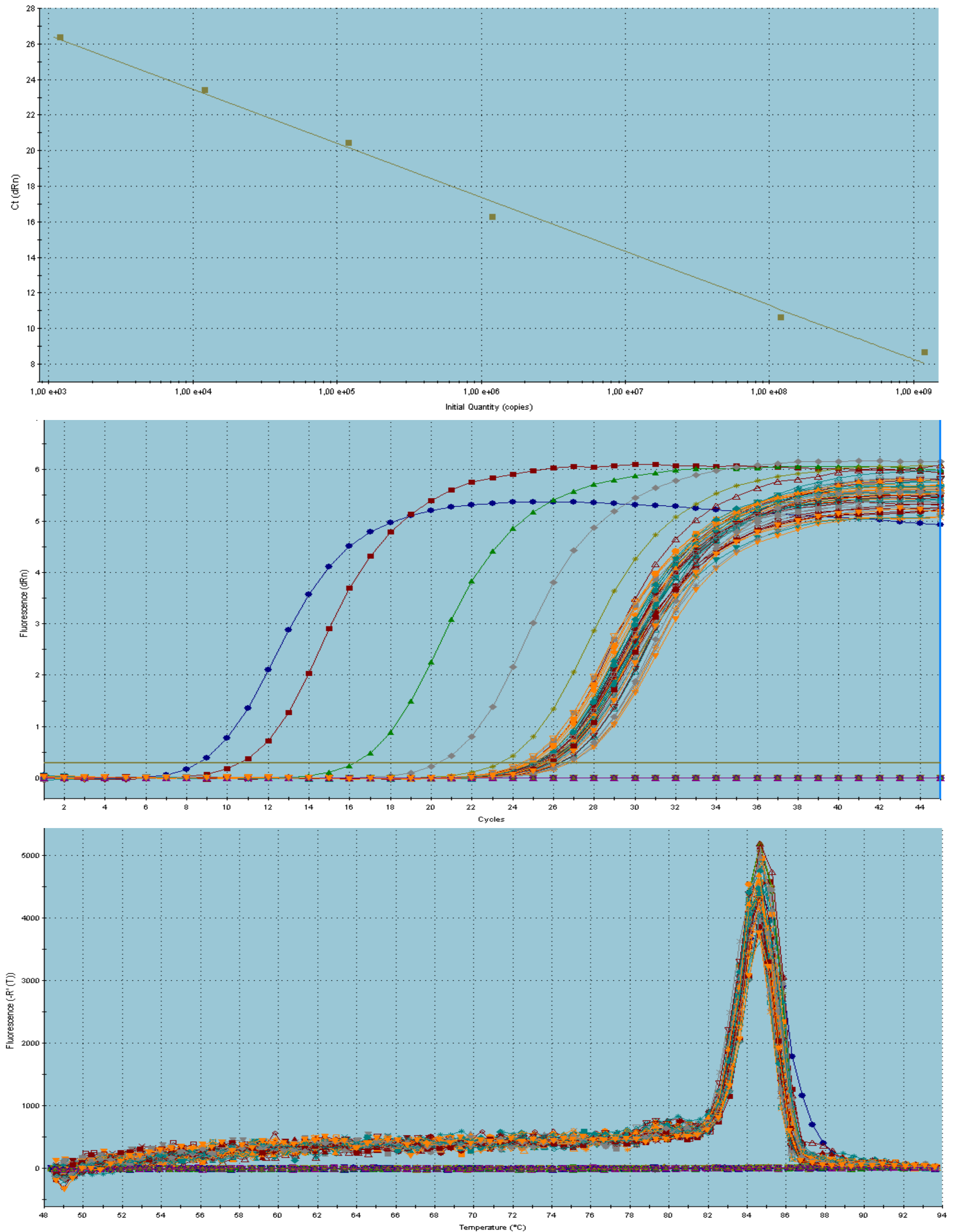

Figure A.1. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for *Achromobacter* (MSMN consortium).

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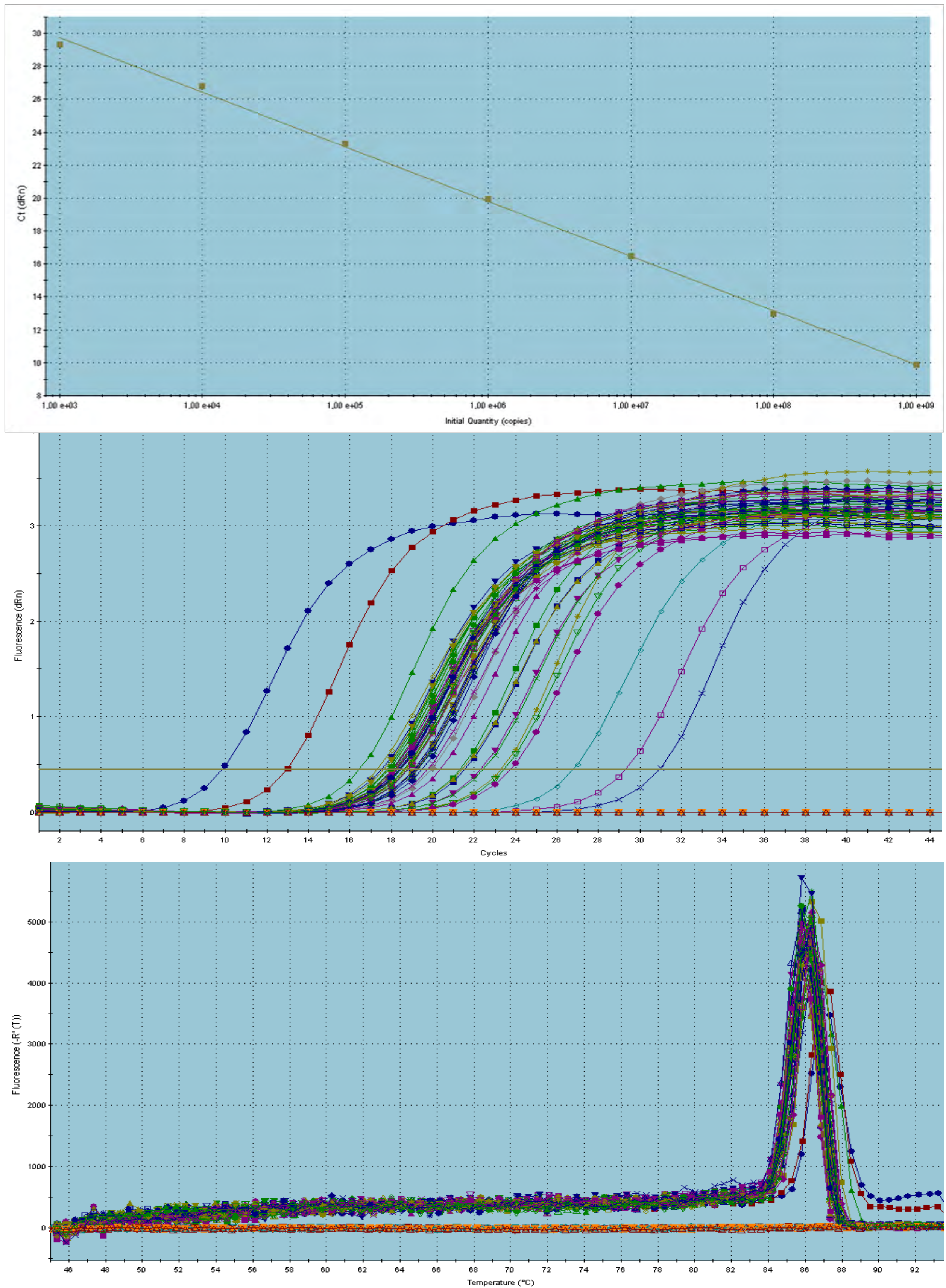

Figure A.2. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for *Methylibium* (MSMN consortium).

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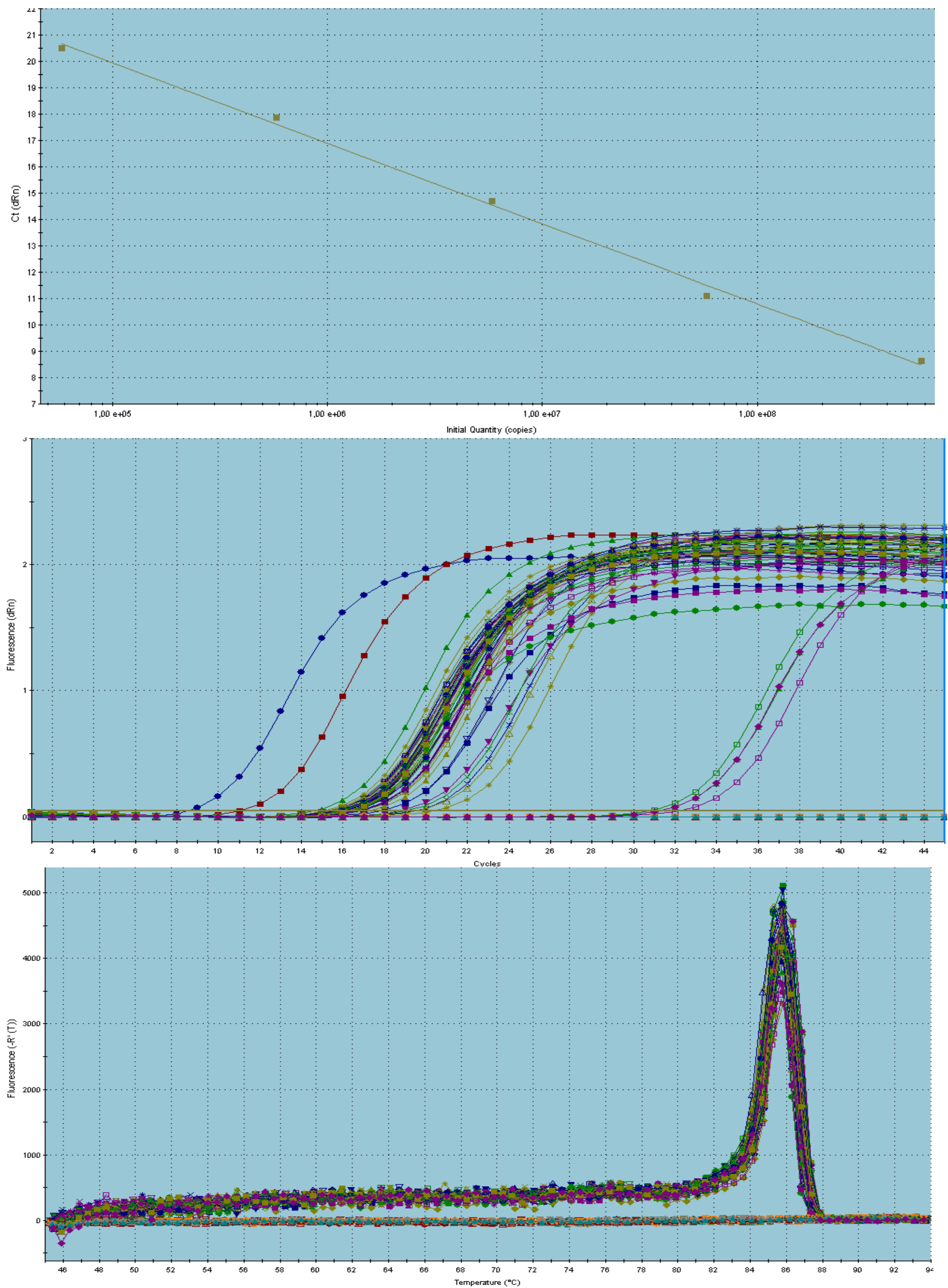

Figure A.3. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for *Methylobacillus* (MSMN consortium).

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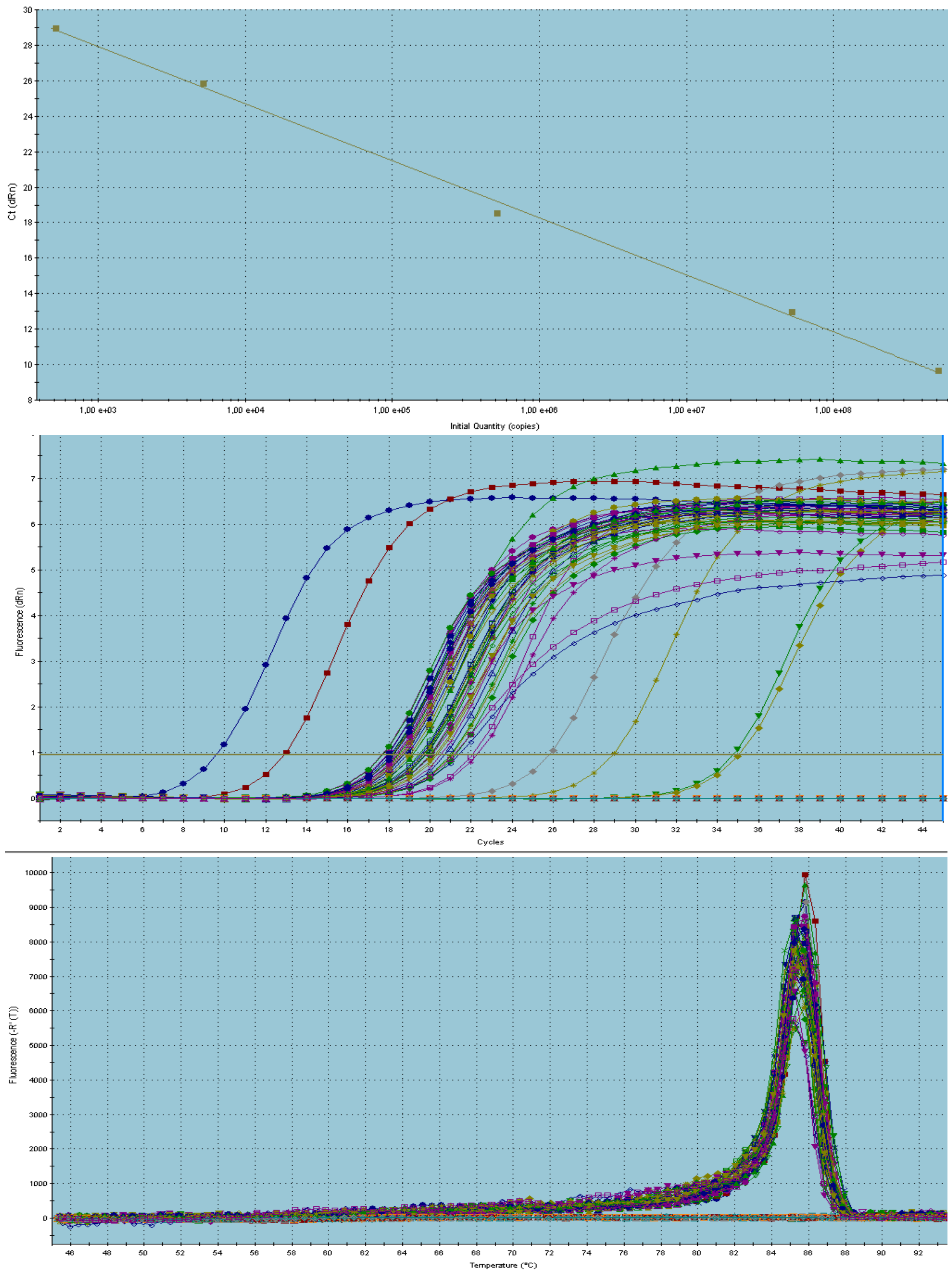

Figure A.4. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for the α-proteobacteria group that comprises *Bosea, Sphingomonas,* and *Shinella.* spp. (MSMN consortium).

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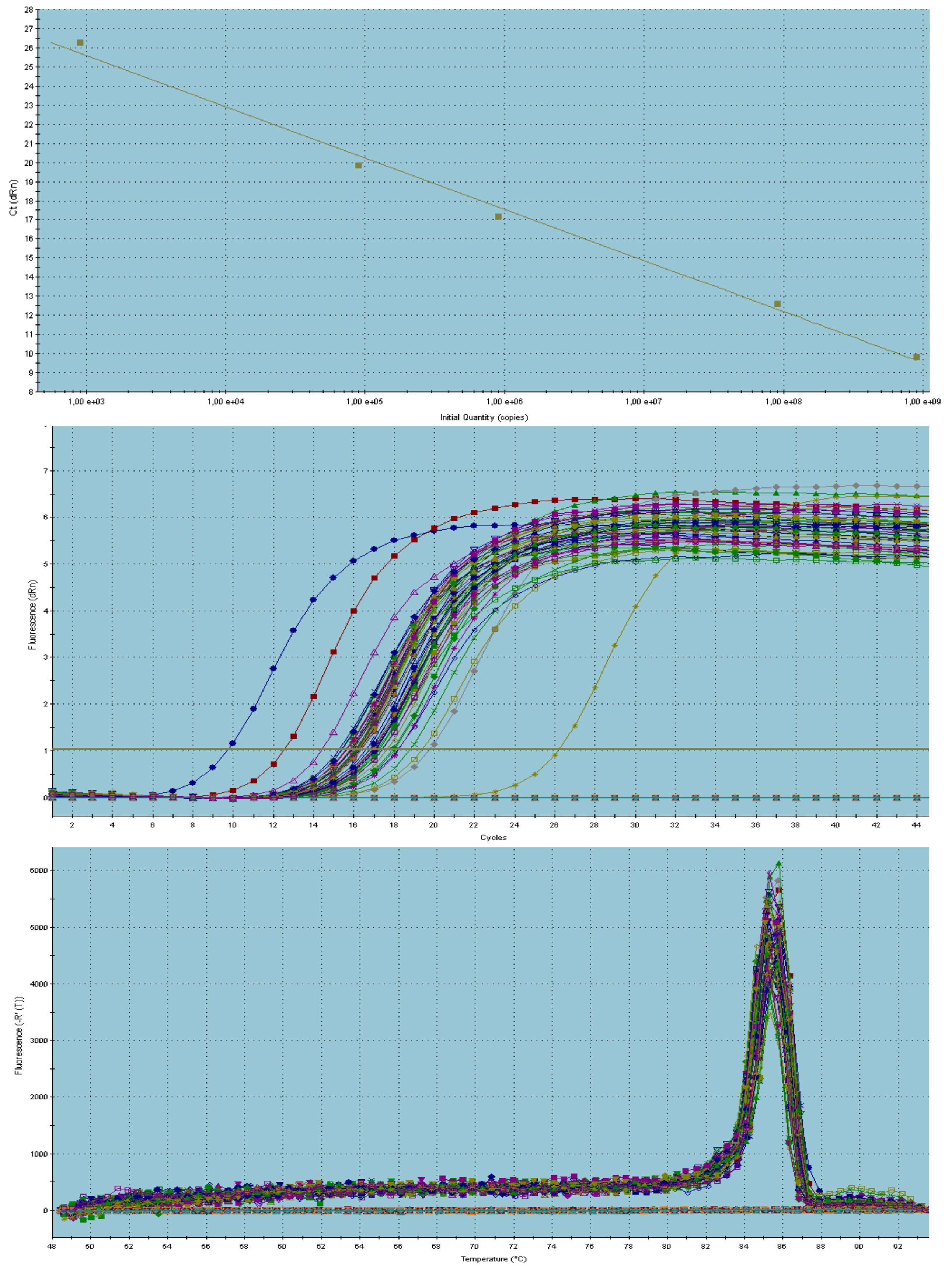

Figure A.5. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for *Pseudomonas* group (MSMN consortium).

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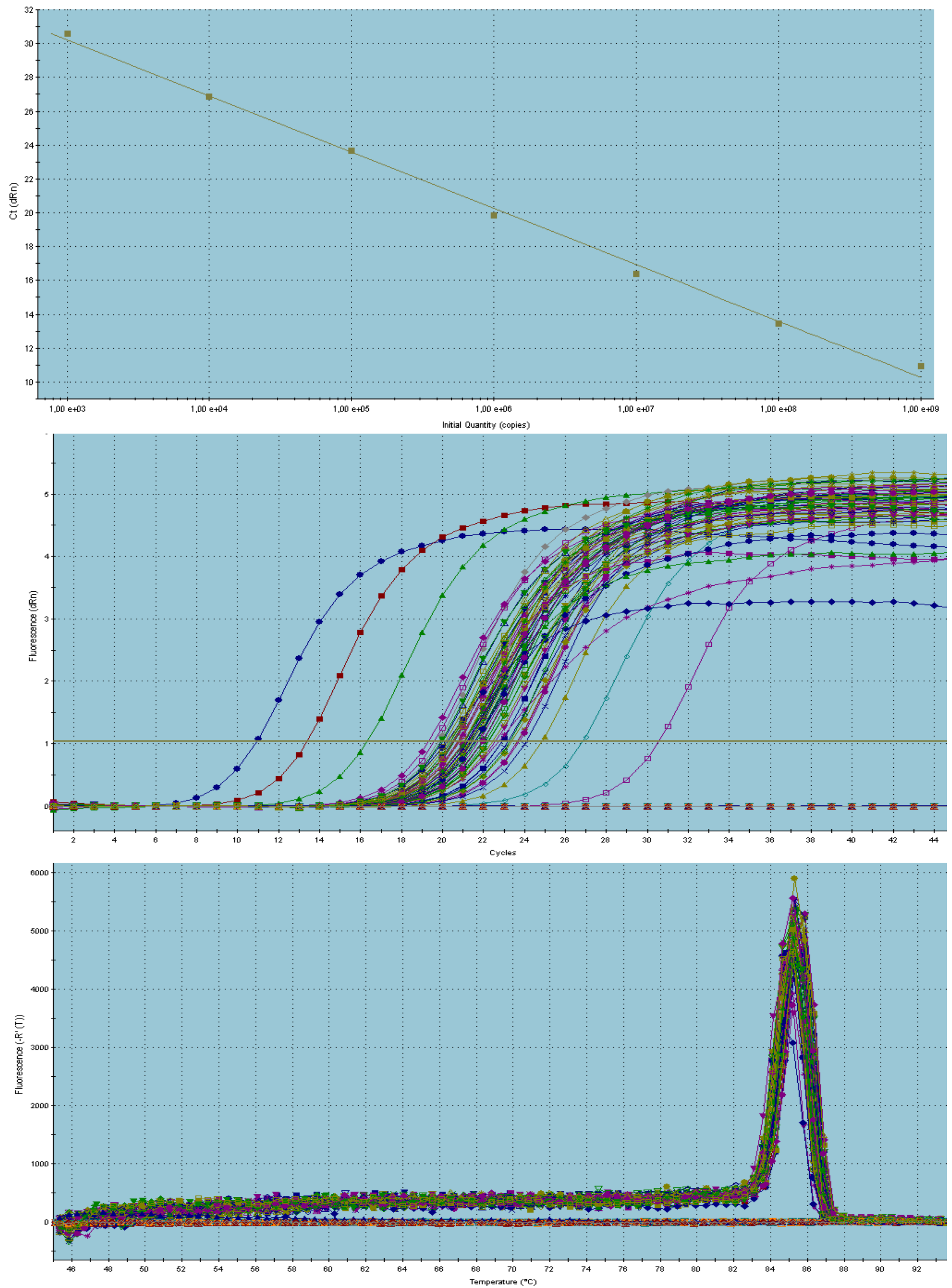

Figure A.6. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for *Hydrocarboniphaga* (MSM consortium).

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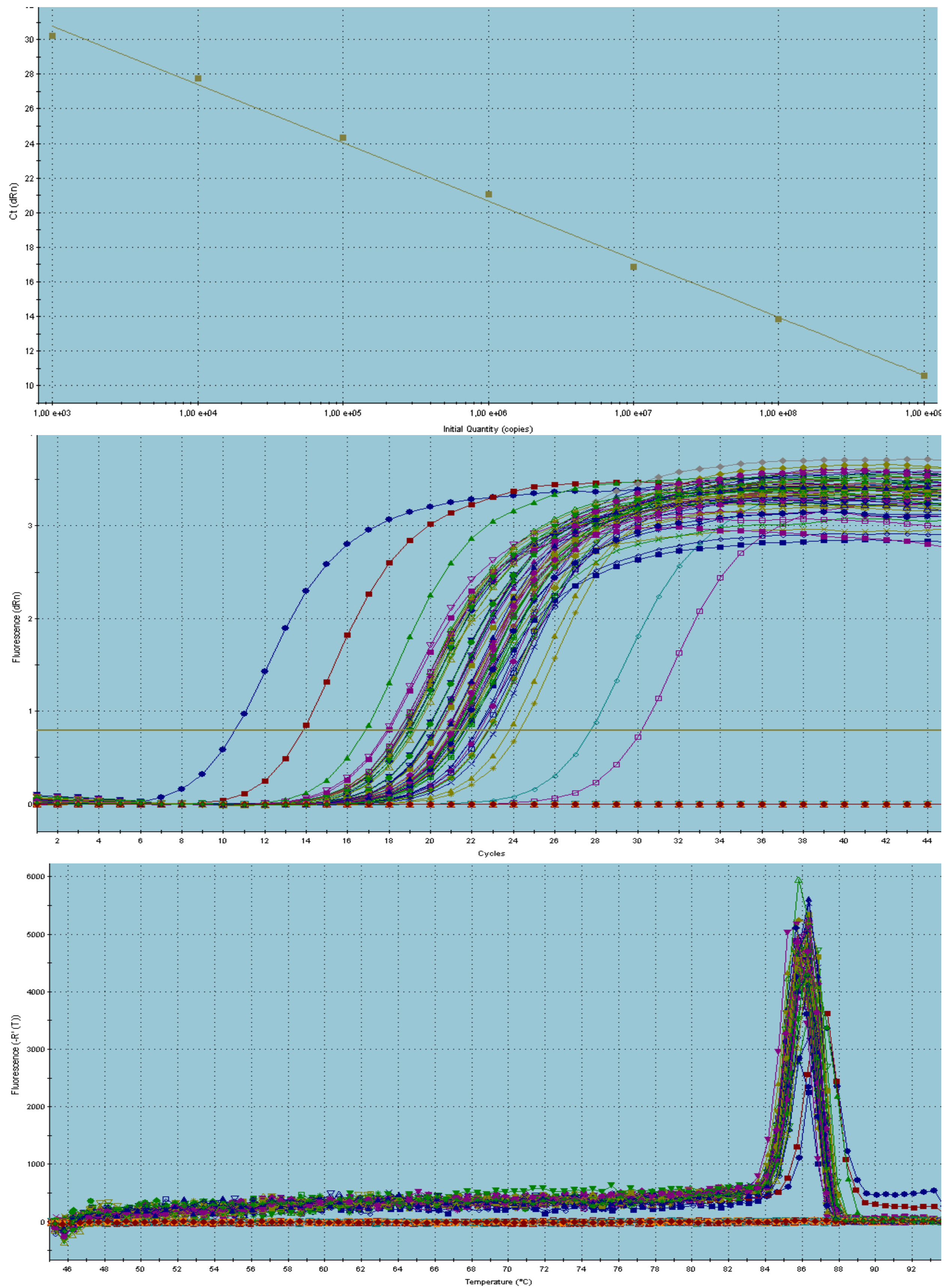

Figure A.7. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for *Methylibium* (MSM consortium).

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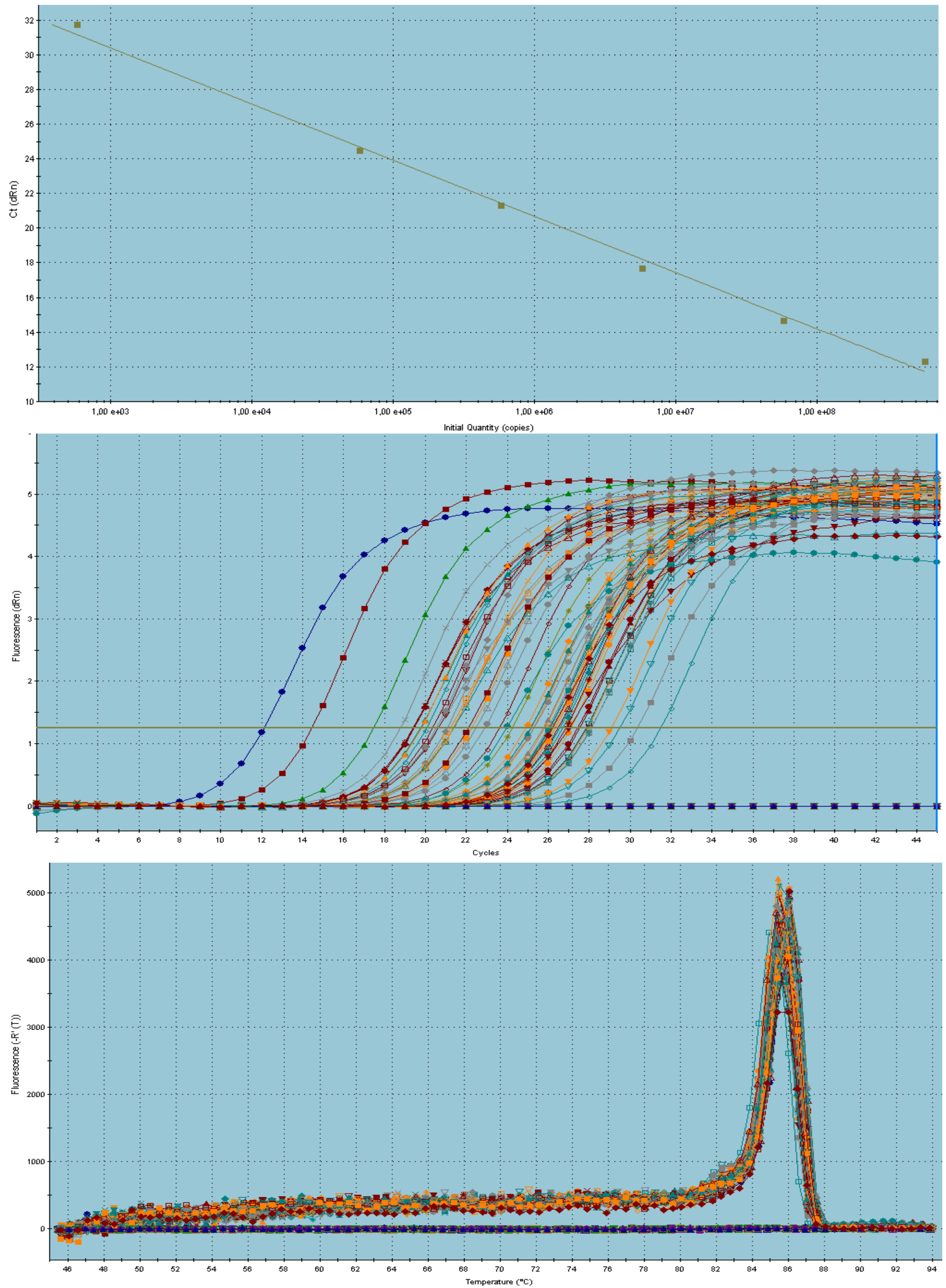

Figure A.8. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for *Methylobacillus* and *Aminomonas* (MSM consortium).

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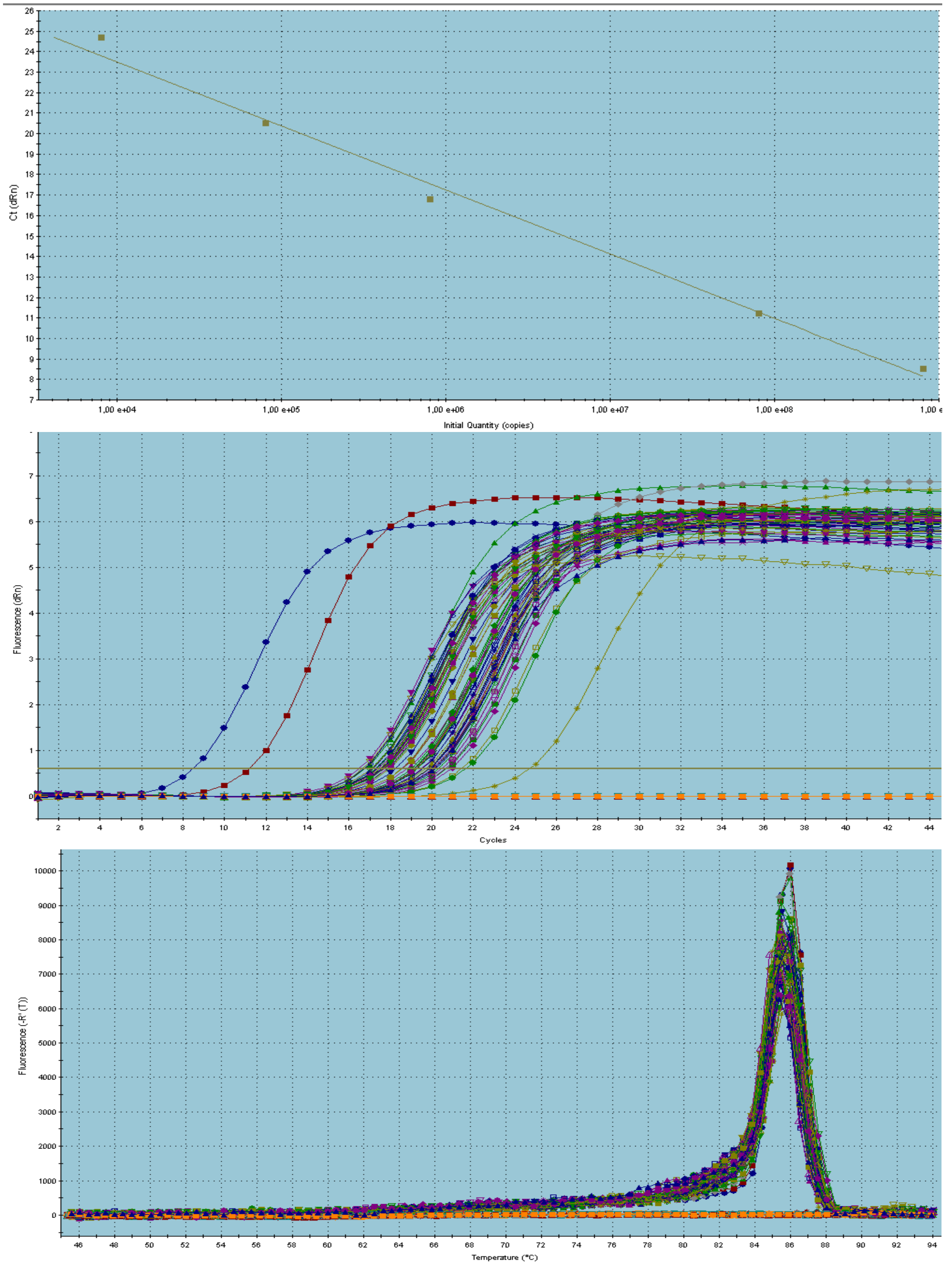

Figure A.9. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for the α-proteobacteria group that comprises *Bosea, Sphingomonas,* and *Oligotropha* spp. (MSM consortium).

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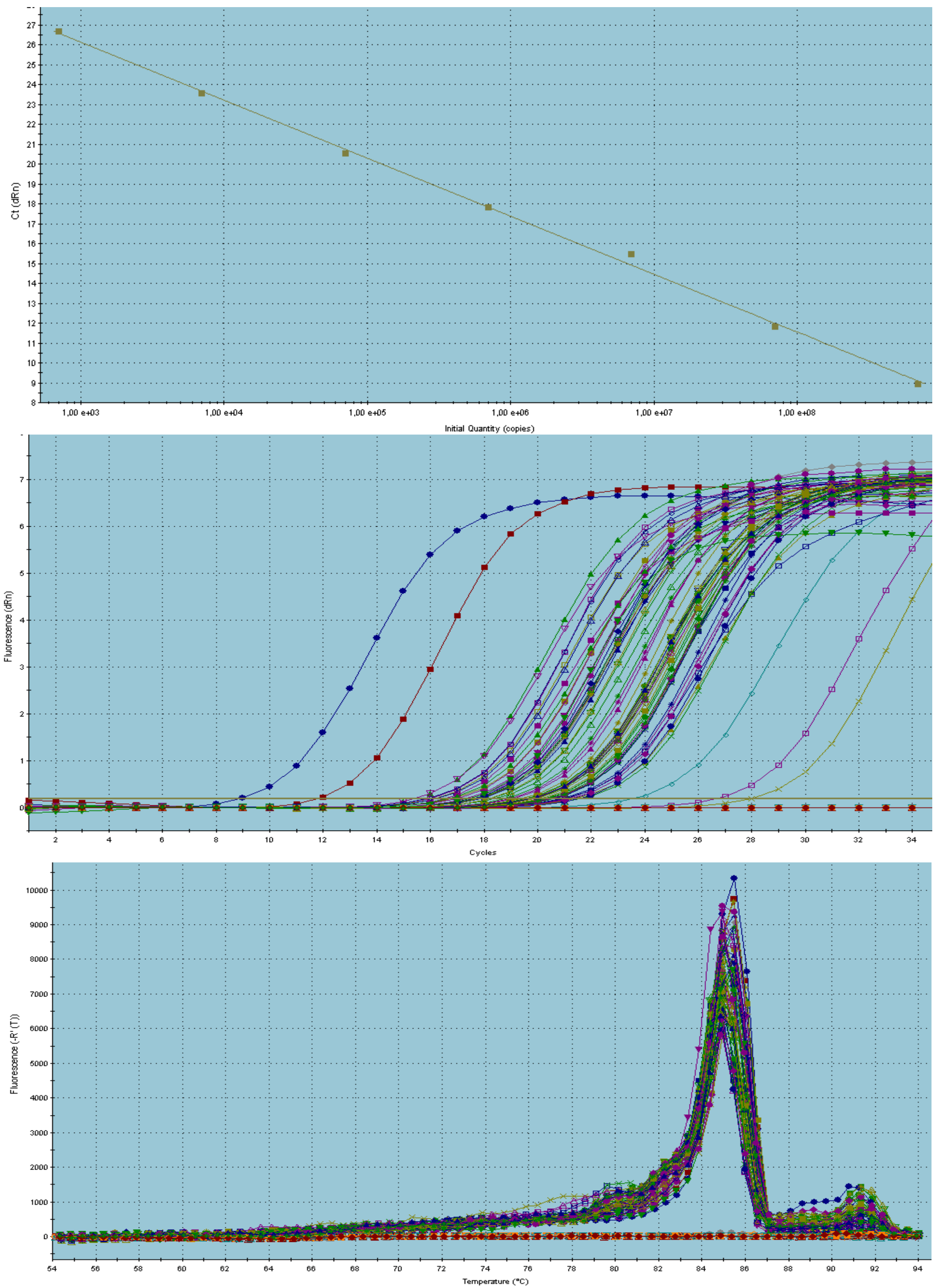

Figure A.10. From top to bottom: q-PCR calibration, amplification, and dissociation curves using specific primers for the *Pseudomonas* group (MSM consortium).

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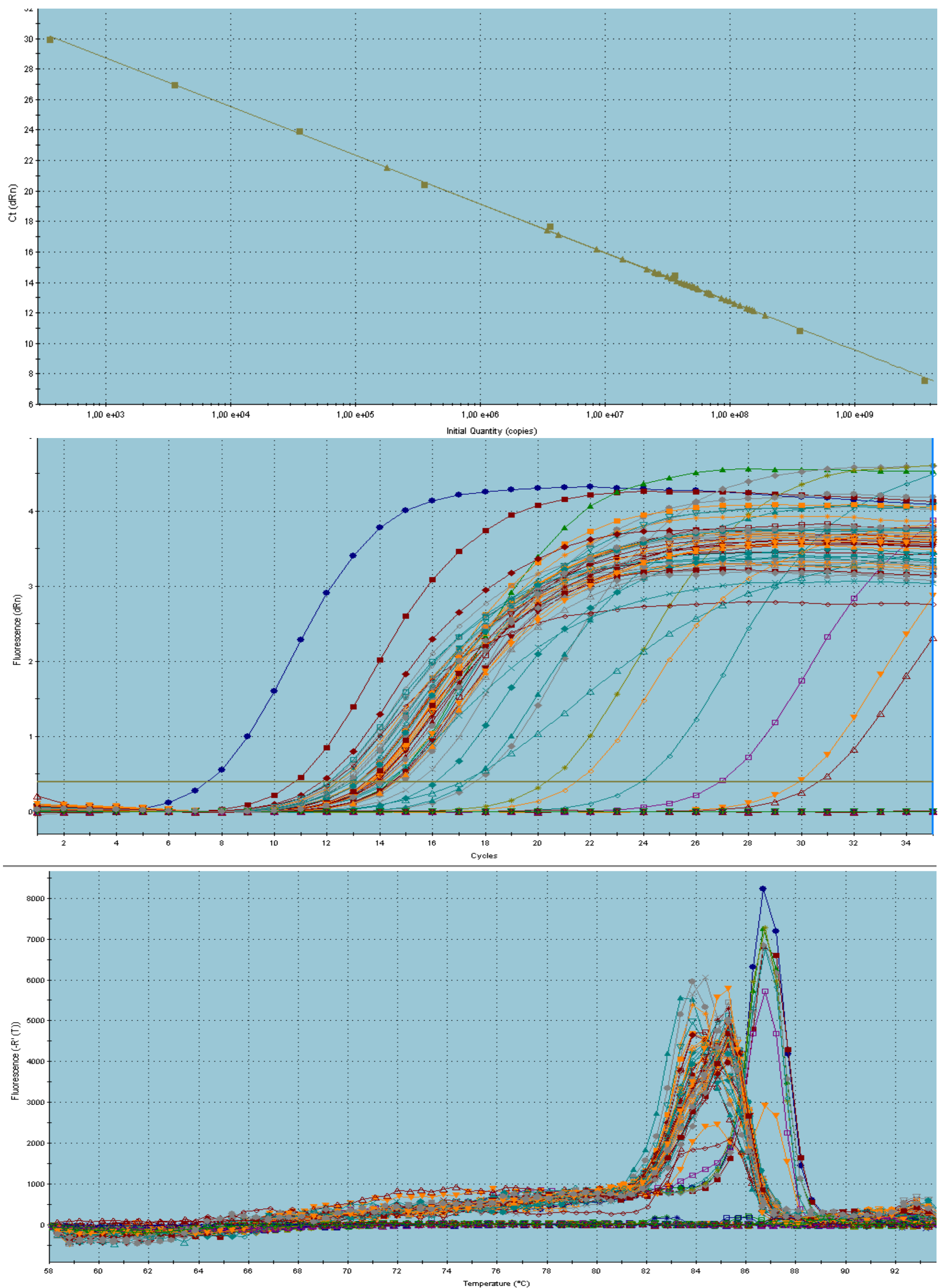

Figure A.11. From top to bottom: q-PCR calibration, amplification, and dissociation curves using the total bacteria primers Eub338/Eub518 (MSMN consortium).

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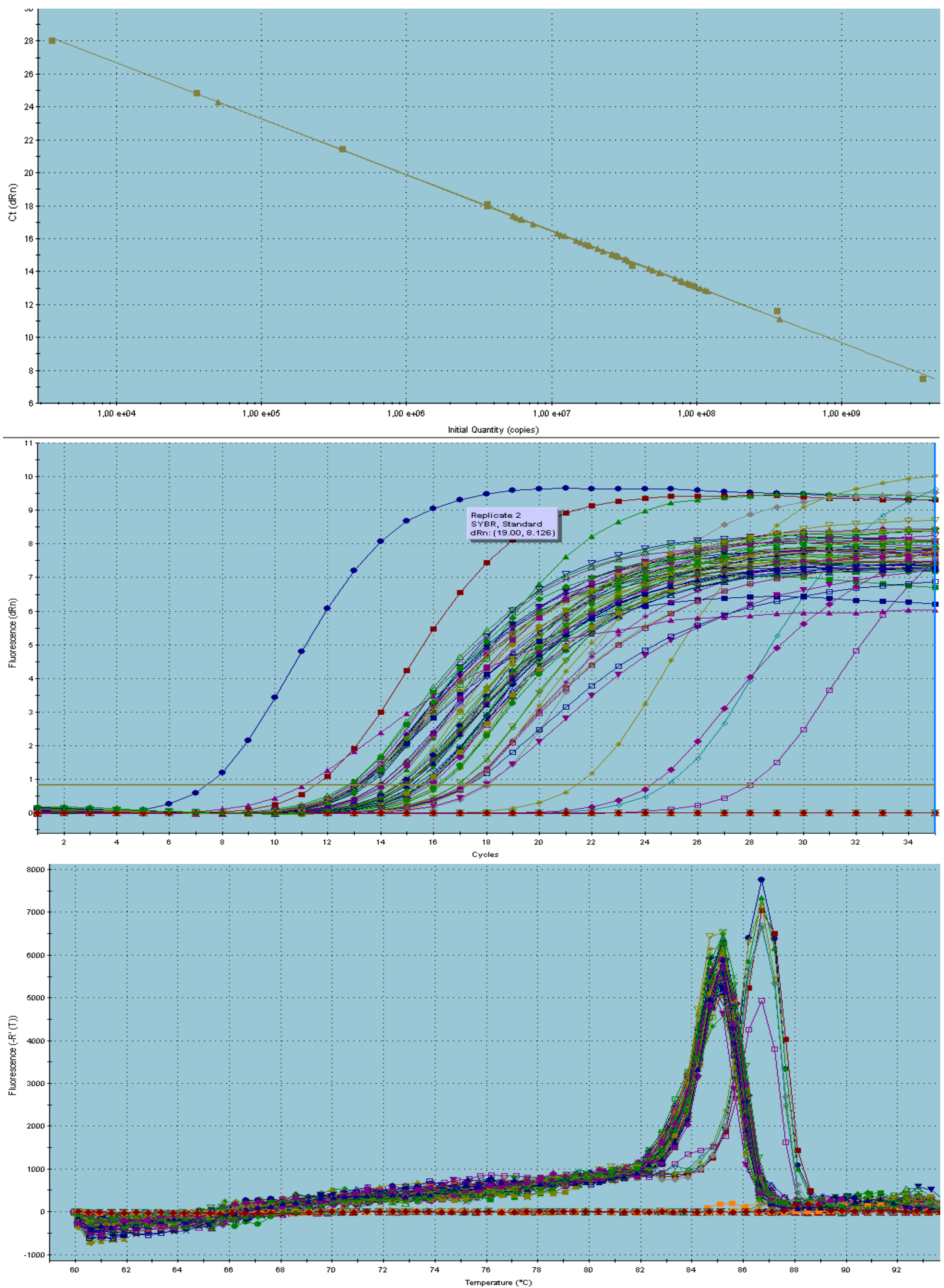

Figure A.12. From top to bottom: q-PCR calibration, amplification, and dissociation curves using the total bacteria primers Eub338/Eub518 (MSM consortium).

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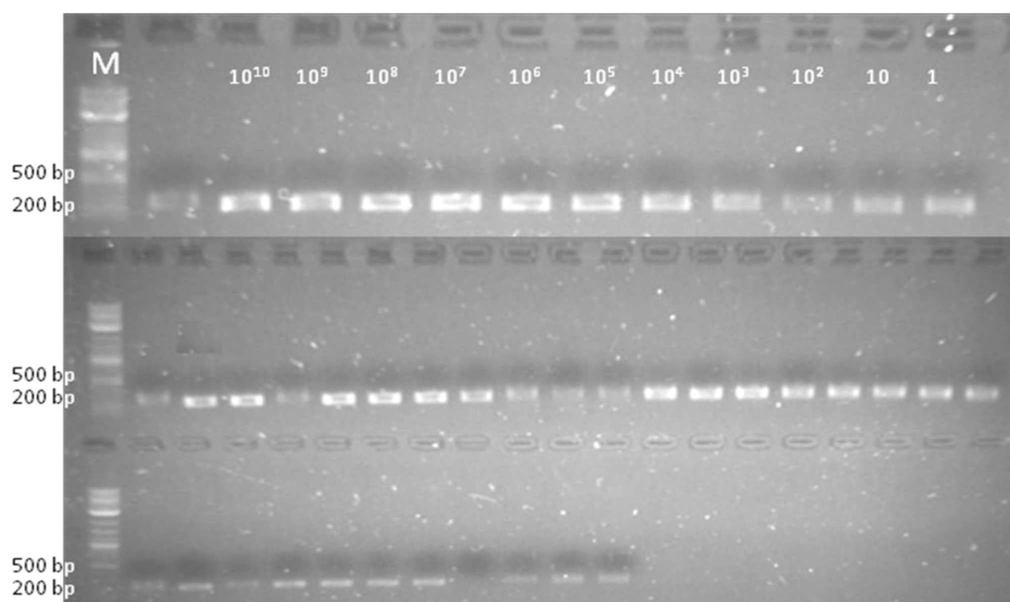

Figure A.13. 1.5% agarose gel electrophoresis showing the q-PCR products amplified using the total bacteria primers Eub338/Eub518. Lane 1: calibration care plasmid dilutions. Lanes 2 and 3: MSMN and MSM DNA samples. In all cases only one fragment of the expected size (200 bp) was produced during the q-PCR reaction. “M” indicates the molecular size marker.

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2. Chapter 3

3.9. Specific q-PCR for the isolated bacterium *Sphingomonas* strain B13/E8/ColC

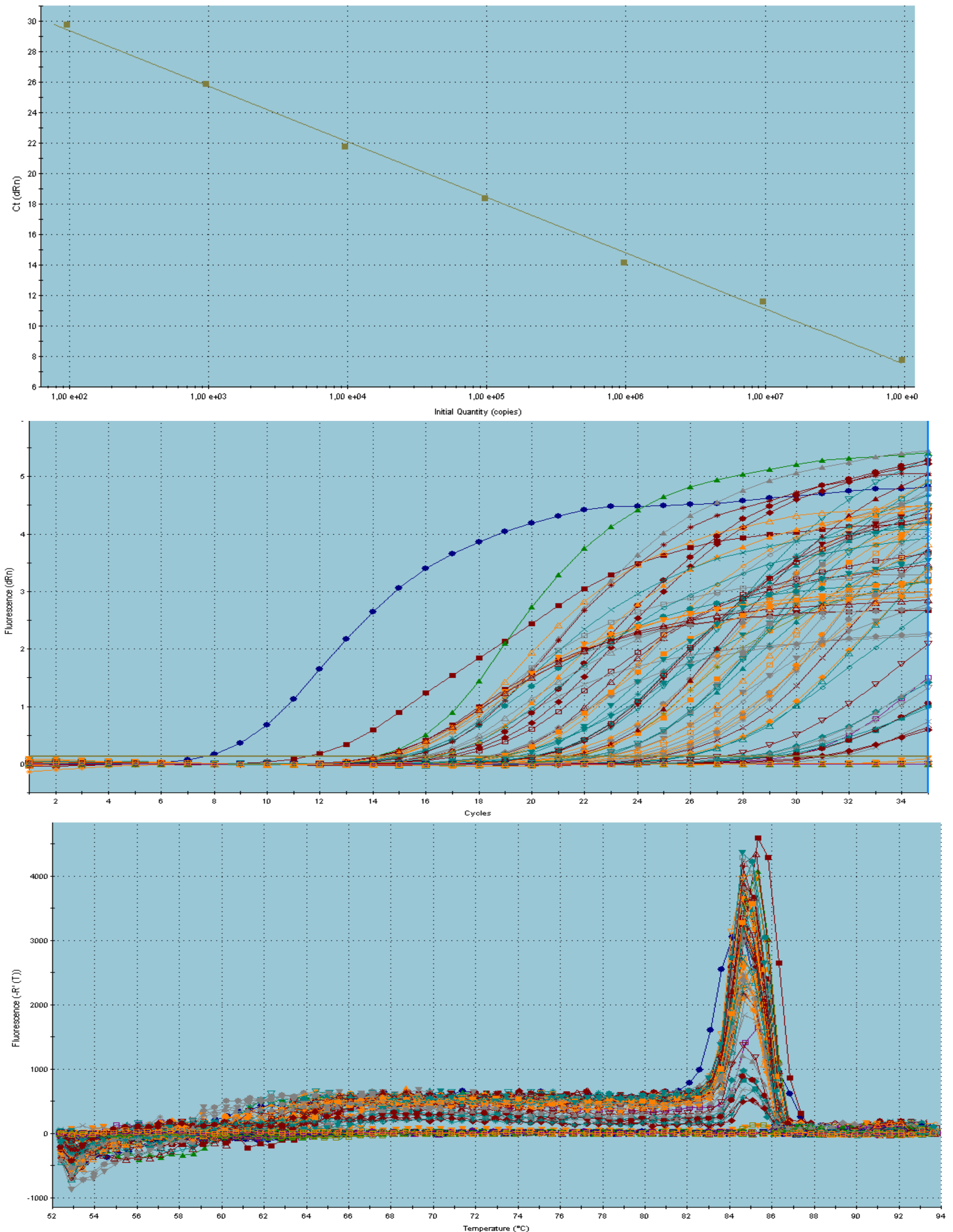

Figure A.14. From top to bottom: q-PCR calibration, amplification, and dissociation curves using the specific primers ColC_f/ColC_r for the detection of the bacterium *Sphingomonas* strain B13/E8/ColC.

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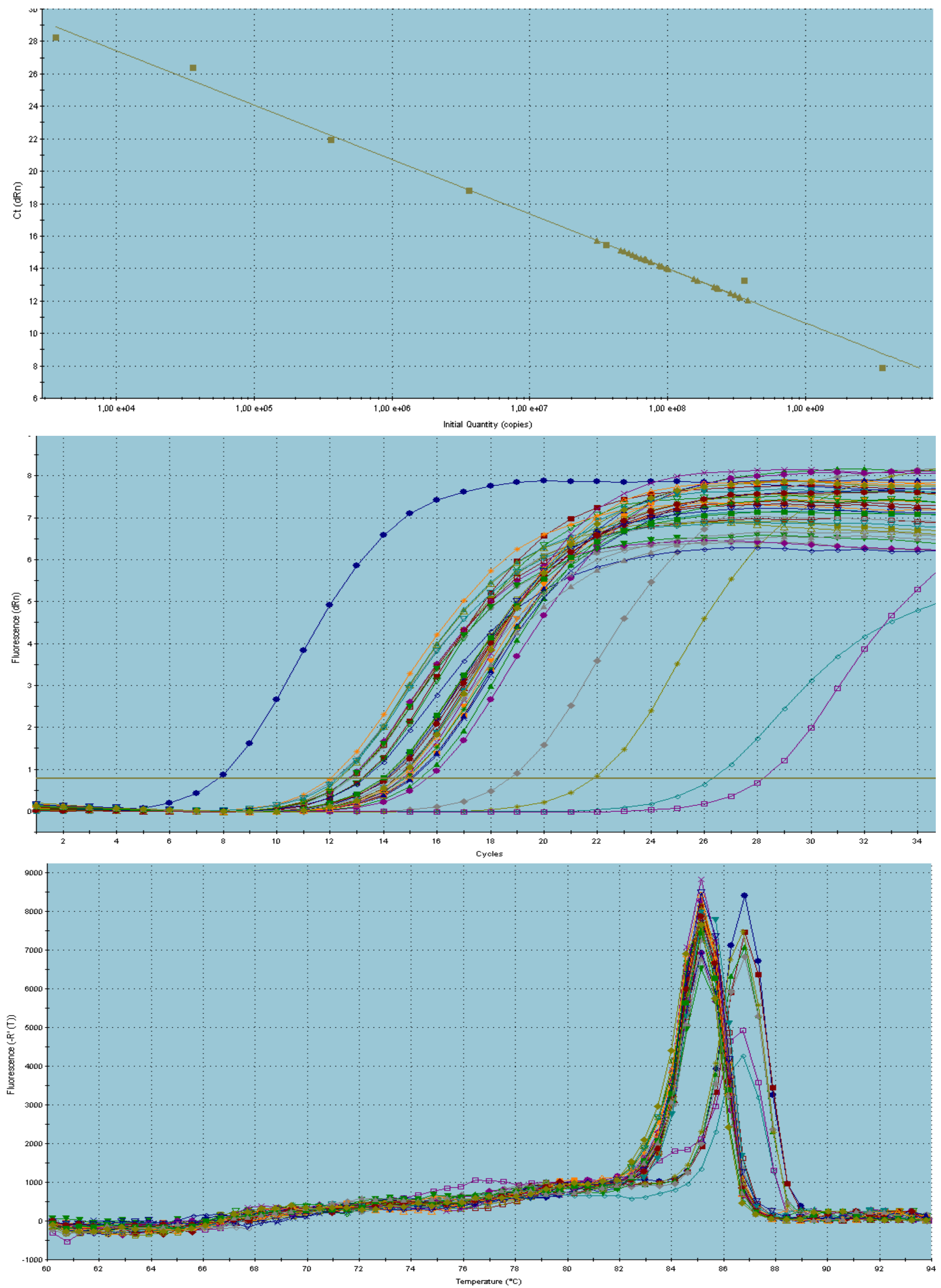

Figure A.15. From top to bottom: q-PCR calibration, amplification, and dissociation curves using the total bacteria primers Eub338/Eub518.

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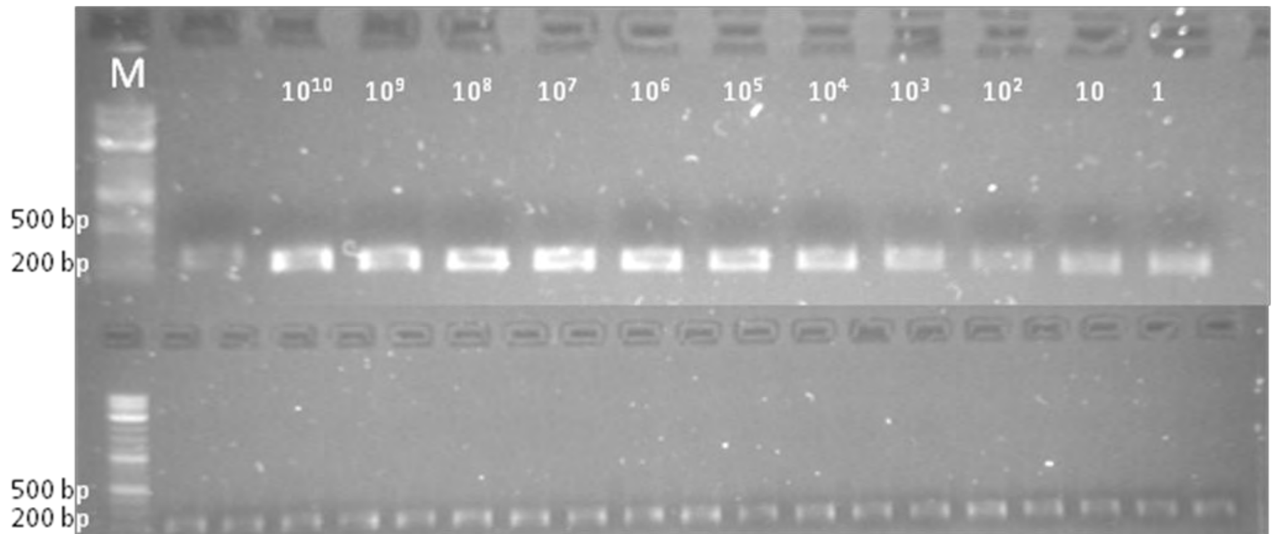

Figure A.16. 1.5% agarose gel electrophoresis showing the q-PCR products amplified using the total bacteria primers Eub338/Eub518. Lane 1: calibration care plasmid dilutions. Lanes 2: DNA samples. In all cases only one fragment of the expected size (200 bp) was produced during the q-PCR reaction. "M" indicates the molecular size marker.

3. Gene sequences of the isolated bacteria and NCBI accession number

The sequences of the main bacteria isolated in the course of this study were registered in the GenBank database and are available at http://www.ncbi.nlm.nih.gov/ providing the accession numbers reported below. Primers sequences are not included.

3.1. Thiabendazole (Chapter 3)

The partial 16S rRNA gene sequences of the main bacteria of the degrading consortia (see Section 3.7 of the Chapter) are listed.

Variovorax paradoxus strain B4, 16S rRNA gene sequence (Accession N° KJ676725)

```
1     gattgaacgc   tggcggcatg   ccttacacat   gcaagtcgaa   cggcagcgcg   ggagcaatcc
61    tggcggcgag   tggcgaacgg   gtgagtaata   catcggaacg   tgcccaatcg   tgggggataa
121   cgcagcgaaa   gctgtgctaa   taccgcatac   gatctacgga   tgaaagcagg   ggatcgcaag
181   accttgcgcg   aatggagcgg   ccgatggcag   attaggtagt   tggtgaggta   aaggctcacc
241   aagccttcga   tctgtagctg   gtctgagagg   acgaccagcc   acactgggac   tgagacacgg
301   cccagactcc   tacgggaggc   agcagtgggg   aattttggac   aatgggcgaa   agcctgatcc
361   agccatgccg   cgtgcaggat   gaaggccttc   gggttgtaaa   ctgcttttgt   acggaacgaa
421   acggtctttt   ctaatacaga   aggctaatga   cggtaccgta   agaataagca   ccggctaact
481   acgtgccagc   agccgcggta   atacgtaggg   tgcaagcgtt   aatcggaatt   actgggcgta
541   aagcgtgcgc   aggcggttat   gtaagacagt   tgtgaaatcc   ccgggctcaa   cctgggaact
601   gcatctgtga   ctgcatagct   agagtacggt   agagggggat   ggaattccgc   gtgtagcagt
661   gaaatgcgta   gatatgcgga   ggaacaccga   tggcgaaggc   aatcccctgg   acctgtactg
721   acgctcatgc   acgaaagcgt   ggggagcaaa   caggattaga   taccctggta   gtccacgccc
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901	cacaagcggt	ggatgatgtg	gtttaattcg	atgcaacgcg	aaaaacctta	cccacctttg
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1141	ggaggaaggt	ggggatgacg	tcaagtcctc	atggccctta	taggtggggc	tacacacgtc
1201	atacaatggc	tggtacaaag	ggttgccaac	ccgcgagggg	gagctaatcc	cataaaacca
1261	gtcgtagtcc	ggatcgcagt	ctgcaactcg	actgcgtgaa	gtcggaatcg	ctagtaatcg
1321	tggatcagaa	tgtcacggtg	aatacgttcc	cgggtcttgt	acacaccgcc	cgtcacacca
1381	tgggagcggg	ttctgccaga	agtagttagc	ttaaccgcaa	ggagggcgat	taccacggca
1441	gggttcgtga	ctggggtgaa				

Hydrocarboniphaga effusa strain B7/E6, 16S rRNA gene sequence (Accession N^{o} KJ676716)

1	gattgaacgc	tggcggcatg	cctaacacat	gcaagtcgaa	cgagggtagc	aataccttag
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121	cccggattaa	taccgcatga	actcgtgaga	gcaaagtcgg	ggaccttcgg	gcctggcgct
181	tttggagaag	cctatgtcgg	attagctagt	tggtgaggta	atggctcacc	aaggctgcga
241	tccgtaactg	gtctgagagg	atgatcagtc	acaccggaac	tgagacacgg	tccggactcc
301	tacgggaggc	agcagtgggg	aatattggac	aatgggcgaa	agcctgatcc	agcaatgccg
361	cgtgtgtgaa	gaaggccttc	gggttgtaaa	gcactttagg	ctggaaagaa	aaagctttgg
421	ctaatatcca	aagccttgac	ggtaccagca	gaataagcac	cggctaactc	tgtgccagca
481	gccgcggtaa	tacagagggt	gcaggcgtta	atcggattta	ctgggcgtaa	agcgtgcgta
541	ggcggctatt	taagtcggat	gtgaaagccc	tgggcttaac	ctgggaactg	cattcgatac
601	tgggtagctg	gagatcggta	gagggcggcg	gaattccggg	tgtagcggtg	aaatgcgtag
661	atatccggag	gaacaccgat	ggcgaaagca	accgcctggg	cctgatctga	cgctgaggca
721	cgaaggcgtg	gggagcaaac	aggattagat	acccttggta	gtccacgctg	taaacgatga
781	gaacttgacg	tccgctctgc	ttcgcgagtg	ggtgtcgaag	ctaacgcgct	aagttctccg
841	cctggggagt	acggccgcaa	ggttgaaact	caaaggaatt	gacgggggcc	cgcacaagcg
901	gtggagtatg	tggtttaatt	cgatgcaacg	cgaagaacct	tactggtctt	gacatgtcga
961	gaatcctgca	gagatgcggg	agtgccttcg	ggaactcgaa	cacaggtgct	gcatggctgt
1021	cgtcagctcg	tgtcgtgaga	tgttgggtta	agtcccgcaa	cgagcgcaac	ccttgccctt
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1141	ggggatgacg	tcaagtcatc	atggccctta	tgaccagggc	tacacacgta	ctacaatggt
1201	cggtacagag	ggttgccaac	ccgcgagggg	gagctaatcc	caaaaagccg	atcgtagtcc
1261	ggattggagt	ctgcaactcg	actccatgaa	gtcggaatcg	ctagtaatcg	cggatcagca
1321	cttgtcgcgg	tgaatacgtt	cccgggcctt	gtacacaccg	cccgtcacac	catgggagtt
1381	gtctgcacca	gaagtaggta	gtctaaccgc	aaggggggcg	cttaccacgg	tgtggtcaat
1441	gactggggtg	aa				

Sphingopyxis sp. strain X3a, 16S rRNA gene sequence (Accession N^{o} KJ676726)

1	gaacgaacgc	tggcggcatg	cctaacacat	gcaagtcgaa	cgagatcttc	ggatctagtg
61	gcgcacgggt	gcgtaacgcg	tgggaatctg	cccttgggta	cggaataact	cagagaaatt

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301 ggggaatatt ggacaatggg cgaaagcctg atccagcaat gccgcgtgag tgatgaaggc
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541 cccggaatag cctttgaaac tggaaaacta gaatcttgga gaggtcagtg gaattccgag
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901 ccagcgtttg acatcctgat cgcggttacc agagatggtt tccttcagtt cggctggatc
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1261 cgctagtaat cgcggatcag catgccgcgg tgaatacgtt cccaggcctt gtacacaccg
1321 cccgtcacac catgggagtt ggtttcaccc gaaggcagtg ctctaacccg caagggagga
1381 agctgaccac ggtgggatca gcgactgggg tgaa

Bosea sp. strain X3b/E16, 16S rRNA gene sequence (Accession N^{o} KJ676723)

1 aggcctaaca catgcaagtc gaacgggcac ttcggtgcta gtggcagacg ggtgagtaac
61 acgtgggaac gtacctttcg gttcggaata attcagggaa acttggacta ataccggata
121 cgcccttcgg gggaaagatt tatcgccgat agatcggccc gcgtctgatt agctagttgg
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721 gaatgccagc cgttggggtg catgcacttc agtggcgcag ctaacgcttt aagcattccg
781 cctggggagt acggtcgcaa gattaaaact caaaggaatt gacgggggcc cgcacaagcg
841 gtggagc

Methylibium petroleiphilum strain X3b/E16, 16S rRNA gene sequence (Accession N^{o} KJ676717)

1 gattgaacgc tggcggcatg ccttacacat gcaagtcgaa cggcagcacg ggagcaatcc
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1321	tcagcttgcc	gcggtgaata	cgttcccggg	tcttgtacac	accgcccgtc	acaccatggg
1381	agcgggttct	gccagaagta	gttagcctaa	ccgcaaggag	ggcgattacc	acggcagggt
1441	ttgtgactgg	ggtgaa				

Hydrogenophaga sp. strain X3c, 16S rRNA gene sequence (Accession N^{o} KJ676727)

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781	aacgatgtca	actggttgtt	gggtctcttc	tgactcagta	acgaagctaa	cgcgtgaagt
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901	caagcggtgg	atgatgtggt	ttaattcgat	gcaacgcgaa	aaaccttacc	cacctttgac
961	atgtacggaa	tttgccagag	atggcttagt	gctcgaaaga	gagccgtaac	acaggtgctg
1021	catggctgtc	gtcagctcgt	gtcgtgagat	gttgggttaa	gtcccgcaac	gagcgcaacc
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1201	tggctggtac	aaagggttgc	caacccgcga	gggggagcta	atcccataaa	gccagtcgta
1261	gtccggatcg	cagtctgcaa	ctcgactgcg	tgaagtcgga	atcgctagta	atcgtggatc
1321	agcatgtcac	ggtgaatacg	ttcccgggtc	ttgtacacac	cgcccgtcac	accatgggag
1381	cgggtctcgc	cagaagtagt	tagcctaacc	gcaaggaggg	cgattaccac	ggcggggttc
1441	gtgactgggg	tgaa				

Sphingomonas wittichii strain B13/E8/ColC, 16S rRNA gene sequence (Accession N° KJ676715)

1	gaacgaacgc	tggcggcatg	cctaacacat	gcaagtcgaa	cgagaccttc	gggtctagtg
61	gcgcacgggt	gcgtaacgcg	tgggaatctg	cccttgggta	cggaataaca	gtgagaaatt
121	actgctaata	ccgtatgatg	acgtaagtcc	aaagatttat	cgcccaagga	tgagcccgcg
181	taggattagc	ttgttggtga	ggtaaaagct	caccaaggcg	acgatcctta	gctggtctga
241	gaggatgatc	agccacactg	ggactgagac	acggcccaga	ctcctacggg	aggcagcagt
301	ggggaatatt	ggacaatggg	cgaaagcctg	atccagcaat	gccgcgtgag	tgatgaaggc
361	cttagggttg	taaagctctt	ttacccggga	agataatgac	tgtaccggga	gaataagccc
421	cggctaactc	cgtgccagca	gccgcggtaa	tacggagggg	gctagcgttg	ttcggaatta
481	ctgggcgtaa	agcgcacgta	ggcggctttg	taagttagag	gtgaaagcct	ggggctcaac
541	tccggaattg	cctttaagac	tgcatcgctt	gaacgtcgga	gaggtgagtg	gaattccgag
601	tgtagaggtg	aaattcgtag	atattcggaa	gaacaccagt	ggcgaaggcg	gctcactgga
661	cgactgttga	cgctgaggtg	cgaaagcgtg	gggagcaaac	aggattagat	accctggtag
721	tccacgccgt	aaacgatgat	aactagctgt	ccgggcactt	ggtgcttggg	tggcgcagct
781	aacgcattaa	gttatccgcc	tggggagtac	ggccgcaagg	ttaaaactca	aagaaattga
841	cgggggcctg	cacaagcggt	ggagcatgtg	gtttaattcg	aagcaacgcg	cagaacctta
901	ccaacgtttg	acatgtccag	tatggtttcc	agagatggat	tccttcagtt	cggctggctg
961	gaacacaggt	gctgcatggc	tgtcgtcagc	tcgtgtcgtg	agatgttggg	ttaagtcccg
1021	caacgagcgc	aaccctcgcc	tttagttgcc	atcatttagt	tgggcactct	aaaggaaccg
1081	ccggtgataa	gccggaggaa	ggtggggatg	acgtcaagtc	ctcatggccc	ttacgcgttg
1141	ggctacacac	gtgctacaat	ggcggtgaca	atgggctgca	aacccgcgag	ggtgagctaa
1201	tctccaaaag	ccgtctcagt	tcggattgtt	ctctgcaact	cgagagcatg	aaggcggaat
1261	cgctagtaat	cgtggatcag	catgccacgg	tgaatacgtt	cccaggcctt	gtacacaccg
1321	cccgtcacac	catgggagtt	ggattcaccc	gaaggcgctg	cgctaaccgc	aaggaggcag
1381	gcgaccacgg	tgggtttagc	gactggggtg	aa		

Oligotropha carboxidovorans strain B10a/E21/ColE, 16S rRNA gene sequence (Accession N° KJ676724)

1	gagcgaacgc	tggcggcagg	cttaacacat	gcaagtcgaa	cgggcgtagc	aatacgtcag
61	tggcagacgg	gtgagtaacg	cgtgggaacg	taccttttgg	ttcggaacaa	cacagggaaa
121	cttgtgctaa	taccggataa	gcccttacgg	ggaaagattt	atcgccgaaa	gatcggcccg
181	cgtctgatta	gctagttggt	gaggtaacgg	ctcaccaagg	cgacgatcag	tagctggtct
241	gagaggatga	tcagccacat	tgggactgag	acacggccca	aactcctacg	ggaggcagca
301	gtggggaata	ttggacaatg	ggcgcaagcc	tgatccagcc	atgccgcgtg	agtgatgaag
361	gccctagggt	tgtaaagctc	ttttgtgcgg	gaagataatg	acggtaccgc	aagaataagc

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421 cccggctaac ttcgtgccag cagccgcggt aatacgaagg gggctagcgt tgctcggaat
481 cactgggcgt aaagggtgcg taggcgggtc tttaagtcag aggtgaaagc ctggagctca
541 actccagaac tgcctttgat actgaggatc ttgagttcgg gagaggtgag tggaactgcg
601 agtgtagagg tgaaattcgt agatattcgc aagaacacca gtggcgaagg cggctcactg
661 gcccgatact gacgctgagg cacgaaagcg tggggagcaa acaggattag ataccctggt
721 agtccacgcc gtaaacgatg aatgccagcc gttggaaagt ttacttttca gtggcgcagc
781 taacgcttta agcattccgc ctggggagta cggtcgcaag attaaaactc aaaggaattg
841 acgggggccc gcacaagcgg tggagcatgt ggtttaattc gacgcaacgc gcagaacctt
901 accagccctt gacatgtcca ggaccggtcg cagagatgtg accttctctt cggagcctgg
961 agcacaggtg ctgcatggct gtcgtcagct cgtgtcgtga gatgttgggt taagtcccgc
1021 aacgagcgca acccccgtcc ttagttgcta ccatttagtt gagcactcta aggagactgc
1081 cggtgataag ccgcgaggaa ggtggggatg acgtcaagtc ctcatggccc ttacgggctg
1141 ggctacacac gtgctacaat ggcggtgaca atgggatgca aaggggcgac ccctagcaaa
1201 tctcaaaaag ccgtctcagt tcggattggg ctctgcaact cgagcccatg aagttggaat
1261 cgctagtaat cgtggatcag catgccacgg tgaatacgtt cccgggcctt gtacacaccg
1321 cccgtcacac catgggagtt ggttttacct gaagacggtg cgctaacccg caagggaggc
1381 agccggccac ggtagggtca gcgactgggg tgaa

Shinella sp. strain B10b, 16S rRNA gene sequence (Accession N^{o} KJ676718)

1 aacgaacgct ggcggcaggc ttaacacatg caagtcgaac gggcatagca atatgtcagt
61 ggcagacggg tgagtaacgc gtgggaacgt accctttact acggaataac gcatggatac
121 gtgtgctaat accgtatgtg cccttcgggg gaaagattta tcggtaaagg atcggcccgc
181 gttggattag ctagttggtg gggtaaaggc ctaccaaggc gacgatccat agctggtctg
241 agaggatgat cagccacatt gggactgaga cacggcccaa actcctacgg gaggcagcag
301 tggggaatat tggacaatgg gcgcaagcct gatccagcca tgccgcgtga gtgatgaagg
361 ccctagggtt gtaaagctct ttcaccggtg aagataatga cggtaaccgg agaagaagcc
421 ccggctaact tcgtgccagc agccgcggta atacgaaggg ggctagcgtt gttcggaatt
481 actgggcgta aagcgcacgt aggcgggtat ttaagtcagg ggtgaaatcc cggagctcaa
541 ctccggaact gcctttgata ctgggtacct agagtatgga agaggtaagt ggaattccga
601 gtgtagaggt gaaattcgta gatattcgga ggaacaccag tggcgaaggc ggcttactgg
661 tccattactg acgctgaggt gcgaaagcgt ggggagcaaa caggattaga taccctggta
721 gtccacgccg taaacgatga atgttagccg tcggcatgca tgcatgtcgg tggcgcagct
781 aacgcattaa acattccgcc tggggagtac ggtcgcaaga ttaaaactca aaggaattga
841 cgggggcccg cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg cagaacctta
901 ccagcccttg acatgtcggt cgcggattac agagatgttt tccttcagtt aggctggacc
961 gaacacaggt gctgcatggc tgtcgtcagc tcgtgtcgtg agatgttggg ttaagtcccg
1021 caacgagcgc aaccctcgcc cttagttgcc agcattcagt tgggcactct aaggggactg
1081 ccggtgataa gccgagagga aggtggggat gacgtcaagt cctcatggcc cttacgggct
1141 gggctacaca cgtgctacaa tggtggtgac agtgggcagc gagacagcga tgtcgagcta
1201 acctccaaaa gccatctcag ttcggattgc actctgcaac tcgagtgcat gaagttggaa
1261 tcgctagtaa tcgcggatca gcatgccgcg gtgaatacgt tcccgggcct tgtacacacc
1321 gcccgtcaca ccatgggagt tggttttacc cgaaggcgat gcgctaaccg caaggaggca
1381 gtcgaccacg gtagggtcag cgactggggt gaa

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Achromobacter sp. strain B10c, 16S rRNA gene sequence (Accession N^{o} KJ676719)

1	gattgaacgc	tagcgggatg	ccttacacat	gcaagtcgaa	cggcagcacg	gacttcggtc
61	tggtggcgag	tggcgaacgg	gtgagtaatg	tatcggaacg	tgcccagtag	cgggggataa
121	ctacgcgaaa	gcgtagctaa	taccgcatac	gccctacggg	ggaaagcagg	ggatcgcaag
181	accttgcact	attggagcgg	ccgatatcgg	attagctagt	ggtggggta	acggctcacc
241	aaggcgacga	tccgtagctg	gtttgagagg	acgaccagcc	acactgggac	tgagacacgg
301	cccagactcc	tacgggaggc	agcagtgggg	aattttggac	aatgggggaa	accctgatcc
361	agccatcccg	cgtgtgcgat	gaaggccttc	gggttgtaaa	gcacttttgg	caggaaagaa
421	acgtcgtggg	ttaatacccc	gcgaaactga	cggtacctgc	agaataagca	ccggctaact
481	acgtgccagc	agccgcggta	atacgtaggg	tgcaagcgtt	aatcggaatt	actgggcgta
541	aagcgtgcgc	aggcggttcg	gaaagaaaga	tgtgaaatcc	cagagcttaa	ctttggaact
601	gcatttttaa	ctaccgagct	agagtgtgtc	agagggaggt	ggaattccgc	gtgtagcagt
661	gaaatgcgta	gatatgcgga	ggaacaccga	tggcgaaggc	agcctcctgg	gataacactg
721	acgctcatgc	acgaaagcgt	ggggagcaaa	caggattaga	taccctggta	gtccacgccc
781	taaacgatgt	caactagctg	ttggggcctt	cgggccttgg	tagcgcagct	aacgcgtgaa
841	gttgaccgcc	tggggagtac	ggtcgcaaga	ttaaaactca	aaggaattga	cggggacccg
901	cacaagcggt	ggatgatgtg	gattaattcg	atgcaacgcg	aaaaacctta	cctacccttg
961	acatgtctgg	aatcctgaag	agatttagga	gtgctcgcaa	gagaaccgga	acacaggtgc
1021	tgcatggctg	tcgtcagctc	gtgtcgtgag	atgttgggtt	aagtcccgca	acgagcgcaa
1081	cccttgtcat	tagttgctac	gaaagggcac	tctaatgaga	ctgccggtga	caaaccggag
1141	gaaggtgggg	atgacgtcaa	gtcctcatgg	cccttatggg	tagggcttca	cacgtcatac
1201	aatggtcggg	acagagggtc	gccaacccgc	gagggggagc	caatcccaga	aacccgatcg
1261	tagtccggat	cgcagtctgc	aactcgactg	cgtgaagtcg	gaatcgctag	taatcgcgga
1321	tcagcatgtc	gcggtgaata	cgttcccggg	tcttgtacac	accgcccgtc	acaccatggg
1381	agtgggtttt	accagaagta	gttagcctaa	ccgcaagggg	ggcgattacc	acggtaggat
1441	tcatgactgg	ggtgaa				

Sphingomonas sp. strain ColB, 16S rRNA gene sequence (Accession N^{o} KJ676720)

1	gaacgaacgc	tggcggcatg	cctaacacat	gcaagtcgaa	cgaagccttc	gggcttagtg
61	gcgcacgggt	gcgtaacgcg	tgggaatctg	cctcttgctt	cggaataaca	gttagaaatg
121	actgctaata	ccggatgatg	tcttcggacc	aaagatttat	cggcaagaga	tgagcccgcg
181	taggattagg	tagttggtgg	ggtaaaggcc	taccaagccg	acgatcctta	gctggtctga
241	gaggatgatc	agccacactg	ggactgagac	acggcccaga	ctcctacggg	aggcagcagt
301	ggggaatatt	ggacaatggg	ggcaaccctg	atccagcaat	gccgcgtgag	tgatgaaggc
361	cttagggttg	taaagctctt	ttaccaggga	tgataatgac	agtacctgga	gaataagctc
421	cggctaactc	cgtgccagca	gccgcggtaa	tacggaggga	gctagcgttg	ttcggaatta
481	ctgggcgtaa	agcgcacgta	ggcggttact	caagtcagag	gtgaaagccc	ggggctcaac
541	cccggaactg	cctttgaaac	taggtaacta	gaatcctgga	gaggtgagtg	gaattccgag
601	tgtagaggtg	aaattcgtag	atattcggaa	gaacaccagt	ggcgaaggcg	gctcactgga
661	caggtattga	cgctgaggtg	cgaaagcgtg	gggagcaaac	aggattagat	accctggtag
721	tccacgccgt	aaacgatgat	aactagctgt	ccgggttcat	ggaatttggg	tggcgcagct
781	aacgcattaa	gttatccgcc	tggggagtac	ggtcgcaaga	ttaaaactca	aaggaattga
841	cgggggcctg	cacaagcggt	ggagcatgtg	gtttaattcg	aagcaacgcg	cagaacctta
901	ccagcgtttg	acatcccgat	cgcggattac	agagatgttt	tccttcagtt	tggctggatc

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961	ggtgacaggt	gctgcatggc	tgtcgtcagc	tcgtgtcgtg	agatgttggg	ttaagtcccg
1021	caacgagcgc	aaccctcgtc	cttagttgcc	atcattaagt	tgggcactct	aaggaaaccg
1081	ccggtgataa	gccggaggaa	ggtggggatg	acgtcaagtc	ctcatggccc	ttacacgctg
1141	ggctacacac	gtgctacaat	ggcggtgaca	gtgggcagcg	acctcgcgag	gggtagctaa
1201	tctccaaaag	ccgtctcagt	tcggattgtt	ctctgcaact	cgagagcatg	aaggcggaat
1261	cgctagtaat	cgcggatcag	cgtgccgcgg	tgaatacgtt	cccaggcctt	gtacacaccg
1321	cccgtcacac	catgggagtt	ggattcactc	gaaggcgttg	agctaacccg	caagggaggc
1381	aggcgaccac	agtgggttta	gcgactgggg	tgaa		

Cupriavidus sp. strain ColD, 16S rRNA gene sequence (Accession N^{o} KJ676721)

1	gattgaacgc	tggcggcatg	ccttacacat	gcaagtcgaa	cggcagcacg	ggagcaatcc
61	tggtggcgag	tggcgaacgg	gtgagtaata	catcggaacg	gccctgtcg	tgggggataa
121	ctagtcgaaa	gattagctaa	taccgcatac	gacctgaggg	tgaaagcggg	ggaccgtaag
181	gcctcgcgcg	ataggagcgg	ccgatgtctg	attagctagt	tggtggggta	aaggcccacc
241	aaggcgacga	tcagtagctg	gtctgagagg	acgatcagcc	acactgggac	tgagacacgg
301	cccagactcc	tacgggaggc	agcagtgggg	aattttggac	aatgggggca	accctgatcc
361	agcaatgccg	cgtgtgtgaa	gaaggccttc	gggttgtaaa	gcacttttgt	ccggaaagaa
421	atcccttgcc	ctaatacggc	gggggatga	cggtaccgga	agaataagca	ccggctaact
481	acgtgccagc	agccgcggta	atacgtaggg	tgcgagcgtt	aatcggaatt	actgggcgta
541	aagcgtgcgc	aggcggtttt	gtaagacagg	cgtgaaatcc	ccgagctcaa	cttgggaatg
601	gcgcttgtga	ctgcaaggct	agagtatgtc	agaggggggt	agaattccac	gtgtagcagt
661	gaaatgcgta	gagatgtgga	ggaataccga	tggcgaaggc	agcccctgg	gacgtcactg
721	acgctcatgc	acgaaagcgt	ggggagcaaa	caggattaga	taccctggta	gtccacgccc
781	taaacgatgt	caactagttg	ttggggattc	atttcttcag	taacgtagct	aacgcgtgaa
841	gttgaccgcc	tggggagtac	ggtcgcaaga	ttaaaactca	aaggaattga	cggggacccg
901	cacaagcggt	ggatgatgtg	gattaattcg	atgcaacgcg	aaaaacctta	cctacccttg
961	acatgccact	aacgaagcag	agatgcatca	ggtgcccgaa	agggaaagtg	gacacaggtg
1021	ctgcatggct	gtcgtcagct	cgtgtcgtga	gatgttgggt	taagtcccgc	aacgagcgca
1081	acccttgtct	ttagttgcta	cgcaagagca	ctctagagag	actgccggtg	acaaaccgga
1141	ggaaggtggg	gatgacgtca	agtcctcatg	gcccttatgg	gtagggcttc	acacgtcata
1201	caatggtgcg	tacagagggt	tgccaacccg	cgaggggag	ctaatcccag	aaaacgcatc
1261	gtagtccgga	tcgtagtctg	caactcgact	acgtgaagct	ggaatcgcta	gtaatcgcgg
1321	atcagcatgc	cgcggtgaat	acgttcccgg	gtcttgtaca	caccgcccgt	cacaccatgg
1381	gagtgggttt	tgccagaagt	agttagccta	accgcaagga	gggcgattac	cacggcaggg
1441	ttcatgactg	gggtgaa				

Hyphomicrobium sp. strain ColF, 16S rRNA gene sequence (Accession N^{o} KJ676722)

1	gaacgaacgc	tggcggcagg	cctaacacat	gcaagtcgaa	cgccccgcaa	ggggagtggc
61	agacgggtga	gtaacacgtg	ggaaccttcc	ctatagtacg	gaatagccca	gggaaacttg
121	gagtaatacc	gtatacgccc	gaaaggggaa	agaatttcgc	tataggatgg	gcccgcgtag
181	gattagctag	ttggtgaggt	aatggctcac	caaggcgacg	atccttagct	ggtttgagag
241	aacgaccagc	cacactggga	ctgagacacg	gcccagactc	ctacgggagg	cagcagtggg
301	gaatattgga	caatgggcgc	aagcctgatc	cagccatgcc	gcgtgagtga	tgaaggcctt

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361 agggttgtaa agctcttttg ccggggacga taatgacggt acccggagaa taagtcccgg
421 ctaacttcgt gccagcagcc gcggtaatac gaaggggact agcgttgttc ggaatcactg
481 ggcgtaaagc gcacgtaggc ggatttgtaa gtcaggggtg aaatcccggg gctcaacctc
541 ggaactgcct ttgatactgc gaatcttgag tccgatagag gtgggtggaa ttcctagtgt
601 agaggtgaaa ttcgtagata ttaggaagaa caccggtggc gaaggcggcc cactggatcg
661 gtactgacgc tgaggtgcga aagcgtgggg agcaaacagg attagatacc ctggtagtcc
721 acgccgtaaa cgatggatgc tagccgtcgg atagcttgct attcggtggc gcagctaacg
781 cattaagcat cccgcctggg gagtacggcc gcaaggttaa aactcaaagg aattgacggg
841 ggcccgcaca agcggtggag catgtggttt aattcgacgc aacgcgaaga accttaccag
901 ctcttgacat tcactgatcg cctggagaga tctgggaatt ccagcaatgg acagtgggac
961 aggtgctgca tggctgtcgt cagctcgtgt cgtgagatgc tgggttaagt cccgcaacga
1021 gcgcaaccct cgccattagt tgccatcatt cagttgggca ctctagtggg actgccggtg
1081 ataagccgga ggaaggtggg gatgacgtca agtcatcatg gcccttacgg gctgggctac
1141 acacgtgcta caatggcggt gacaatgcgc agccacctag caatagggag ctaatcgcaa
1201 aaagccgtct cagttcagat tgaggtctgc aactcgacct catgaagtcg gaatcgctag
1261 taatcgcgca tcagcatggc gcggtgaata cgttcccggg ccttgtacac accgcccgtc
1321 acaccatggg agttggtctt accctaaaac ggtgcgctaa ccgcaaggag gcagccggcc
1381 acggtaaggt cagcgactgg ggtg

3.2. *Ortho*-phenyl-phenol (Chapter 5)

The partial 16S rRNA gene sequences of the isolated degrader *S. haloaromaticamans* strain P3 and of the two other main member of the community *P. stutzeri* strain P1 and *S. novella* strain P10 are reported (see Section 3.3).

Sphingomonas haloaromaticamans strain P3, 16S rRNA gene sequence (Accession N^{o} KJ676712)

1 ttcaccccag tcgctaaacc caccgtggtc gcctgcctcc cttgcgggtt agcgcagcgc
61 cttcgggtga atccaactcc catggtgtga cgggcggtgt gtacaaggcc tgggaacgta
121 ttcaccgcgg catgctgatc cgcgattact agcgattccg ccttcatgct ctcgagttgc
181 agagaacaat ccgaactgag acaacttttg gagattagct caccctcgcg agttcgctgc
241 ccactgtagt tgccattgta gcacgtgtgt agcccaacgc gtaagggcca tgaggacttg
301 acgtcatccc caccttcctc cggcttatca ccggcggttc ctttagagtg cccaacttaa
361 tgatggcaac taaaggcgag ggttgcgctc gttgcgggac ttaacccaac atctcacgac
421 acgagctgac gacagccatg cagcacctgt cacctatcca gccgaactga aggaaagtgt
481 ctccactaac cgcgataggg atgtcaaacg ttggtaaggt tctgcgcgtt gcttcgaatt
541 aaaccacatg ctccaccgct tgtgcaggcc cccgtcaatt tctttgagtt ttaaccttgc
601 ggccgtactc cccaggcgga taacttaatg cgttagctgc gccacccaac caccaagtgc
661 ccggacagct agttatcatc gtttacggcg tggactacca gggtatctaa tcctgtttgc
721 tccccacgct ttcgcacctc agcgtcaaca gtcgtccagt gagccgcctt cgccactggt
781 gttcttccga atatctacga atttcacctc tacactcgga attccactca cctctccgac
841 gttcaagcga ggcagtctta aaggctattc cggagttgag ctccgggctt tcacctctaa
901 cttaccaagc cgcctacgtg cgctttacgc ccagtaattc cgaacaacgc tagccccctc
961 cgtattaccg cggctgctgg cacggagtta gccggggctt attctcccgg tacagtcatt

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1021	atcttcccgg	gtaaaagagc	tttacaaccc	tagggccttc	atcactcacg	cggcattgct
1081	ggatcaggct	ttcgcccatt	gtccaatatt	ccccactgct	gcctcccgta	ggagtctggg
1141	ccgtgtctca	gtcccagtgt	ggctgatcat	cctctcagac	cagctaagga	tcgtcgcctt
1201	ggtgggcctt	taccccacca	actagctaat	cctacgcggg	ctcatccttt	ggcgataaat
1261	ctttggtctc	gcgacatcat	acggtattag	cagtaatttc	tcactgttat	tccgtaccaa
1321	agggcagatt	cccacgcgtt	acgcacccgt	gcgccactaa	ggccgaagcc	ttcgttcgac
1381	ttgcatgtgt	taggcatgcc	gccagcgttc	gttc		

Pseudomonas stutzeri strain P1, 16S rRNA gene sequence (Accession N^{o} KJ676713)

1	tgaacgctgg	cggcaggcct	aacacatgca	agtcgagcgg a	tgaagagag	cttgctctct
61	gattcagcgg	cggacgggtg	agtaatgcct	aggaatctgc	ctggtagtgg	gggacaacgt
121	ttcgaaagga	acgctaatac	cgcatacgtc	ctacgggaga	aagtggggga	tcttcggacc
181	tcacgctatc	agatgagcct	aggtcggatt	agctagttgg	cgaggtaaag	gctcaccaag
241	gcgacgatcc	gtaactggtc	tgagaggatg	atcagtcaca	ctggaactga	gacacggtcc
301	agactcctac	gggaggcagc	agtggggaat	attggacaat	gggcgaaagc	ctgatccagc
361	catgccgcgt	gtgtgaagaa	ggtcttcgga	ttgtaaagca	ctttaagttg	ggaggaaggg
421	agtaagtta	ataccttgct	gttttgacgt	taccgacaga	ataagcaccg	gctaacttcg
481	tgccagcagc	cgcggtaata	cgaagggtgc	aagcgttaat	cggaattact	gggcgtaaag
541	cgcgcgtagg	tggttcgtta	agttggatgt	gaaagccccg	ggctcaacct	gggaactgca
601	tccaaaactg	gcgagctaga	gtatggcaga	gggtggtgga	atttcctgtg	tagcggtgaa
661	atgcgtagat	ataggaagga	acaccagtgg	cgaaggcgac	cacctgggct	aatactgaca
721	ctgaggtgcg	aaagcgtggg	gagcaaacag	gattagatac	cctggtagtc	cacgccgtaa
781	acgatgtcga	ctagccgttg	ggatccttga	gatcttagtg	gcgcagctaa	cgcattaagt
841	cgaccgcctg	gggagtacgg	ccgcaaggtt	aaaactcaaa	tgaattgacg	ggggcccgca
901	caagcggtgg	agcatgtggt	ttaattcgaa	gcaacgcgaa	gaaccttacc	aggccttgac
961	atgctgagaa	cctgccagag	atggcggggt	gccttcggga	actcagacac	aggtgctgca
1021	tggctgtcgt	cagctcgtgt	cgtgagatgt	tgggttaagt	cccgtaacga	gcgcaaccct
1081	tgtccttagt	taccagcacg	ttatggtggg	cactctaagg	agactgccgg	tgacaaaccg
1141	gaggaaggtg	gggatgacgt	caagtcatca	tggcccttac	ggcctgggct	acacacgtgc
1201	tacaatggtc	ggtacaaagg	gttgccaagc	cgcgaggtgg	agctaatccc	ataaaaccga
1261	tcgtagtccg	gatcgcagtc	tgcaactcga	ctgcgtgaag	tcggaatcgc	tagtaatcgt
1321	gaatcagaat	gtcacggtga	atacgttccc	gggccttgta	cacaccgccc	gtcacaccat
1381	gggagtgggt	tgctccagaa	gtagctagtc	taaccttcgg	ggggacggtt	accacggagt
1441	gattcatgac	tggggt				

Starkeya novella strain P10, 16S rRNA gene sequence (Accession N^{o} KJ676714)

1	gaacgaacgc	tggcggcagg	cttaacacat	gcaagtcgaa	cgcaccgcaa	ggtgagtggc
61	agacgggtga	gtaacacgtg	gggatctgcc	caatggtacg	gaatagctcc	gggaaactgg
121	aattaatacc	gtatgagccc	gcaaggggaa	agatttatcg	ccattggatg	aacccgcgtc
181	ggattagcta	gttggtgtgg	taaaggcgca	ccaaggcgac	gatccgtagc	tggtctgaga
241	ggatgatcag	ccacactggg	actgagacac	ggcccagact	cctacgggag	gcagcagtgg
301	ggaatattgg	acaatgggcg	caagcctgat	ccagccatgc	cgcgtgagtg	atgaaggcct
361	tagggttgta	aagctctttc	gccgacgaag	ataatgacgg	tagtcggaga	agaagccccg

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421	gctaacttcg	tgccagcagc	cgcggtaata	cgaagggggc	tagcgttgtt	cggaatcact
481	cagaactgcc	cttgatactg	gcaatctcga	gtccggaaga	ggtaagtgga	actgcgagtg
601	tagaggtgaa	attcgtagat	attcgcaaga	acaccagtgg	cgaaggcggc	ttactggtcc
661	ggtactgacg	ctgaggtgcg	aaagcgtggg	gagcaaacag	gattagatac	cctggtagtc
721	cacgccgtaa	acgatggagg	ctagccgttg	gtgagcatgc	tcatcagtgg	cgcagctaac
781	gcattaagcc	tcccgcctgg	ggagtacggt	cgcaagatta	aaactcaaag	gaattgacgg
841	gggcccgcac	aagcggtgga	gcatgtggtt	taattcgaag	caacgcgcag	aaccttacca
901	gcctttgaca	tgccccggaa	ttggatcaga	gatgaaccaa	gctcttcgga	gccggggaca
961	caggtgctgt	atggctgtcg	tcagctcgtg	tcgtgagatg	ttgggttaag	tcccgcaacg
1021	agcgcaaccc	tcgcccttag	ttgccatcat	tcagttgggc	actctagggg	gactgccggt
1081	gataagccga	gaggaaggtg	gggatgacgt	caagtcctca	tggcccttac	gggctgggct
1141	acacacgtgc	tacaatggcg	gtgacagtgg	gacgcgaacc	cgcgagggtg	agcaaatctc
1201	caaaagccgt	ctcagttcgg	attgcactct	gcaactcgag	tgcatggagt	tggaatcgct
1261	agtaatcgtg	gatcagcacg	ccacggtgaa	tacgttcccg	ggccttgtac	acaccgcccg
1321	tcacaccatg	ggagttggct	ttacccgaag	gcgctgcgct	aacccgcaag	ggaggcaggc
1381	gaccacggta	gggtcagcga	ctggggt			

3.3. Diphenylamine (Chapter 6)

The four polymorphic 16S rRNA gene sequences of the isolated DPA degrader *P. monteilii* strain DPA are listed (see Section 3.1 of the Chapter).

Pseudomonas monteilii strain DPA_a, 16S rRNA gene sequence (Accession N^{o} KJ676706)

1	gattgaacgc	tggcggcagg	cctaacacat	gcaagtcgag	cggatgacgg	gagcttgctc
61	cttgattcag	cggcggacgg	gtgagtaatg	cctaggaatc	tgcctggtag	tgggggacaa
121	cgtttcgaaa	ggaacgctaa	taccgcatac	gtcctacggg	agaaagcagg	ggaccttcgg
181	gccttgcgct	atcagatgag	cctaggtcgg	attagctagt	tggtggggta	atggctcacc
241	aaggcgacga	tccgtaactg	gtctgagagg	atgatcagtc	acactggaac	tgagacacgg
301	tccagactcc	tacgggaggc	agcagtgggg	aatattggac	aatgggcgaa	agcctgatcc
361	agccatgccg	cgtgtgtgaa	gaaggtcttc	ggattgtaaa	gcactttaag	ttgggaggaa
421	gggcagtaag	ctaatacctt	gctgttttga	cgttaccgac	agaataagca	ccggctaact
481	ctgtgccagc	agccgcggta	atacagaggg	tgcaagcgtt	aatcggaatt	actgggcgta
541	aagcgcgcgt	aggtggtttg	ttaagttgga	tgtgaaagcc	ccgggctcaa	cctgggaact
601	gcatccaaaa	ctggcaagct	agagtacggt	agagggtggt	ggaatttcct	gtgtagcggt
661	gaaatgcgta	gatataggaa	ggaacaccag	tggcgaaggc	gaccacctgg	actgatactg
721	acactgaggt	gcgaaagcgt	ggggagcaaa	caggattaga	taccctggta	gtccacgccg
781	taaacgatgt	caactagccg	ttggaatcct	tgagatttta	gtggcgcagc	taacgcatta
841	agttgaccgc	ctggggagta	cggccgcaag	gttaaaactc	aaatgaattg	acgggggccc
901	gcacaagcgg	tggagcatgt	ggtttaattc	gaagcaacgc	gaagaacctt	accaggcctt
961	gacatgcaga	gaactttcta	gagatggatt	ggtgccttcg	ggaactctga	cacaggtgct
1021	gcatggctgt	cgtcagctcg	tgtcgtgaga	tgttgggtta	agtcccgtaa	cgagcgcaac
1081	ccttgtcctt	agttaccagc	acgttatggt	gggcactcta	aggagactgc	cggtgacaaa
1141	ccggaggaag	gtggggatga	cgtcaagtca	tcatggccct	tacggcctgg	gctacacacg
1201	tgctacaatg	gtcggtacag	agggttgcca	agccgcgagg	tggagctaat	ctcacaaaac

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1261	cgatcgtagt	ccggatcgca	gtctgcaact	cgactgcgtg	aagtcggaat	cgctagtaat
1321	cgcgaatcag	aatgtcgcgg	tgaatacgtt	cccgggcctt	gtacacaccg	cccgtcacac
1381	catgggagtg	ggttgcacca	gaagtagcta	gtctaacctt	cgggaggacg	gttaccacgg
1441	tgtgattcat	gactggggtg	aa			

Pseudomonas monteilii strain DPA_b, 16S rRNA gene sequence (Accession N^{o} KJ676707)

1	gattgaacgc	tggcggcagg	cctaacacat	gcaagtcgag	cggatgacgg	gagcttgctc
61	cttgattcag	cggcggacgg	gtgagtaatg	cctaggaatc	tgcctggtag	tgggggacaa
121	cgtttcgaaa	ggaacgctaa	taccgcatac	gtcctacggg	agaaagcagg	ggaccttcgg
181	gccttgcgct	atcagatgag	cctaggtcgg	attagctagt	tggtggggta	atggctcacc
241	aaggcgacga	tccgtaactg	gtctgagagg	atgatcagtc	acactggaac	tgagacacgg
301	tccagactcc	tacgggaggc	agcagtgggg	aatattggac	aatgggcgaa	agcctgatcc
361	agccatgccg	cgtgtgtgaa	gaaggtcttc	ggattgtaaa	gcactttaag	ttgggaggaa
421	gggcagtaag	ttaatacctt	gctgttttga	cgttaccgac	agaataagca	ccggctaact
481	ctgtgccagc	agccgcggta	atacagaggg	tgcaagcgtt	aatcggaatt	actgggcgta
541	aagcgcgcgt	aggtggtttg	ttaagttgga	tgtgaaagcc	ccgggctcaa	cctgggaact
601	gcatccaaaa	ctggcaagct	agagtacggt	agagggtggt	ggaatttcct	gtgtagcggt
661	gaaatgcgta	gatataggaa	ggaacaccag	tggcgaaggc	gaccacctgg	actgatactg
721	acactgaggt	gcgaaagcgt	ggggagcaaa	cagggattag	ataccctggt	agtccacgcc
781	gtaaacgatg	tcaactagcc	gttggaatcc	ttgagatttt	agtggcgcag	ctaacgcatt
841	aagttgaccg	cctggggagt	acggccgcaa	ggttaaaact	caaatgaatt	gacgggggcc
901	cgcacaagcg	gtggagcatg	tggtttaatt	cgaagcaacg	cgaagaacct	taccaggcct
961	tgacatgcag	agaactttcc	agagatggat	tggtgccttc	gggaactctg	acacaggtgc
1021	tgcatggctg	tcgtcagctc	gtgtcgtgag	atgttgggtt	aagtcccgta	acgagcgcaa
1081	cccttgtcct	tagttaccag	cacgttatgg	tgggcactct	aaggagactg	ccggtgacaa
1141	accggaggaa	ggtggggatg	acgtcaagtc	atcatggccc	ttacggcctg	ggctacacac
1201	gtgctacaat	ggtcggtaca	gagggttgcc	aagccgcgag	gtggagctaa	tctcacaaaa
1261	ccgatcgtag	tccggatcgc	agtctgcaac	tcgactgcgt	gaagtcggaa	tcgctagtaa
1321	tcgcgaatca	gaatgtcgcg	gtgaatacgt	tcccgggcct	tgtacacacc	gcccgtcaca
1381	ccatgggagt	gggttgcacc	agaagtagct	agtctaacct	tcggggggac	ggttaccacg
1441	gtgtgattca	tgactggggt	gaa			

Pseudomonas monteilii strain DPA_c, 16S rRNA gene sequence (Accession N^{o} KJ676708)

1	gattgaacgc	tggcggcagg	cctaacacat	gcaagtcgag	cggatgacgg	gagcttgctc
61	cttgattcag	cggcggacgg	gtgagtaatg	cctaggaatc	tgcctggtag	tgggggacaa
121	cgtttcgaaa	ggaacgctaa	taccgcatac	gtcctacggg	agaaagcagg	ggaccttcgg
181	gccttgcgct	atcagatgag	cctaggtcgg	attagctagt	tggtggggta	atggctcacc
241	aaggcgacga	tccgtaactg	gtctgagagg	atgatcagtc	acactggaac	tgagacacgg
301	tccagactcc	tacgggaggc	agcagtgggg	aatattggac	aatgggcgaa	agcctgatcc
361	agccatgccg	cgtgtgtgaa	gagggtcttc	ggattgtaaa	gcactttaag	ttgggaggaa
421	gggcagtaag	ttaatacctt	gctgttttga	cgttaccgac	agaataagca	ccggctaact
481	ctgtgccagc	agccgcggta	atacagaggg	tgcaagcgtt	aatcggaatt	actgggcgta
541	aagcgcgcgt	aggtggtttg	ttaagttgga	tgtgaaagcc	ccgggctcaa	cctgggaact

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601	gcatccaaaa	ctggcaagct	agagtacggt	agagggtggt	ggaatttcct	gtgtagcggt
661	gaaatgcgta	gatataggaa	ggaacaccag	tggcgaaagc	gaccacctgg	actgatactg
721	acactgaggt	gcgaaagcgt	ggggagcaaa	caggattaga	taccctggta	gtccacgccg
781	taaacgatgt	caactagccg	ttggaatcct	tgagatttta	gtggcgcagc	taacgcatta
841	agttgaccgc	ctggggagta	cggccgcaag	gttaaaactc	aaatgaattg	acgggggccc
901	gcacaagcgg	tggagcatgt	ggtttaattc	gaagcaacgc	gaagaacctt	accaggcctt
961	gacatgcaga	gaactttcca	gagatggatt	ggtgccttcg	ggaactctga	cacaggtgct
1021	gcatggctgt	cgtcagctcg	tgtcgtgaga	tgttgggtta	agtcccgtaa	cgagcgcaac
1081	ccttgtcctt	agttaccagc	actcaagtca	tcatggccct	tacggcctgg	gctacacacg
1141	tgctacaatg	gtcggtacag	agctgcaact	cgactgcgtg	aagtcggaat	cgctagtaat
1201	cgcgaatcag	aatgtcgcgg	tgaatacgtt	cccgggcctt	gtacacaccg	cccgtcacac
1261	catgggagtg	ggttgcacca	gaagtagcta	gtctaacctt	cgggaggacg	gttaccacgg
1321	tgtgattcat	gactggggtg	aa			

Pseudomonas monteilii strain DPA_d, 16S rRNA gene sequence (Accession N^{o} KJ676709)

1	gattgaacgc	tggcggcagg	cctaacacat	gcaagtcgag	cggatgacgg	gagcttgctc
61	cttgattcag	cggcggacgg	gtgagtaatg	cctaggaatc	tgcctggtag	tgggggacaa
121	cgtttcgaaa	ggaacgctaa	taccgcatac	gtcctacggg	agaaagcagg	ggaccttcgg
181	gccttgcgct	atcagatgag	cctaggtcgg	attagctagt	tggtggggta	atggctcacc
241	aaggcgacga	tccgtaactg	gtctgagagg	atgatcagtc	acactggaac	tgagacacgg
301	tccagactcc	tacgggaggc	agcagtgggg	aatattggac	aatgggcgaa	agcctgatcc
361	agccatgccg	cgtgtgtgaa	gaaggtcttc	ggattgtaaa	gcactttaag	ttgggaggaa
421	gggcagtaag	ctaatacctt	gctgttttga	cgttaccgac	agaataagca	ccggctaact
481	aagcgcgcgt	aggtggtttg	ttaagttgga	tgtgaaagcc	ccgggctcaa	cctgggaact
541	gcatccaaaa	ctggcaagct	agagtacggt	agagggtggt	ggaatttcct	gtgtagcggt
601	gaaatgcgta	gatataggaa	ggaacaccag	tggcgaaggc	gaccacctgg	actgatactg
661	acactgaggt	gcgaaagcgt	ggggagcaaa	caggattaga	taccctggta	gtccacgccg
721	taaacgatgt	caactagccg	ttggaatcct	tgagatttta	gtggcgcagc	taacgcatta
781	agttgaccgc	ctggggagta	cggccgcaag	gttaaaactc	aaatgaattg	acgggggccc
841	gcacaagcgg	tggagcatgt	ggtttaattc	gaagcaacgc	gaagaacctt	accaggcctt
901	gacatgcaga	gaactttcca	gagatggatt	ggtgccttcg	ggaactctga	cacaggtgct
961	gcatggctgt	cgtcagctcg	tgtcgtgaga	tgttgggtta	agtcccgtaa	cgagcgcaac
1021	ccttgtcctt	agttaccagc	acgttatggt	gggcactcta	aggagactgc	cggtgacaaa
1081	ccggaggaag	gtggggatga	cgtcaagtca	tcatggccct	tacggcctgg	gctacacacg
1141	tgctacaatg	gtcggtacag	agggttgcca	agccgcgagg	tggagctaat	ctcacaaaac
1201	cgatcgtagt	ccggatcgca	gtctgcaact	cgactgcgtg	aagtcggaat	cgctagtaat
1261	cgcgaatcag	aatgtcgcgg	tgaatacgtt	cccgggcctt	gtacacaccg	cccgtcacac
1321	catgggagtg	ggttgcacca	gaagtagcta	gtctaacctt	cgggaggacg	gttaccacgg
1381	tgtgattcat	gactggggtg	aa			

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The sequences of the *gyrB* and *rpoD* genes of the isolated *P. monteilii* are also reported (see Section 3.2 of Chapter 6).

Pseudomonas monteilii strain DPA, *gyrB* gene sequence (Accession N^{o} KJ676710)

1	cgacaactcc	tacaaagtat	ccggcggtct	gcacggtgta	ggtgtgtcgg	ttgtgaacgc
61	cctgtccgag	aagctggttc	tgaccgttcg	ccgtagcggc	aagatctggg	aacagactta
121	cgttcacggt	gttccacaag	cgcctatggc	ggttgtcggt	gacagtgaaa	ccacgggtac
181	ccacatccac	ttcaagccat	cggctgaaac	cttcaaaaac	attcacttca	gctgggacat
241	cctggccaag	cgcatccgcg	agctgtcgtt	ccttaactcg	ggcgttggca	ttctgctgaa
301	ggacgagcgc	agcggtaagg	aagagttctt	caagtacgaa	ggcggtctgc	gtgcgttcgt
361	cgagtacttg	aacaccaaca	agacgccggt	caactcccag	gtgttccact	tcaacgttca
421	gcgtgacgat	ggcgtgggtg	ttgaagtcgc	cctgcaatgg	aacgacagct	tcaacgaaaa
481	cctgctgtgc	tttaccaaca	atattccgca	gcgtgatggc	ggtacccacc	tggtgggctt
541	ccgttcctcg	ctgacccgta	gccttaacag	ctacatcgag	caggaaggcc	tggccaagaa
601	gaacaaggtg	gcaaccactg	gcgacgacgc	ccgtgaaggc	ctgaccgcaa	tcatctcggt
661	gaaggtaccg	gacccgaagt	tcagctcgca	gaccaaggac	aagctggtct	cctcggaggt
721	gaaaaccgcc	gtggaacagg	agatgaacaa	gtacttcgcc	gatttcctcc	tggaaaaccc
781	gaacgaggcg	aaggccgtcg	ttggcaagat	gatcgacgcg	gctcgcgccc	gtgaagccgc
841	ccgtaaagcc	cgtgagatga	cccgccgtaa	aggtgcgctg	gatatcgcgg	gtctgccggg
901	caagctggcc	gac				

Pseudomonas monteilii strain DPA, *rpoD* gene sequence (Accession N^{o} KJ676711)

1	tctcggcaac	ggtcaggccg	gtttcggtct	caaggtcgat	cagcttctgt	tggcaagcga
61	cgatggcagc	gttcttttca	cccagggcgg	cagcccactt	ggtgttgcgc	ttggccaggt
121	caccgctcca	ggtctggtcg	gtttcgttgc	ttgggaacat	gcgcaggaag	tcggctcgcg
181	gcatgcgggc	gtcacgtacg	cagagctgca	tgatggcgcg	ttcttgctgc	cgcagacggt
241	tcagggcatc	acgtacacgc	tcgaccagta	cctcgaactg	cttcggtacc	agcttgatcg
301	gcatgaacag	gtcagccagg	gcctgcaggg	cctcgatgct	ttccttgtgg	ttacgaccat
361	ttttcttcag	gaccttggag	gtcgcctgaa	gctgatcgga	tactgcaccg	aagcgttggg
421	ctgcgactac	cgggtccggg	ccgctctcgg	cctcttcctc	gtcgtcaccg	ctttcggatt
481	cttcttcgtc	gtcgtcggac	tcttccttcg	ccgcagcggc	cttggcgcct	gggatcggca
541	cttcttcggt	tggcgcggca	atgttgtcgt	cagggtcgat	gtaaccgctg	agaacgtccg
601	acaggcggcc	accctcggtg	gtgacgcgat	catattcgcc	gagaatgtag	tcgacagtgc
661	ccgggaagtg	ggcgatagcg	cccatgactt	cacgaatgcc	ttcctcgat	

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