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Isolation of bacteria that degrade carbamate insecticides and characterization of the functional and ecological role of bacterial genes involved in their hydrolysis in soil

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Isolation of bacteria that degrade carbamate insecticides and characterization of the functional and ecological role of bacterial genes involved in their hydrolysis in soil

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To my family

A Ship in Harbor Is Safe,

But that Is Not What Ships Are Built For

John A. Shedd

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Abstract

Synthetic carbamates constitute a significant pesticide group with oxamyl being a leading compound in the insecticide/nematicide market. Microbial degradation constitutes one of the main processes controlling the environmental dissipation of oxamyl. Paradoxically, microbial degradation, which was initially viewed as a desirable process for reducing environmental hazards, has turned into a double-edged sword with the development of the phenomenon of enhanced microbial degradation, which under conducive condition can lead to loss of the biological efficacy of pesticides. Considering the importance of biodegradation in the environmental fate and efficacy of soil-applied carbamate insecticides we aimed (1) to isolate and identify bacteria degrading the carbamate oxamyl and to characterize the genes involved in its transformation, (2) to explore the ecology, distribution and function of carbamate hydrolase genes in soils and (3) to get insights into their origin and evolution

As a source for the isolation of oxamyl-degrading bacteria we used a soil from a commercial banana plantation located in the area of Sitia, northeast Crete, in Greece with history of previous treatment with oxamyl. A rapid microbially driven hydrolysis of oxamyl to oxamyl oxime was observed in the studied soil which is in line with the reduced biological efficacy of oxamyl in the given field. Subsequent enrichment cultures inoculated with the studied soil resulted in the isolation of four oxamyl-degrading bacterial strains which identified, based on multilocus sequence analysis (MLSA), as *Pseudomonas*. The isolates were able to metabolize oxamyl to oxamyl oxime which was not further transformed by our strains in contrast to its gradual dissipation in soil. Soil sterilization resulted in a complete halting of the degradation of oxamyl oxime suggesting that its transformation was biologically driven. However, our repeated attempts to isolate oxamyl oxime degraders following the same enrichment cultures method failed, suggesting that its transformation in soil is probably a co-metabolic process performed by non-specialized soil bacteria or fungi. All the isolated strains carried the carbamate-hydrolase gene *cehA* which was shown, via transcription analysis, to be responsible for the hydrolysis of oxamyl. Our isolates were able to utilize the methyl carbamate moiety (released during hydrolysis of oxamyl) as a C and N source, in agreement with the high mineralization levels of the

^{14}C-carbomoyl-labelled oxamyl by all isolates, and their capacity to grow on methylamine, that is released from the decomposition of the unstable methyl-carbamate moiety.

We extended our investigations from the *in vitro* bacterial hydrolysis of oxamyl to the role of the soil microbiota in the *in situ* biodegradation of carbamates in soils. We studied the degradation of oxamyl and we determined the abundance of the three most studied carbamate hydrolase genes *cehA*, *mcd* and *cahA* in 16 soils from a potato monoculture area in Greece where oxamyl is regularly used. Oxamyl showed low persistence (DT_{50} = 2.4-26.7 days) and qPCR detected the *cehA* and *mcd* genes in 10 and three of the studied soils, respectively. The abundance of the *cehA* gene was positively correlated with pH, while both *cehA* abundance and pH were negatively correlated with oxamyl DT_{50}. In light of the detection of *mcd* in the studied soils, despite the absence of carbofuran, its main substrate which has been banned and has not been used in the studied region for over 10 years, we tested the hypothesis that other carbamates used in the region might serve as substrates for *mcd*. None of the alternative carbamates tested increased the abundance of *cehA* and *mcd* apart from (i) oxamyl which stimulated the abundance and expression only of the *cehA* gene and (ii) carbofuran that stimulated the abundance and expression of both genes suggesting an interesting catabolic functional redundancy first time reported for carbamates. The *cehA* gene was also detected in pristine soils upon repeated treatments with oxamyl and carbofuran and only in soils with pH $\geq$ 7.2, where the most rapid degradation of oxamyl was observed. The occurrence of *cehA* gene in agricultural and pristine soils suggested its widespread distribution that could be a result of a parallel evolutionary mechanism from a common ancestor, probably involved in the detoxification of natural carbamate soil compounds produced by soil microorganisms and plant roots.

Overall, we studied the degradation of oxamyl by oxamyl-degrading bacteria and its biodegradation in soils. We reported the isolation and identification of four oxamyl-degrading *Pseudomonas* strains and we determined the microbial transformation pathway of oxamyl. All isolates carried the *cehA* gene which was shown to be responsible for the hydrolysis of oxamyl. Soil microcosm studies further reinforced the role of *cehA* gene in the biodegradation of oxamyl, whereas both the *cehA* and *mcd* genes are involved in the biodegradation of carbofuran. The detection

of the *cehA* gene in agricultural and pristine soils, after oxamyl and carbofuran application suggests its widespread occurrence and stresses the significant role of pH as a driver of the distribution of the *cehA* in soils. These results have major implications regarding the maintenance of carbamate hydrolase genes in soils, have practical applications regarding the agricultural use of carbamates and provide insights into the evolution of *cehA* gene.

Περίληψη

Τα συνθετικά καρβαμιδικά αποτελούν μια σημαντική ομάδα γεωργικών φαρμάκων με το oxamyl να είναι ο κύριος εκπρόσωπος τους σήμερα στην αγορά εντομοκτόνων/νηματοδοκτόνων. Η μικροβιακή αποδόμηση αποτελεί μία από τις κύριες διεργασίες που ελέγχει την περιβαλλοντική τύχη του oxamyl. Παραδόξως, η μικροβιακή αποδόμηση, η οποία αρχικά θεωρήθηκε ως μια επιθυμητή διεργασία για τη μείωση των περιβαλλοντικών κινδύνων, έχει μετατραπεί σε δίκοπο μαχαίρι με την ανάπτυξη του φαινομένου της επιταχυνόμενης μικροβιακής αποδόμησης, η οποία υπό ευνοϊκές συνθήκες μπορεί να οδηγήσει σε απώλεια της αποτελεσματικότητας των γεωργικών φαρμάκων. Λαμβάνοντας υπόψη τη σημασία της βιοαποδόμησης στην περιβαλλοντική τύχη και στην αποτελεσματικότητα των καρβαμιδικών εντομοκτόνων που εφαρμόζονται στο έδαφος στοχεύσαμε (1) στην απομόνωση και ταυτοποίηση βακτηρίων που αποδομούν το καρβαμιδικό oxamyl και στον χαρακτηρισμό των γονιδίων που εμπλέκονται στη αποδόμησή του, (2) στην διερεύνηση της οικολογίας, της κατανομής και της λειτουργίας των γονιδίων που κωδικοποιούν υδρολάσες των καρβαμιδικών σε γεωργικά εδάφη και (3) στη διερεύνηση της προέλευσης και των μηχανισμών εξέλιξης των συγκεκριμένων γονιδίων.

Ως πηγή για την απομόνωση βακτηρίων που αποδομούν το oxamyl χρησιμοποιήσαμε έδαφος από μια εμπορική καλλιέργεια μπανάνας που βρίσκεται στην περιοχή της Σητείας, βορειοανατολικά της Κρήτης, στην Ελλάδα με ιστορικό προηγούμενων εφαρμογών oxamyl. Παρατηρήθηκε ταχύτατη μικροβιακή υδρόλυση του oxamyl σε oxamyl oxime στο υπό μελέτη έδαφος η οποία είναι σε συμφωνία με την μειωμένη αποτελεσματικότητα του oxamyl στον συγκεκριμένο αγρό. Επακόλουθες καλλιέργειες εμπλουτισμού εμβολιασμένες με το υπό μελέτη έδαφος οδήγησαν στην απομόνωση τεσσάρων βακτηριακών στελεχών που αποδομούν το oxamyl τα οποία ταυτοποιήθηκαν, με βάση την ανάλυση αλληλουχίας πολλαπλών γενετικών τόπων (MLSA), ως *Pseudomonas*. Τα απομονωθέντα βακτήρια ήταν ικανά να μεταβολίζουν το oxamyl σε oxamyl oxime το οποίο δεν μετασχηματίστηκε περαιτέρω από τα στελέχη μας σε αντίθεση με την σταδιακή του αποδόμηση του στο έδαφος. Η αποστείρωση του εδάφους είχε ως αποτέλεσμα την πλήρη ανάσχεση της αποδόμησης του oxamyl oxime υποδηλώνοντας ότι ο μετασχηματισμός του ήταν βιολογικά εξαρτώμενος. Ωστόσο, οι επαναλαμβανόμενες προσπάθειες μας να

απομονώσουμε βακτήρια που διασπούν το oxamyl oxime ακολουθώντας την ίδια μέθοδο εμπλουτισμένων καλλιεργειών απέτυχαν, υποδηλώνοντας ότι ο μετασχηματισμός του στο έδαφος είναι πιθανώς μια συμμεταβολική διεργασία που εκτελείται από μη εξειδικευμένα βακτήρια ή μύκητες. Όλα τα απομονωθέντα βακτηριακά στελέχη έφεραν το γονίδιο *cehA* που κωδικοποιεί μια καρβαμιδική υδρολάση και το οποίο δείχθηκε, μέσω μεταγραφικής ανάλυσης, να είναι υπεύθυνο για την υδρόλυση του oxamyl. Τα απομονωθέντα στελέχη μπορούσαν να χρησιμοποιούν την ομάδα του μεθυλοκαρβαμικού οξέος (που απελευθερώνεται κατά την διάρκεια της υδρόλυσης του oxamyl) ως πηγή C και N, σε συμφωνία με τα υψηλά επίπεδα ανοργανοποίησης του σημασμένου oxamyl με ^{14}C στο καρβαμικό τμήμα από όλα τα στελέχη, και την ικανότητά τους να αναπτύσσονται σε μεθυλαμίνη, η οποία ελευθερώνεται από την διάσπαση της ασταθούς ομάδας του μεθυλοκαρβαμικού οξέος.

Επεκτείναμε τις έρευνές μας από την *in vitro* βακτηριακή υδρόλυση του oxamyl στο ρόλο της μικροβιακής κοινότητας του εδάφους στην *in situ* βιοδιάσπαση των καρβαμιδικών στο έδαφος. Μελετήσαμε την διάσπαση του oxamyl και καθορίσαμε την αφθονία των τριών πιο μελετημένων γονιδίων που κωδικοποιούν υδρολάσες καρβαμιδικών *cehA, mcd* και *cahA* σε 16 εδάφη από μια περιοχή μονοκαλλιέργειας πατάτας στην Ελλάδα, όπου το oxamyl χρησιμοποιείται συχνά. Το oxamyl έδειξε χαμηλή υπολειμματικότητα (DT_{50} = 2.4-26.7 ημέρες) και μέσω qPCR ανιχνεύσαμε τα γονίδια *cehA* και *mcd* σε 10 και 3 από τα εδάφη που μελετήθηκαν, αντίστοιχα. Η αφθονία του *cehA* γονιδίου εμφάνισε σημαντική θετική συσχέτιση με το pH, ενώ και η αφθονία του *cehA* και το pH εμφάνισαν σημαντική αρνητική συσχέτιση με το DT_{50} του oxamyl. Λαμβάνοντας υπόψη την ανίχνευση του *mcd* στα μελετούμενα εδάφη, παρά την απουσία του carbofuran, του κυριότερου υποστρώματός του, του οποίου η χρήση έχει απαγορευθεί και δεν χρησιμοποιήθηκε στην υπό μελέτη περιοχή τουλάχιστον τα τελευταία 10 έτη, εξετάσαμε την υπόθεση ότι άλλα καρβαμιδικά που χρησιμοποιήθηκαν στη περιοχή θα μπορούσαν να χρησιμεύσουν ως υποστρώματα για το *mcd*. Κανένα από τα εναλλακτικά καρβαμιδικά που δοκιμάστηκαν δεν οδήγησε σε αύξηση της αφθονίας των γονιδίων *cehA* και *mcd* εκτός από (i) το oxamyl το οποίο προκάλεσε αύξηση της αφθονίας και της έκφρασης μόνο του *cehA* γονιδίου και (ii) το carbofuran που οδήγησε σε αύξηση της αφθονίας και της έκφρασης και των δύο γονιδίων υποδηλώνοντας έναν

ενδιαφέρον καταβολικό λειτουργικό πλεονασμό ο οποίος αναφέρεται πρώτη φορά για καρβαμιδικά εντομοκτόνα εδάφους. Το *cehA* γονίδιο ανιχνεύθηκε επίσης σε μη γεωργικά εδάφη (που δεν είχαν εκτεθεί ποτέ σε συνθετικά καρβαμιδικά) μετά από επαναλαμβανόμενες εφαρμογές oxamyl και carbofuran και μόνο σε εδάφη με pH $\geq$ 7.2, όπου παρατηρήθηκε η πιο ταχεία αποδόμηση του oxamyl. Η ύπαρξη του γονιδίου *cehA* σε γεωργικά που έχουν εκτεθεί σε συνθετικά καρβαμιδικά εντομοκτόνα και σε μη γεωργικά εδάφη που δεν έχουν εκτεθεί σε συνθετικά καρβαμιδικά υποδεικνύει την ευρεία κατανομή του συγκεκριμένου γονιδίου που θα μπορούσε να είναι αποτέλεσμα ενός παράλληλου εξελικτικού μηχανισμού από έναν κοινό πρόγονο, πιθανότατα εμπλεκόμενου στην αποτοξικοποίηση φυσικών καρβαμιδικών ενώσεων που παράγονται στο έδαφος από μικροοργανισμούς και φυτά.

Συνολικά, μελετήσαμε την αποδόμηση του oxamyl από βακτήρια που έχουν την ικανότητα να διασπούν το oxamyl και την μικροβιακή αποδόμηση του σε εδάφη. Αναφέραμε την απομόνωση και την ταυτοποίηση τεσσάρων στελεχών *Pseudomonas* με την ικανότητα να αποδομούν το oxamyl και προσδιορίσαμε το μονοπάτι του μικροβιακού μετασχηματισμού του oxamyl. Όλα τα στελέχη έφεραν το γονίδιο *cehA* το οποίο δείχθηκε να είναι υπεύθυνο για την υδρόλυση του oxamyl. Περαιτέρω μελέτες σε δείγματα εδάφους επιβεβαίωσαν το ρόλο του γονιδίου *cehA* στη διάσπαση του oxamyl, ενώ και τα δύο γονίδια *cehA* και *mcd* εμπλέκονται στην αποδόμηση του carbofuran. Η ανίχνευση του γονιδίου *cehA* σε γεωργικά και μη γεωργικά εδάφη, μετά από εφαρμογή oxamyl υποδηλώνει την ευρεία εμφάνισή του και τονίζει τον σημαντικό ρόλο του pH στην κατανομή του *cehA* στα εδάφη. Τα αποτελέσματα της παρούσας διατριβής αναμένεται να έχουν σημαντικές συνέπειες σχετικά με την διατήρηση γονιδίων που κωδικοποιούν υδρολάσες καρβαμιδικών σε εδάφη, έχουν πρακτικές εφαρμογές σχετικά με την γεωργική χρήση των καρβαμιδικών και παρέχουν πληροφορίες σχετικά με την εξέλιξη του γονιδίου *cehA*.

CHAPTER 1

General Introduction

1.1 PESTICIDES AND PESTICIDES USE

According to FAO and WHO (2014) pesticide is any substance, or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest, or regulating plant growth. The definition of pesticide varies with times and countries. However, the essence of pesticide remains basically the same: it is a (mixed) substance that is poisonous and efficient to target organisms and is safe to not-target organisms and environments (Zhang et al. 2011). The most common use of pesticides is in the form of plant protection products which in general protect plants from weeds, pathogenic microorganisms or insects. This use of pesticides is so common that the term "pesticide" is often treated as synonymous with plant protection products, although it is in fact a broader term, as pesticides are also used for non-agricultural purposes.

The historic background of pesticides use in agriculture is dated back to the beginning of agriculture itself. People have been using pesticides to try to control pests that may be harmful to crop quality and yields. The first recorded use of insecticides is about 4500 years ago by Sumerians who used sulphur compounds to control insects and mites. The Greeks and Romans used oil, ash sulfur while the Chinese used mercury and arsenic compounds to control various pests (Hansen 2014). Pyrethrum, which is derived from the dried flowers of *Chrysanthemum cinerariaefolium* has been used as an insecticide for over 2000 years. Up until the 1940s inorganic substances or organic chemicals derived from natural sources were still widely used in pest control but their use was abandoned because of their toxicity or limited efficacy (Unsworth 2010). During World War II (1939-1945), the development of pesticides increased, because it was urgent to maximize food production. Consequently, the 1940s witnessed a marked growth in synthetic pesticides like DDT, aldrin, dieldrin, endrin, parathion, and 2,4-D. In the 1950s, the application of pesticides in agriculture was considered advantageous, and no concern about the potential risks of these chemicals to the environment and the human health existed. In 1962, Rachel Carson published the book "Silent Spring", in which she mentioned problems that could arise from the indiscriminate use of pesticides. This book inspired widespread concern about the impact of pesticides on the human health and the environment (Bernardes et al. 2015).

From an idealistic perspective, the easiest way to reduce or eliminate risks associated with pesticide application is to stop using them. But this is easier said than done because their application brings substantial direct benefits to humans by protecting crops from weeds, insects, and other pests (Storck et al. 2017). Meantime, the risks of using pesticides are serious as well. In fact, it has been estimated that less than 0.1% of the pesticide applied to crops actually reaches the target pest; the rest enters the environment gratuitously, contaminating soil, water and air, where it can adversely affect non target organisms (Arias-Estevez et al. 2008).

Pesticides must be regulated to ensure that they do not present unacceptable risks to humans, animals, or the environment. Pesticide legislation varies greatly worldwide, because countries have different requirements, guidelines, and legal limits for plant protection. Developed nations have more stringent regulations than developing countries, which lack the resources and expertise to adequately implement and enforce legislation (Handford et al. 2015). In the EU, active substances (i.e. the molecule with pesticidal effects) are approved at the EU level, while plant protection products (i.e. the commercial formulation containing the active substance and other chemicals) are authorized at the national level of each EU Member State (Storck et al. 2017).

1.2 ENVIRONMENTAL FATE OF PESTICIDES

Once a pesticide is introduced into the environment it is prone to many processes. These processes determine a pesticide's persistence and mobility/transportation, if any, and its ultimate fate. These fate processes can have both positive (they can move a pesticide to the target area) and negative (leading to injury of non-target organisms) influence on the efficacy of a pesticide or its impact on the environment. To conciliate agricultural and environmental interests, a good understanding of the fate of pesticides is needed.

The soil-pesticide-plant interactions are quite complex (Fig. 1.1). The dynamics of pesticides in soils are controlled by processes that are responsible for **accumulation, transportation** and **disappearance** of pesticides in the soil (Navarro

et al. 2007). Pesticide properties, soil properties, site conditions and management practices can all affect these processes.

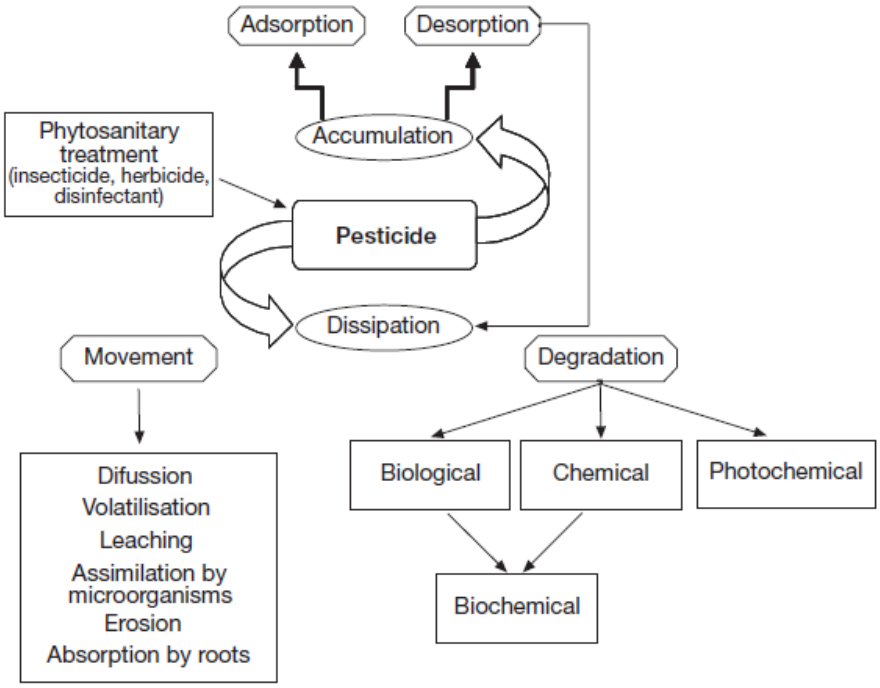


Figure 1.1. Schematic representation of pesticide dynamics and processes affecting its fate in soil (Navarro et al. 2007)

1.2.1 Processes Responsible for the Accumulation of Pesticides in Soil: Adsorption-Desorption

Adsorption refers to the attraction and accumulation of molecules at the soil–water or soil–air interface, resulting in molecular layers on the surface of soil particles (Harper 1994). Adsorption–desorption is a dynamic process in which molecules are continually transferred between the bulk liquid and solid surface. Adsorption of pesticides by soil particles occurs through a number of mechanisms involving varying bond strengths. Mechanisms of binding may include ionic, hydrogen and covalent bonding, charge transfer or electron donor acceptor mechanisms, van der Walls forces, ligand exchange, and hydrophobic bonding or partitioning (Gevao et al. 2000). Adsorption processes may vary from complete reversibility to total irreversibility. The extent of adsorption depends on soil characteristics (texture, moisture, organic matter,

pH, temperature) and the nature of the pesticide (molecular structure, electrical charge, solubility) (Gavrilescu 2005).

Adsorption plays an important role in determining the fate of a pesticide as it reduces pesticide's bioavailability and mobility and, consequently, its environmental and health potential impact. Therefore, quantification of the concentration of pesticides in the adsorbed phase in soil is of considerable importance for determining their fate. The soil sorption coefficient (K_d) and the soil organic carbon sorption coefficient (K_{oc}), are basic environmental fate parameters for pesticides (Wauchope et al. 2002). These parameters constitute measures of the strength of pesticide sorption onto soils and other sorbent surfaces at the water/solid interface, and therefore are directly related to environmental mobility and persistence. A high K_d value implies that the pesticide is strongly adsorbed onto soil; while, a low K_d value indicates that more of the pesticide exists in the soil solution. K_{oc} is used for comparing the relative sorption of pesticides normalizing adsorption strength for the soil organic carbon content, which constitutes the main adsorbent for non-polar pesticides (Ahangar and Shabani 2014). The most commonly used method for the measurement of K_d and K_{oc} is the batch equilibrium method developed by OECD (2000). Problems associated with adsorption of pesticides to soil are the reduced pest control and plant phytotoxicity. Pests may not be controlled if a pesticide is tightly adsorbed onto soil particles. Plant phytotoxicity might be observed when a pesticide used along the cultivation of one crop is later released from the soil particles (desorption) in amounts great enough to cause injury to a sensitive rotational crop (Gavrilescu 2005).

1.2.2 Processes Responsible for Pesticides Transportation in Soil

The processes that can result in pesticide transportation from their site of application are volatilization, erosion and run-off, leaching, assimilation by microorganisms, and plant uptake.

Volatilization

Volatilization is defined as the process by which a compound evaporates to the atmosphere from another environmental compartment (Navarro et al. 2007). The likelihood of a pesticide to volatilize is a function of both its vapor pressure and its solubility. This function is expressed by Henry's Law Constant, H=vapor pressure/solubility (Gavrilescu 2005). A high value of Henry's Law Constant indicates a tendency for the pesticide to volatilize. Volatilization of pesticides is controlled by pesticide properties (vapor pressure, solubility etc.), soil properties (water content, organic matter content etc.), climate conditions (temperature, wind, humidity etc.) and agricultural practices (application dose, mode of pesticide application etc.) (Bedos et al. 2002).

Leaching

Leaching is the vertical movement of pesticides through the soil. Leaching of pesticides is a process which has raised enviromental concerns since it often leads to the contamination of the phreatic horizon and groundwater resources (Flury 1996). Many factors determine whether a pesticide will leach to groundwater, including pesticide properties, soil characteristics, site conditions, and management practices (Gavrilescu 2005). The pesticides most susceptible to leaching are those characterized by high water solubility, low soil adsorption affinity, and long-term persistence.

Runoff and erosion

Runoff is the movement of pesticides in water over a sloping surface. The pesticides are either resolved in water or bound onto eroded soil particles. Runoff can also occur when water is added to a field faster than it can be absorbed by the surface soil. Pesticides may move with runoff as compounds dissolved in the water or attached to the soil particles. The severity of pesticide runoff depends on the slope of an area; the erodibility, texture and moisture content of the surface soil layer and the amount and timing of precipitation and irrigation (Srivastava et al. 2010).

Uptake by organisms

Absorption of pesticides by living organisms (microorganism, mesofauna, plants) is quite variable and is influenced by species characteristics, environmental conditions, and by the physicochemical properties of both pesticides and soils. To determine the probability of a pesticide being absorbed and distributed by a given organism, it is

useful to know its coefficient of partition between octanol and water (K_{ow}). K_{ow} is a measure of the lipophilicity of a substance and is therefore assumed as an index of the ability to pass through biological membranes and bioaccumulate in living organisms (Navarro et al. 2007).

1.2.3 Processes Responsible for Pesticides Disappearance in Soil

Pesticide transformation or degradation is one of the key processes resulting in a reduction in their environmental concentration after their application. The extent to which degradation proceeds varies widely, from minor alterations of the pesticide molecule to complete mineralization to carbon dioxide, ammonia, water and inorganic salts. The degradation of pesticides involves both biotic and abiotic processes. The degradation processes are broadly categorized to photodegradation, chemical degradation and biological degradation (biodegradation).

Photodegradation

Photochemical reactions, *i.e.* reactions induced by UV or visible light, may lead to the degradation of pesticides according to two types of processes, known as direct photolysis and indirect photodegradation (Chaplain et al. 2011). In direct photolysis, the pesticide itself absorbs light energy, becomes excited and, depending on the reaction activation energy, may undergo a transformation reaction. Most pesticides show UV-Vis absorption band at relatively short UV wavelengths. Since sunlight reaching the Earth's surface (mainly UV-A, with varying amounts of UV-B) contains only a very small amount of short wavelength UV radiation, the direct photodegradation is expected to be, in general, of only limited importance (Burrows et al. 2002). Indirect photodegradation occurs when a chemical species other than the compound of interest absorbs light energy and initiates a series of reactions that eventually degrades the pesticide. These other chemical species are known as photosensitizers and can include naturally occurring organic and inorganic species, including humic materials, clay minerals, transition metals, ozone and various free radical produces by the interaction of sunlight with air and water (Havens et al. 1995). An important advantage of the indirect photodegradation is the possibility of using

light of wavelengths longer than those corresponding to the absorption characteristics of the pollutants (Burrows et al. 2002).

Photodegradation is possible to occur on the surface of vegetation, topsoil, water and in the atmosphere (Al-Mamun 2017). Pesticides that are applied to the surface of vegetation and soil are more prone to photodegradation than pesticides that are incorporated into soil. All pesticides are subject to photolysis to a certain extent. Factors affecting pesticide photolysis are the intensity of sunlight, time exposure, the properties of the sites, the method of application, and the properties of pesticides (Al-Mamun 2017).

Chemical degradation

The most common reactions that are involved in the degradation of pesticides are hydrolysis, oxidation/reduction, and ionization. Hydrolysis is the reaction of a pesticide with water (neutral hydrolysis), usually resulting in the cleavage of the molecule into smaller, more water-soluble portions and in the formation of new C-OH or C-H bonds. In addition to neutral hydrolysis, hydrolytic reactions can be acid- or base-catalyzed (*i.e.* by H_3O^+ or OH^- ions, respectively) (Nowell et al. 1999). Therefore, water availability and pH are key-factors for reaction occurrence and kinetics. Temperature is also an important factor. There is generally a two to four-fold increase in the reaction rate for a temperature increase of 10°C (Mabey and Mill 1978). Some functional groups that are susceptible to hydrolytic reactions include amides, carbamates, carboxylic acid esters, epoxides, lactones, phosphoric acid esters, and sulfonic acid esters (Neely and Blau 1985).

Oxidation and reduction correspond to reactions in which the oxidation number (state) changes in a molecule (or ion, or atom): oxidation for its increase and reduction for its decrease. Because oxidation and reduction reactions are always coupled, they are often referred to as ‘’redox reactions’’. The rate of redox reaction depends on the redox potential of the couple (oxidation/reduction), the number of electrons transfers, temperature, pH, and composition of metal ions present in the electrolytic media (soil and water) (Al-Mamun 2017).

The fate of organic pesticides that are either acids or bases can be strongly affected by the concentration of hydrogen ions in a water body. An organic acid or

base that is extensively ionized could differ substantially from its corresponding neutral counterpart regarding solubility, adsorption, bio-concentration, and toxicity characteristics (Gavrilescu 2005).

Biodegradation

Microbial degradation of pesticides applied to soil is a principle mechanism which prevents the accumulation of these chemicals in the environment. Among the microbial communities, bacteria, fungi and actinomycetes are the main pesticide degraders (Briceño 2007). The abundance of microorganisms, together with their great ability for horizontal gene transfer and their high growth rates, allows them to evolve quickly and to adapt to environmentally changing conditions, even to extreme environments that do not allow proliferation of other living organisms (Diaz 2004). Soil organic matter content, moisture, temperature, aeration, and pH all affect the microbial degradation of pesticides. The next section provides a more detailed literature analysis of the microbial degradation of pesticides in soil.

1.3 BIODEGRADATION OF PESTICIDES IN SOIL

The importance of the soil microbial community in mediating pesticide degradation has long been recognized. In fact, it was for one of the first synthetic organic pesticides, 2,4-D, that biodegradation was first shown to be an important factor (Racke 1990). Since this early discovery there has been considerable appreciation for the role of the soil microbial community in pesticide transformations. The role of microorganisms in pesticide degradation has been stressed by comparison of degradation rates in natural and sterilized systems and by the isolation of pesticide-degrading microorganisms from a variety of sites contaminated with pesticides (Racke 1990). Because pesticides are mainly applied to agricultural crops, soil is the main environmental depot of these chemicals, besides pesticide industry's effluent, sewage sludge, activated sludge, wastewater, natural waters, sediments, areas surrounding the manufacture of pesticides, and even some living organisms. New analytical and molecular tools have deepened our understanding of the mechanisms

(how), the occurrence (what) and the identity (who) of active players that affect the biodegradation of organic environmental pollutants (Jeon and Madsen 2013)

1.3.1 Microorganisms Involved in the Biodegradation of Pesticides

Several studies have reported that native microorganisms from soils and sediment are capable of degrading pesticides (Al-Mamun et al. 2017). Among the microbial communities, bacteria, actinobacteria and fungi are the main pesticide degraders (Ortiz-Hernandez et al. 2013). The bacteria and fungi seem to play different roles. Fungi are often responsible for detoxification reactions associated with small changes in the molecular structure that often result in solubility increase and a decrease in biological activity. By comparison, bacteria appear to be involved in transformations associated in complex metabolic schemes catalyzed by more specific enzymes (Bertrand et al. 2015). Products may enter central biochemical pathways to generate energy and new biomass. Large number of bacterial strains have been isolated from different parts of the world with the ability to degrade pesticides and the list is expanding rapidly. Some examples include bacteria belonging to genera like *Pseudomonas, Bacillus, Alcaligenes, Flavobaterium Arthobacter, Rhodococcus Ralstonia* (Aislabie et al. 1995; Huang et al. 2018). Several fungi showing high pesticide degrading potential have also been identified, including members of the following genera: *Aspergillus, Candida, Fusarium, Penicillium, Trichoderma, Rhodotorula, Pleurotus, Phaenerochaete* (Tewari and Saini 2012).

The isolation of microorganisms responsible for pesticide biodegradation can be invaluable because studying these isolates provides the opportunity to investigate not only their biodegradation reactions, but also other aspects of their physiology that are likely to control their growth and activity in contaminated environments (Lovley 2003). Unfortunately, current estimates indicate that 95% of the microorganisms present in many natural environments are not readily culturable and therefore not accessible for basic research (Janssen et al. 2002). The recent development of advanced methods for isolating nucleic acids from environmental sources has opened a window to a previously unknown microbial diversity and enabled the study of natural microbial communities without the need for cultivation.

1.3.2 Microbial Adaptation to Pesticides

Adaptation describes the process whereby microorganisms gain an increased ability to degrade a compound as a result of prior exposure to this compound. There are several mechanisms or combinations thereof, by which microbial communities can adapt to the presence of xenobiotics in their environment. Adaptation does not always mean evolution (Mattes et al. 2010). Certain microbial populations can adapt, after an exposure period, to xenobiotic pollutants without requiring genetic changes. This could involve the induction of specific enzymes in members of the microbial community or the growth of a specific subpopulation of the microbial community able to transform the given compound (van der Meer et al. 1992). However, in most cases adaptation to xenobiotics involves changes to specific catabolic or regulatory genes, and may require multiple novel enzyme activities to come together in a single cell to facilitate productive and heritable growth on xenobiotics (Mattes et al. 2010). While the evolution of microbial genes for the catabolism of novel compounds released into the environment was originally thought to take long period of time (on an evolutionary scale), recent evidence indicates that microbes and their genomes are relatively plastic and as such can evolve the ability to utilize new carbon and energy sources in a relatively short time frame from years to tens of years (Sadowsky 2010). Microbial adaptation for the degradation of pesticides is an ongoing process. Three general strategies can contribute to the overall production of alterations in DNA sequences: (1) small local changes in the nucleotide sequence of the genome (2) intragenomic rearrangement of DNA segments and (3) the acquisition of a DNA segment from another organism by horizontal gene transfer (Arber 2008).

Local small changes in the genome

Many mutagenesis events result in DNA sequence alterations affecting only one or a few adjacent bases in the linear genetic script. A possible source of such mutations can be an encounter with a chemical mutagen, but local mutations go probably most often back to a replication infidelity or to a low degree of chemical instability of nucleotides (Arber 2008). Several studies have illustrated that single-site mutations can alter substrate specificities of enzymes or effector specificities. Noor et al. (2014), for example, identified atrazine chlorohydrolase, AtzA, homologues in different

triazine degrading bacteria in which single amino acid substitutions determined triazine substrate range.

Intragenomic DNA rearrangement

Larger-scale genetic changes such as gene duplications, insertions and deletions are also important to the development of xenobiotic degradations pathways (Mattes et al. 2010). These changes can be mediated by chromosomally encoded replication and repair systems (homologous recombination) or by the activity of mobile genetic elements (e.g. insertion sequences, transposon and integrons). Clément et al. (2001), for example, reported a deletion/duplication rearrangement of plasmid pPJ4 produced after subculture of a strain of *Ralstonia* in liquid medium containing 3-chlorobenzoate (3-CB). The host strain was able to grow on minimal media containing the pollutants 3-CB or 2,4-dichlorophenoxyacetate (2,4-D). During the growth of the strain in liquid medium containing 3-CB, the strain lost the ability to grow on 2,4-D due to a deletion of about 16 kb of DNA that includes genes involved in the catabolism of 2,4-D. However, the ability of this strain to grow on 3-CB was increased due to duplication of an approximately 23 kb region that includes the gene cluster involved in the catabolism of 3-CB.

DNA acquisition

The strategy of DNA acquisition allows microorganisms to share the evolutionary success of others. This strategy is very efficient and can result in essential new capacities in a single step of acquisition. Horizontal gene transfer (HGT) in microbial communities is attributable to three main mechanisms: (a) transformation, a process whereby cell-free DNA is taken up and integrated into the genome; (b) transduction, wherein gene transfer is mediated by bacteriophages; and (c) conjugation, a process whereby plasmids or conjugative transposon mediate cell-to-cell contact and transfer DNA from donor to recipient (Sota and Top. 2008). Recent studies have uncovered a newly identified mechanism for DNA transfer utilizing ubiquitously produced extracellular vesicles (Tran and Boedicker 2017). These vesicles coordinate many forms of intercellular communication and facilitate the exchange of small molecules, proteins, and nucleic acids, including RNA and DNA. Mobile genetic elements (MGEs) such as plasmids, bacteriophages, integrative conjugative elements, transposons, IS (insertion sequence) elements, integrons, gene cassettes and genomic

islands are the important vehicles in HGT (OECD, 2010). HGT by MGEs is an ongoing process that plays a primary role in the ecological adaptation of bacteria.

Microorganisms respond differently to various kinds of stresses and gain fitness in the polluted environment. This process can be accelerated by applying genetic engineering techniques. The detoxification of organophosphate pesticides was first demonstrated by genetically engineered microorganisms and the genes encoding these hydrolases have been cloned and expressed in *Pseudomonas pseudolcaligenes*, *Escherichia coli*, *Streptomyces lividans*, *Yarrowia lipolytica* and *Pichia pastoris* (Ortiz-Hernández et al. 2013).

1.3.3 Mechanisms of Pesticide Biodegradation

There are two major strategies that microorganisms follow to degrade organic pollutants including pesticides. In the first, **catabolism**, the pesticide or a primary metabolite is completely degraded with concomitant benefit to the microbe, which utilizes the compounds as carbon, energy, or nutrient source (Racke 1992). In many cases this type of degradation results in complete mineralization of the compound with production of carbon dioxide. The capability of certain microorganisms to grow with pesticides as a sole source of carbon and energy has been reported in many circumstances and this property is used for the isolation of active strains by soil enrichment studies. In the soil environment, catabolism may contribute to a stepwise increase of the microbial community as applications of the chemical are repeated (Bertrand 2015). This microbial enrichment is the basis of the phenomenon of accelerated degradation and it may be detrimental for chemicals which are directly applied to the soil as they show a progressive loss of efficacy. The list of pesticides affected by accelerated degradation is long and is constantly growing. It includes herbicides, insecticides, nematicides, fungicides and fumigants (Arbeli 2007)

The second strategy employed by microorganisms to degrade pesticides is called **co-metabolism** which often involves an incomplete degradation of the pesticide by the microorganisms, with the transient accumulation of certain metabolites. The microorganisms that initiate the degradation process obtain no benefit from the degradation process, and thus this process is often termed incidental

degradation (Racke 1992). Although, in co-metabolism the pesticide in itself does not support microbial proliferation most examples of pesticide co-metabolism involve microbial growth at the expense of a co-substrate that provides carbon and energy. In the soil environment, fresh or humidified organic matter is the main source of co-substrates. Microbial co-metabolism provides an important approach for the degradation of environmental pollutants such as petroleum hydrocarbons, aromatic compounds, halogenated hydrocarbons and pesticides (Luo 2014). Isolation of co-metabolic microorganisms can be difficult because specific substrates cannot generally be used for microbial enrichment procedures (Fournier et al. 1997)

1.3.4 Microbial Enzymes Involved in Pesticides Biodegradation

Due to the diversity of chemistries used in pesticides, the biochemistry of pesticide biodegradation requires a wide range of catalytic mechanisms, and therefore a range of enzyme classes. The most important classes of enzymes involved in the catalysis of pesticides' conversion are oxidoreductases, hydrolases and lyases.

Oxidoreductases are a broad group of enzymes that catalyze the transfer of electrons from one molecule to another. Many of these enzymes require additional cofactors, to act as either electron donors, electron acceptors or both. Some subclasses that play a role in biodegradation pathways include oxidases, monooxygenases and dioxygenases. **Oxidases** are enzymes that catalyze oxidation/reduction reactions involving molecular oxygen as the electron acceptor, whereby oxygen is reduced to water (H_2O) or hydrogen peroxide (H_2O_2). One of the best characterized oxidases with an involvement in pesticide biodegradation is glyphosate oxidase (GOX) (Scott et al. 2008). **Monooxygenases** generally catalyze the transfer of one atom of O_2 to an organic compound and the other gets reduced by electrons from co-factors to yield water. Monooxygenases often play a role in the metabolism of xenobiotics by increasing either their reactivity and/or the water solubility through the addition of an oxygen atom. The cytochrome P450 family is a large and well characterized group of monooxygenase enzymes that have been found across various prokaryotes and eukaryotes (Jaiswal 2016). **Dioxygenases** catalyze the incorporation of both atoms of molecular oxygen into substrates. Cleavage of aromatic rings is one of the most important functions of dioxygenases, which play key roles in the degradation of

biogenic and xenobiotic aromatic compounds. One example of dioxygenase is the toluene dioxygenases enzyme which has been isolated from *P. putida* strain F1 and is highly used for the degradation of toluene, polychlorinated hydrocarbons, ethylbenzene and p-xylene (Jaiswal 2016)

Hydrolases are another class of enzymes involved in the degradation of pesticides. Hydrolytic enzymes cleave the bonds of a substrate by adding –H or –OH groups from H_2O to the substrate and they generally operate in the absence of redox cofactors. Hydrolases catalyze the hydrolysis of several major pesticides' classes (esters, peptide bonds, carbon-halide bonds, thioesters, etc.). Among the most studied pesticide degrading enzymes are the phosphotriesterases which hydrolyze and detoxify organophosphate pesticides. The best characterized phosphotriesterases have been isolated from *Pseudomonas diminuta* (OPH) and *Agrobacterium radiobacter* (OpdA) and, despite high sequence homology (>90% identity) and conserved metal ion coordination, these enzymes display considerable variations in substrate specificity, metal ion affinity and reaction mechanism (Pedroso et al. 2014). There are several examples of hydrolases that target the C-N bond. One of them is the amidase CahA from the bacterium *Arthrobacter* sp. RC100 which is able to hydrolyze aryl N-methylcarbamates, such as the insecticides carbaryl, xylycarb and metolcarb (Hayatsu and Nagata 1993). For the halogenated pesticides the enzymatic removal of halogen substituents is often an important initial step in the removal of biological activity and their degradation. Hydrolytic dehalogenation is a reaction catalyzed by halidohydrolases in which, a halogen substituent is replaced in a nucleophilic substitution reaction with a hydroxyl group derived from water. One example of a hydrolytic dehalogenase is LinB that is involved in the early steps of the transformation of the chlorinated insecticide γ-HCH (Nagata et al. 2005).

Lyases are a less populated group of enzymes compared to oxidoreductases and hydrolases. They are enzymes cleaving C-C, C-O, C-N and other bonds by other means than by hydrolysis or oxidation. Such examples are the dehydroclorinases, carbon halide lyases involved in the degradation of lindane and DDT. In this reaction an HCl molecule is released with both atoms being derived from the pesticide molecule (Bertrand 2015).

1.3.5 Factors Influencing the Biodegradation of Pesticides in Soils

Microbial degradation depends not only on the presence of microorganisms with the appropriate degradative enzymes, but also on physicochemical properties of pesticides and on soil characteristics.

Pesticide Structure

The structure of a pesticide molecule determines its physical and chemical properties and its degradation potential. Any type of change in the chemical structure can cause a drastic change in the susceptibility of compounds to biotransformation. For example, introduction of polar groups such as OH, COOH and NH_2 may provide to the microbial system a site of attack, while halogen or alkyl substituents tend to make molecules more resistant to biodegradation (Cork and Krueger 1991).

Soil properties

Soil plays a critical role in pesticide biodegradation since it hosts a versatile microbial community including pesticide degraders. Soil properties (clay content, organic matter, pH etc.) greatly affect the rate of pesticide degradation in soil, because its physio-chemical properties govern the sorption, bioavailability and persistence of a pesticide in the soil (Jaiswal et al. 2016). Gupta and Gajbhiye (2002) stated that the degradation of flufenacet was greatly influenced by soil types, and Jones and Ananyeva (2001) reported that the degradation of metalaxyl and propachlor occurred at different rates in different soils. Pantelelis et al. (2006) showed that pH was the most critical factor influencing the biodegradation of the organophosphorus nematicide fosthiazate in soil. Soil pH may affect pesticide sorption, abiotic and biotic degradation processes. It affects the sorptive behavior of pesticide molecules on clay and organic matter surfaces and thus, the chemical speciation, mobility and bioavailability (Hicks 1990). The biodegradation of a compound is dependent on specific enzymes secreted by microorganisms. These enzymes are largely pH-dependent and bacteria tend to have optimum pH between 6.5 and 7.5 which equals their intracellular pH (Anjum et al. 2012)

Soil Moisture

Variations in soil moisture affect the diffusion of soluble substrates and the mobility of microorganisms at lower soil water content, while at higher soil moisture diffusion of oxygen can become restricted, both conditions affecting the activity of soil microbial communities (Skopp et al. 1990). Generally, aerobic microbial activity increases with soil water content up to a maximum point before decreasing (Linn and Doran 1984). For example, Schroll et al. (2006) showed an optimum mineralization for isoproturon, benazolin-ethyl, and glyphoshate at a soil water potential of -0.015 MPa, whereas, pesticide mineralization was considerably reduced when soil moisture approximated water holding capacity.

Temperature

The effect of temperature on pesticide biodegradation depends on the molecular structure of the pesticide. Temperature affects pesticide sorption in soil and microbial activity. High temperatures increase the degradation rate because the substances become more soluble and adsorb less onto the soil colloids being more available to the soil microorganisms, and because the abundance and metabolic activity of soil microorganisms increase (Stenersen 2004). The maximum growth and activity of microorganisms in soils occur at 25-35℃ and the pesticide degradation is optimal at a mesophilic temperature range of 25-37℃ (Pal et al. 2006)

1.4 CLASSIFICATION OF PESTICIDES

Pesticides can be grouped or classified in several different ways. However, there are three most popular ways of classifying pesticides: classification based on the mode of action, classification based on the targeted pest species and classification based on the chemical structure.

1.4.1 Classification by Mode of Action

Under this type of classification, pesticides are grouped based on the way in which they act to bring about the desired effect (Zacharia and Tano 2011). In this way pesticides are classified as contact (non-systemic) and systemic pesticides. Non-systemic pesticides are those that do not appreciably penetrate plant tissues and consequently not transported within the plant vascular system. On the contrary, systemic pesticides are those that effectively penetrate plant tissues and move through the plant vascular system in order to bring about the desired effect**.**

1.4.2 Classification by Target Pest

In this type of classification, pesticides are categorized according to their target pest as shown in the Table 1.1:

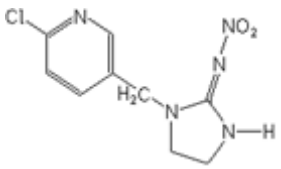
Table 1.1. Classification of pesticides according to their target

Pesticide	**Target**
Bactericides	Bacteria
Fungicides	Fungi
Herbicides	Weeds
Insecticides	Insects
Nematicides	Nematodes

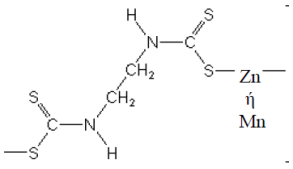
1.4.3 Classification by Chemical Structure

Under chemical classification, pesticides are categorized according to the chemical nature of the active ingredients. This classification allows to group pesticides in a uniform and scientific way and to establish a correlation between structure, activity, toxicity and degradation mechanisms, among others. Pesticides are classified into several groups according to their chemical structures (Figure 1.2) and the most important groups are listed below (abbreviation in brackets denotes the target pest IN: insecticides, FUN: fungicide, HER: herbicide, NEM: nematicide):

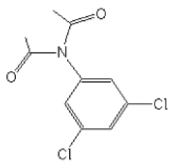
- Organophosphates (IN, NEM)
- Carbamates (IN, NEM, FUN)
- Pyrethroids (IN)
- Organochlorines (IN)
- Neonicotinoids (IN)
- Strobilurins (FUN)
- Benzimidazoles (FUN)
- Dicarboxamides (FUN)
- Thiocarbamates (FUN, HER)
- Anilinopyrimidines (FUN)
- Triazoles, Imidazoles (FUN)
- Phenoxy-alkanoics (HER)
- Triazines (HER)
- Substituted Phenylureas (HER)
- Sulfonylureas (HER)
- Triketones (HER)
- Diphenylethers (HER)
- Chloroacetamides (HER)



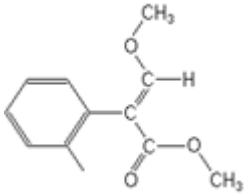
Neonicotinoids



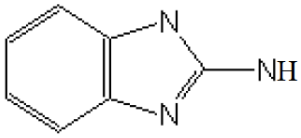
Thiocarbamates



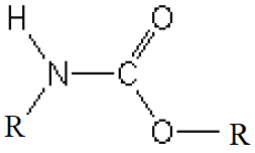
Dicarboxamides



Strobilurins



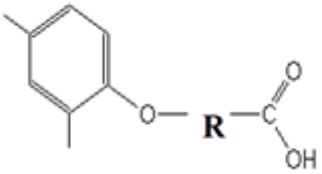
Benzimidazoles



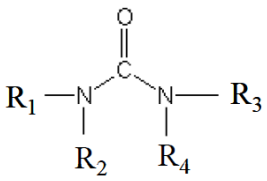
Carbamates

Triazoles

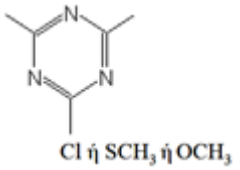
Imidazoles



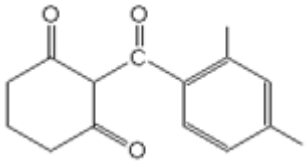
Phenoxyalkanoics



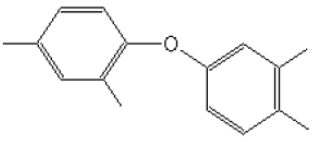
Phenylureas



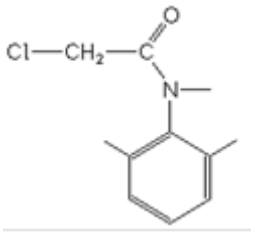
Triazines



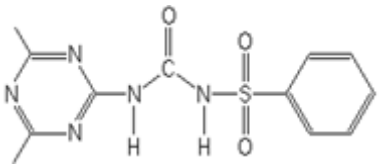
Triketones



Diphenylethers



Chloroacetamides



Sulfonylureas

Figure 1.2. Chemical structures of the most common pesticide groups as classified according to their chemical structure

1.5 MAIN INSECTICIDE GROUPS

According to their structures insecticides comprise four major groups which constitute the most traditional and historic insecticide groups named: organochlorines, organophosphates, carbamates and pyrethrin/pyrethoroids (Zacharia and Tano 2011). In addition to these a number of novel molecules belonging, most of them, to the group of neonicotinoids have been introduced in the market the last 20 years with high market shares.

Organochlorine pesticides are organic compounds with five or more chlorine atoms. Most of them were widely used as insecticides and they have a long-term residual effect in the environment since they are resistant to most chemical and microbial degradation reactions (Zacharia and Tano 2011). Due to their persistence and toxicity, most of these organochlorine compounds have been banned, or their use as pesticides has been restricted (Tadeo et al. 2008). Most common examples of these pesticides include: DDT, lindane, endosulfan, aldrin, and dieldrin.

Organophosphate pesticides are esters, amides, or thiol derivatives of phosphoric, phoshponic, phosphorothioic or phosphonothioic acids (Kavvalakis and Tsatsakis 2012). Organophosphates degrade rapidly by hydrolysis when exposed to sunlight, air, and soil. These chemical compounds inhibit the acetylcholinesterase enzyme, which hydrolyses acetylcholine in the nervous system of a number of species, including humans (Özkara et al. 2016). Although they are easier to be degraded than organochlorines, organophosphates residues are one of the biggest threats to the ecosystem and food industry due to the irreversibility of their high acute toxic effects. Some of the widely used organophosphorous insecticides include chlorpyrifos, dimethoate, parathion, malathion.

Carbamates are organic compounds derived from carbamic acid (NH_2COOH). They are used in agriculture as insecticides, fungicides, herbicides or nematicides although their primary use is as insecticides. Carbamate insecticides inhibit acetylcholinesterase, in the same way as organophosphate insecticides. However, compared to organophosphates, the carbamate pesticides action is reversible, shorter in duration and milder in intensity (Coman et al. 2013). The carbamates are transformed into various products in consequence of several processes

such as hydrolysis, biodegradation, oxidation, photolysis, biotransformation and metabolic reaction in living organisms (Porto et al. 2011). Some of the widely used insecticides of this group include oxamyl, carbofuran and its derivatives, carbaryl, aldicarb and methomyl.

Pyrethroids are synthetic analogues of the naturally occurring **pyrethrins**; a product contained in extracts of flowers of pyrethrum plants (*Chrysanthemum cenerariaefolium*). Synthetic pyrethroids are more stable exhibiting longer residual effects than natural pyrethrins (Parween and Jan 2019). Pyrethroids are highly toxic to insects and fishes but slightly toxic to mammals and birds. These pesticides are non-persistent sodium channel modulators and are much less toxic than carbamates and organophosphates to mammals (Özkara et al. 2016). The most widely used synthetic pyrethroids include permenthrin, cypermenthrin, cyhalothrin and deltamethrin.

Neonicotinoids are synthetic analogues of nicotine. The name literally means new nicotine-like insecticides. Neonicotinoids are insecticides that target insect nicotinic acetylcholine receptors, exhibiting high selective toxicity to insects over vertebrates and good systemic activity in crop plants (Ihara and Matsuda 2018). However, they have important negative side effects, especially for pollinators and other beneficial insects feeding on nectar and honeydew (Calvo-Agudo et al. 2019). Due to the demonstrated negative effects of neonicotinoids on non-target organisms, the European Commission has recently banned the use of the neonicotinoids imidacloprid, thiamethoxam and clothianidin in open agroecosystems in the member states after a risk assessment report of the European Food Safety Authority (EFSA 2018).

1.6 CARBAMATES

Carbamates were introduced as pesticides in the early 1950s and are still used extensively in pest control due to their effectiveness and broad spectrum of biological activity (insecticide, fungicides, herbicides, nematicides). Carbamates are N-substituted esters of carbamic acid with the general formula as illustrated in Figure 1.3.

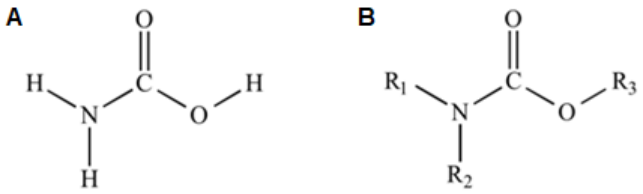


Figure 1.3. Chemical structure of **(A)** carbamic acid and **(B)** carbamates where **R_1**: is a methyl group or hydrogen **R_2**: is a methyl group or aromatic moiety or benzimidazole moiety and **R_3**: alkyl or aryl group.

Carbamic acid is a highly unstable compound decomposing into carbon dioxide and ammonia. It may be stabilized by forming salts such as ammonium carbamate or by forming alkyl or aryl esters (R_3: alkyl or aryl group). The nature of the substituent groups alters both the physicochemical properties of the carbamate compound and dictates its biological activity (Ecobichon 2001).

Depending on the chemical nature of R_2, there are three main classes of carbamate pesticides (WHO 1986):

(a) Carbamate insecticides and nematicides; R_2 is a methyl group;

(b) Carbamate herbicides; R_2 is an aromatic moiety; and

(c) Carbamate fungicides; R_2 is a benzimidazole moiety

Carbamate insecticides and nematicides constitute the most popular group of carbamate pesticides in the global market. Thiocarbamates and dithiocarbamates are sulfur analogues of carbamates. Thiocarbamates have one sulfur atom substituted for an oxygen atom; dithiocarbamates, have two oxygen atoms replace by sulfur. The thio- and dithiocarbamates are used as fungicides.

1.7 CARBAMATE INSECTICIDES

1.7.1 History and Classification of Carbamates Insecticides

Carbamate insecticides were the result of a continuous effort and monitoring to identify novel insecticide compounds exerting anticholinesterase action on the nerve system, similar to that of organophosporous insecticides, but with reduced mammalian toxicity. The structure of these compounds was inspired by the carbamate alkaloid, physostigmine, which occurs in calabar beans (*Physostigma venenosum*). Although physostigmine is an effective inhibitor of insect cholinesterase, it is unsuitable as insecticide due to its low lipophilicity which restricts its penetration through the insect cuticle (Matsamura 1985). Hence modern synthetic carbamate insecticides have been modified by eliminating the polar moiety of physostigmine so that they can easily penetrate the insect cuticle. According to their structure the carbamate insecticides can be classified to three subgroups (Figure 1.4):

a) **Aryl N-methylcarbamate insecticides**. Aryl N-methylcarbamates became one of the first efficient groups of insecticides in the 1950s. This group comprises phenyl methylcarbamates (e.g. propoxur), naphthyl methylcarbamates (e.g. carbaryl) and benzofuranyl methylcarbamates (e.g. carbofuran).

b) **N,N-dimethylcarbamate insecticides.** The N,N-dimethylcarbamate family of compounds is generally more stable than N-methylcarbamates although their insecticidal properties are considerably lower. Pirimicarb is considered the most important representative of this group.

c) **Oxime carbamate insecticides.** Oxime-based insecticides are the most recent carbamates, whose characteristic feature is the presence of an oximino ester scaffold. Representative compounds of this group are aldicarb, methomyl and oxamyl.

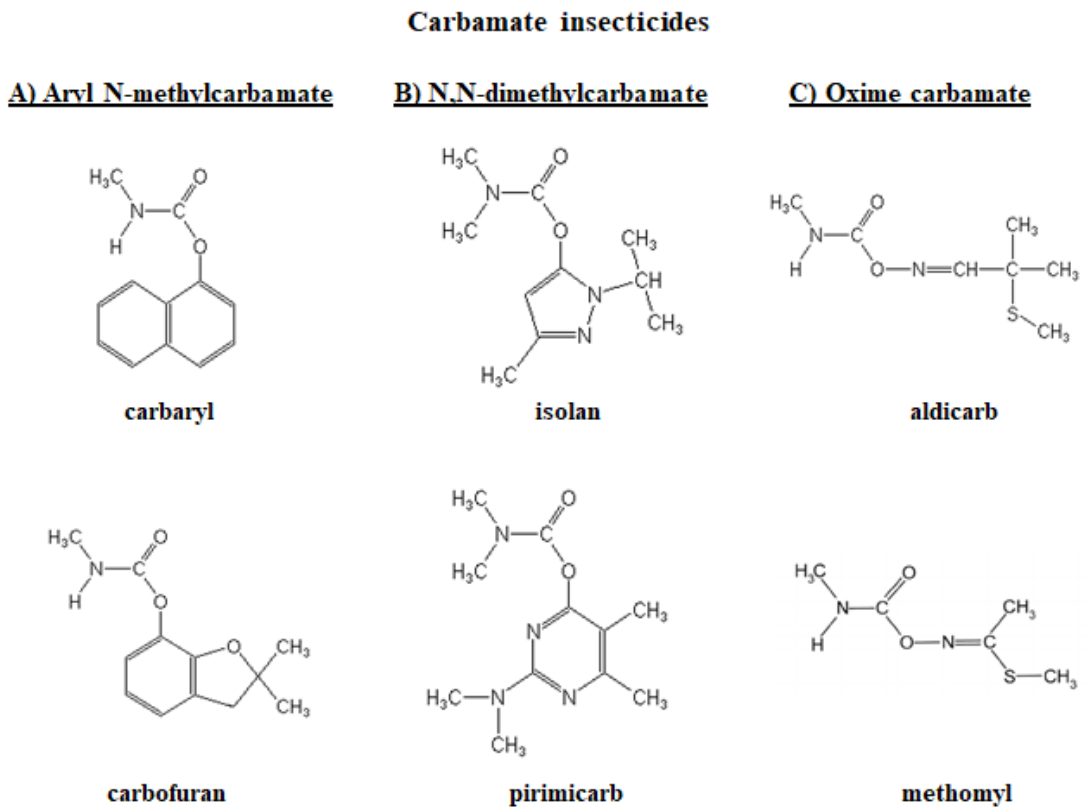


Figure 1.4 Examples of pesticides from the three groups of carbamate insecticides

The synthesis and commercialization of carbamate insecticides has been in progress since the 1950s. Since early 1990´s, a thorough EU review involving the European Commission, EFSA and Member States has been performed on all the substances used in Europe. As a result, many active substances have been banned and among them several carbamate insecticides.

1.7.2 Mode of Action and Toxicity

Carbamates are effective insecticides by virtue of their ability to inhibit acetylcholinesterase (AChE, EC 3.1.1.7) in the nervous system of insects (WHO 1986). AChE is a key enzyme in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. The inhibition of AChE causes excessive excitement in nerves, a blockage of neurotransmission, and the death of insects (Kostic et al. 2016). Anticholinesterase

carbamates react with, and are hydrolyzed by, acetylcholinesterases in a pattern analogous to the normal biological mechanism of acetylcholine-acetylcholinesterase activity. First, a reversible carbamate-acetylcholinesterase complex is formed, next follows a carbamylation reaction with the enzyme and finally decarbamylation frees the enzyme and reactivates the original acetylcholinesterase (Baron 1991). Carbamylation therefore appears to be reversible from the point of view of the enzyme, however, it is not reversible from the point of view of the carbamate, which is cleaved and loses its anticholinesterase potency in the process.

Given their mode of action, carbamate insecticides, are toxic also to non-target organisms, such as beneficial insects and other invertebrate and vertebrate organisms. According to WHO hazard classification the carbamate insecticides categorized as moderately hazardous (LD_{50} oral-rat = 50-5000 mg/kg) like bendiocarb, propoxur, and carbaryl or as highly hazardous (LD_{50} oral-rat = 5-50 mg/kg) like oxamyl, methomyl or as extremely hazardous (LD_{50} oral-rat < 5 mg/kg) like aldicarb (WHO, 2010). Short- and long-term toxicity studies have been carried out. Research based on animal experiments, particularly mammals, show that a number of carbamates have led to a broad spectrum of adverse health effects on different tissues, organs, and systems (hepatic, renal, developmental, and reproductive) in a dose dependent manner with obvious implication on functions (Dias et al. 2015).

1.7.3 Environmental Fate

Soil

In general, carbamate insecticides are not particularly persistent in soil. Because of the many factors affecting the environmental fate of carbamates in soil and the fact that many carbamates have largely variable properties, it is clear that results with one soil type and one carbamate cannot be extrapolated to others. However, there are certain rules that seem to apply to most carbamates in soil. Alkaline hydrolysis and biodegradation constitute the most important processes driving the degradation of carbamates (Karpouzas and Giannakou 2002). Carbamates exhibit more rapid degradation in neutral to alkaline soils, a mechanism driven by a combination of alkaline hydrolysis and microbial biodegradation (Smelt et al. 1987; Harvey and Han

1978; Suett et al. 1986). Regarding sorption and mobility, carbamates are not strongly sorbed in soil with aldicarb and oxamyl being weakly sorbed (K_{om} = 11.8-45.4 ml g^{-1}) and mobile in soil (Gerstl et al. 1984), and carbofuran exhibiting intermediate to high sorption (K_{oc} = 41-1279 ml g^{-1}) (Caceres et al. 2019).

Air

In general, the vapor pressure of carbamates is rather low. Some of them may sublimate slowly at room temperature, and this may also explain their loss from soil surfaces. However, distribution via air is expected to be a less important route for the dispersal in the environment (WHO 1986)).

Water

Carbamates may enter surface water bodies through discharge of industrial wastes, accidental industrial or agricultural spillages, and dumping. However, the risk of contamination of surface water resources by carbamates is limited because of their rapid decomposition in water. Thus, although long-term contamination with carbamates is unlikely to occur, the possibility of adverse effects on aquatic organisms through direct addition to surface water systems or through run-off shortly after application cannot be excluded (WHO 1986)

Biota

The transformation of carbamates in plants, insects, and mammals follows the same general patterns. Accumulation might take place in certain cases, but it is of minor importance. The first step in the metabolism of carbamates is hydrolysis to carbamic acid, which is further decomposed to carbon dioxide (CO_2) and the corresponding amine (WHO 1986). Alternatively carbamates can be oxidized by mixed-function oxidase (MFO) enzymes through reactions like: hydroxylation of the aromatic ring, O-dealkylation, N-methyl hydroxylation, N-dealkylation, oxidation of aliphatic side chains, and sulfoxidation to the corresponding sulfone. In mammals and plants carbamates are prone to conjugation which leads to the formation of O- and N-glucoronides, sulfates, and mercapturic derivatives in mammals and of glycosides and phosphate derivates in plants.

1.7.4 Biodegradation

Early studies provided evidence that microorganisms play an important role in enhancing carbamate degradation in soils in which repeated pesticide applications lead to a greatly reduced persistence of these compounds (Cheesman et al. 2007). This phenomenon has been called enhanced biodegradation and has been reported to seriously affect the efficacy of most soil applied carbamates like carbofuran and other N-methyl carbamates, such as oxamyl and aldicarb (Read 1986; Smelt et al. 1987; Felsot et al. 1982; Suett et al. 1993).

The majority of studies have focused on the isolation of bacteria degrading aryl methylcarbamates like carbofuran and carbaryl. On the contrary, only a few bacterial strains able to degrade the oximino carbamates oxamyl, aldicarb, and methomyl have been isolated to date. The reported carbofuran-degrading microorganisms belong to the bacterial genera *Achromobacter* (Karns et al. 1986), *Pseudomonas*, *Flavobacterium* (Chaudhry and Ali, 1988; Karpouzas et al. 2000), *Arthrobacter* (Ramanand et al. 1988), *Sphingomonas* (Feng et al. 1997), *Novosphingobium* (Yan et al. 2007) and to the fungal genera *Aspergillus* and *Fusarium* (Salama, 1998). Also, reports show that bacterial strains belonging to the genera *Pseudomonas*, *Arthrobacter*, *Rhizobium*, and *Micrococcus* (Phale et al. 2019) are able to degrade carbaryl. Similar studies for the oxime carbamates reported the isolation of two methomyl-degrading bacteria belonging to the genera *Paracoccus* and *Stenotrophomonas* (Xu et al. 2009; Mohamed, 2009) and two aldicarb-degrading bacterial strains which were identified as *Stenotrophomonas maltophilia* and *Methylosinus* sp. (Karayilanoglu,et al. 2008; Kok et al. 1999). Osborn et al. (2010), first reported the isolation of 27 oxamyl-degrading bacteria identified as *Aminobacter* and *Mesorhizobium* spp.

In some cases, microbial adaptation to rapidly degrade a specific pesticide in soil has resulted in the ability to degrade a variety of related pesticides. Cross-accelerated degradation of carbamate insecticides is soils is well known. For example, soil in which accelerated degradation of carbofuran has been induced by previous treatment is known to accelerate the degradation of other carbamate pesticides such as bendiocarb, carbaryl, cloethocarb, isoprocarb and propoxur (Arbeli et al. 2007). Cross acclimation can be explained either by a broad specificity of the degrading enzyme or

by high similarity in the chemical structures of the chemicals for which cross-acclimation has occurred. The primary step in the carbamate degradation process is often hydrolysis of the carbamate linkage. Many of the enzymes that hydrolyze carbamate compounds are either esterases or amidases (Cheesman et al. 2007). Esterases attack the C-O bond adjacent to the carbonyl group, whereas amidases hydrolyze the N-C bond next to this group (Figure 1.5). The reaction mechanisms of the esterases and amidases is similar in that it requires nucleophilic attack at the carbonyl carbon atom.

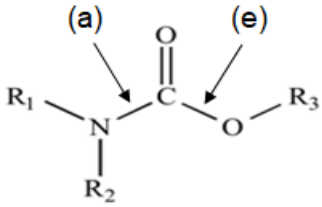


Figure 1.5. General structure of carbamate compounds. The arrows indicate amidase (a) and esterase (e) cleavage sites.

Studies with carbamate-degrading bacteria have identified the genes involved in the hydrolysis of methyl carbamates. Tomasek and Karns (1989) first isolated a plasmid-encoded carbofuran-hydrolase gene *mcd* from a carbofuran-degrading *Acrhomobacter* strain. The *mcd* gene was shown to be present in many bacteria and to be encoded on plasmids found in diverse bacteria isolated from geographically distant areas (Desaint et al. 2000; Parekh et al. 1995). Mcd hydrolase showed activity also against carbaryl and aldicarb (Karns and Tomasek 1991).

Later studies by Hashimoto et al. (2002) reported the isolation of a carbaryl-hydrolase gene *cehA* from a *Rhizobium* strain. This gene was shown to be located in a transposon (Tnceh) on a plasmid. The enzyme CehA was less reactive with the carbamate pesticides propoxur, fenobucarb and isoprocarb and was not able to degrade carbofuran. A close homologue to *cehA* gene, *cfdJ*, was detected in a carbofuran-degrading *Novosphingobium* strain (Öztürk et al. 2016). Although the rhizobial enzyme CehA and the sphingomonad enzyme CfdJ differ by only four amino acids they have different carbamate substrate specificity. Ceha is more efficient in degrading the oxime carbamate nematicide oxamyl and does not recognize

carbofuran as a substrate, whereas CfdJ is more efficient in degrading methylcarbamate pesticides with an aromatic side chain like carbofuran while oxamyl appeared to be a relatively poor substrate. In contrast to the two previous reports (Hashimoto et al. 2002; Öztürk et al. 2016), more recent studies by Yan et al. (2018) demonstrated that rhizobial CehA was able to recognize carbofuran as a substrate, albeit with low catabolic efficiency.

In 2006 Hashimoto et al. reported the isolation of an amidase-encoding gene *cahA* from an *Arthrobacter* strain, which was able to degrade carbaryl. CahA hydrolyzed also the carbamate insecticides xylycarb, metolcarb and XMC but was less reactive with fenobucarb, isoprocarb and propoxur (Hayatsu 2001)

Another carbamate hydrolase encoding gene *mcbA* has been isolated from two carbaryl-degrading *Pseudomonas* strains and reported as a carbaryl esterase (Zhu et al. 2018; Trivedi et al. 2016). The amino acid sequence of McbA of the two strains differ in one amino acid and displayed only 24% identity with CehA from *Rhizobium* sp. AC100. In addition to carbaryl, McbA was also able to hydrolyze different carbamate pesticides with a phenyl group in their structure, including isoprocarb, fenocarb, carbofuran and propoxur with different levels of efficiency but showed low or no activity for the aliphatic substrates aldicarb and oxamyl, respectively.

1.8 OXAMYL

1.8.1 Physicochemical Properties and Uses

Oxamyl is the ISO common name for (*EZ*)-*N*,*N*-dimethyl-2-methylcarbamoyl oxyimino-2-(methylthio)acetamide (IUPAC). Its structure is shown in Figure 1.6. Oxamyl has the empirical formula $C_7H_{13}N_3O_3S$ and a molecular weight of 219.259 g/mol. The pure compound is a white crystalline solid with a slightly sulfurous odor. It melts at 100-102℃, changing to a different crystalline form with a melting point of 108-110℃. The vapor pressure of oxamyl is 0.00023 mm Hg at 25℃. Its solubility (w/w at 25℃) is 28% in water, 1% in toluene, 11% in isopropanol, 33% in ethanol, 67% in acetone (Baron 1991).

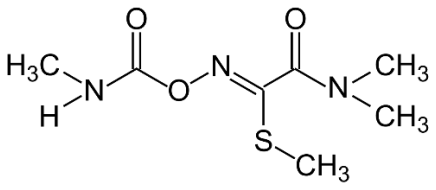


Figure 1.6. Chemical structure of oxamyl

Oxamyl belongs to the oxime N-methylcarbamate class of carbamates. It is a contact and systemic insecticide, acaricide, and nematicide. It is used to control chewing and sucking insects (including soil insects, but not wireworms), spider mites, and nematodes in ornamentals, fruit trees, vegetables, cucurbits, beets, bananas, pineapples, peanuts, cotton, soy beans, tobacco, potatoes and other crops. It is applied directly onto plants or in soil. It is fully systemic since it is absorbed by plant foliage and roots and translocated to the other plant parts (Hazardous Substances Data Bank). Oxamyl was introduced by the E.I. du Pont de Nemours and Company, Inc. Available formulations include a water-soluble liquid and granules but the granular form is banned in the U.S.A. The relatively recent withdrawal or restricted use of several nematicides like aldicarb, cadusafos, and fenamiphos, made oxamyl one of the leading synthetic non-fumigant nematicides in the global market.

1.8.2 Environmental Fate

Oxamyl is not considered a persistent chemical in soil. It dissipates in the soil environment by chemical and microbial degradation and by leaching. Oxamyl has a high leaching potential given its high-water solubility and low organic carbon partition coefficient (K_{OC} = 25 mL g^{-1}) (Wauchope et al. 1992). Harvey and Han (1978) studied the mobility of oxamyl in soils under laboratory and field conditions. They showed that although oxamyl was mobile in soil, based on laboratory evaluation, in field studies oxamyl degradation took place so rapidly that no major residues were available shortly after its application to be translocated vertically into the soil even under the higher precipitation conditions of the study. Therefore, oxamyl leaching to groundwater can be thought of as a race in time between its degradation and its

transportation to groundwater. Sites where the water table is very close to the surface, where large volumes of water are applied to the site, or where there are sandy soils low in organic matter, are sites where oxamyl contamination of the groundwater may occur. In fact, oxamyl has been detected by groundwater surveys conducted throughout the U.S.A. (Strathmann and Stone 2001). Concentrations of oxamyl in the air compartment are expected to be negligible, due to low volatility and short persistence in the atmosphere (EFSA 2005).

Laboratory and field dissipation studies show that oxamyl degrades rapidly in soil with estimated half-lives of several days to weeks. Under laboratory aerobic conditions, Harvey and Han (1978) incubated a loamy sand and a fine sand soil with [^{14}C] oxamyl at 25℃ and reported half-time values of 11 and 15 days, respectively. In similar laboratory studies, Bromilow et al. (1980) incubated [^{14}C] oxamyl in several soils at 15℃ and reported half-time values of 6-18 days. In field experiments, Haydock et al. (2012) studied the degradation of oxamyl in ten potato fields and reported half-lives from 10 to 24 days.

Some factors that have been found to influence oxamyl degradation include temperature, soil moisture, aerobic/anaerobic conditions, pH and microorganisms. Oxamyl half-time values in an Israeli soil varied from 4 to 33 days depending upon soil-water content and soil temperature (Gerstl 1984). The degradation rate of oxamyl increased with increasing moisture content of the soil until field capacity was reached, at which point it levelled off. Also, degradation proceeded more rapidly at higher temperatures. More specifically, the observed half-time values were 32.7, 8.8 and 3.8 days at 15, 25 and 35℃, respectively. Smelt et al. (1983) found that oxamyl degradation in anaerobic subsoils proceeded faster than degradation in the aerobic topsoils from the same sites. In follow-up incubation studies, the ferrous ions that occur frequently in anaerobic reduced subsoil conditions proved to be involved in the rapid transformation of oxamyl (Bromilow et al. 1986).

Soil pH is one of the major determinants of oxamyl degradation. Oxamyl degrades rapidly in neutral to alkaline environments but persist in acidic conditions. Smelt et al. (1983) found half-lives of 26, 21 and 415 days after oxamyl incubation under aerobic conditions in three soils with pH 7.9, 8.0 and 5.0, respectively. Harvey et al. (1978) found that the stability of oxamyl is markedly influenced by pH in

aqueous solutions. Oxamyl was stable in water at pH 5 or lower but hydrolyzed rapidly at pH 9. Another important factor that have a key role on the degradation of oxamyl in soils is the presence of microorganisms which is discussed in the next section. Photolysis appears to be significant in acidic near-surface water (half-life of 14 days at pH 5) but not in soil (Hazardous Substances Data Bank)

The primary degradation pathway of oxamyl in aerobic and anaerobic oxidizing topsoils is via microbial decomposition and abiotic hydrolysis (via base-catalyzed elimination) to form oxamyl oxime which is further degraded to N,N-dimethyloxamic acid (DMOA) and then extensive mineralization to CO_2 and bound residues (European Commission 2017). In anaerobic reducing subsoils where ferrous iron is present at sufficient levels oxamyl is readily converted to the nitrile metabolite N,N-dimethyl-1-cyanoformamid (DMCF) by Fe^{2+}-catalyzed reduction (European Commission, 2017; Strathmann, 2001). According to this mechanism oxamyl undergoes a net two-electron reduction that is coupled with the one-electron oxidation of two Fe^{2+} ions. Depending on the pH and the Fe^{2+} level in the environement oxamyl degradation may proceed in parallel via reduction to the nitrile and hydrolysis to oxime. Figure 1.7 shows the proposed pathways.

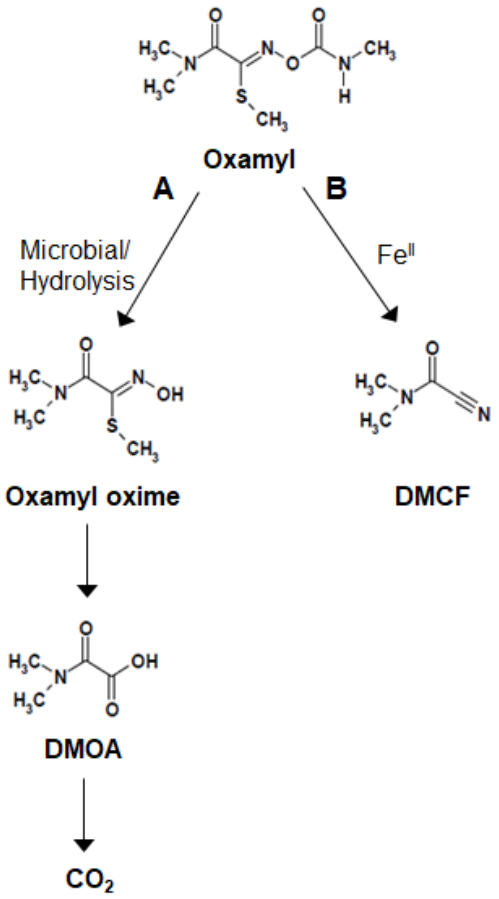


Figure 1.7. Pathway of oxamyl degradation in (A) aerobic and anaerobic oxidizing topsoils and (B) anaerobic reducing subsoils (adopted from the European Commission, 2017)

The major metabolic pathways of oxamyl in plants and animals are similrar. Oxamyl is extensively metabolised in livestock (goats and poultry), laboratory animals (rats), and in various crops . The pathway involves initial hydrolysis to the non-insecticidal oxamyl oxime. This is further metabolised to DMCF with subsequent hydrolysis to DMOA (European Commission, 2017). Further breakdown products or conjugates of these main metabolites differ between animals and plants. Oxamyl residues may utlimately be reincorporated into plant and animal natural products.

1.8.3 Biodegradation

Microorganisms play an important role in the degradation of oxamyl. The microbial involvement in oxamyl degradation is well-documented based on observations of faster degradation of oxamyl in non-sterilized vs sterilized soils and

the faster degradation of oxamyl in soils with history of prior oxamyl use (Smelt et al. 1987; Osborn et al. 2010). Osborn et al. (2010) showed that oxamyl degradation was accelerated at the second and third laboratory applications in agricultural soils, in nine of the ten investigated soils. These results provided evidence that enhanced degradation of oxamyl had occurred in these soils after repeated application. Smelt et al. (1996) demonstrated that strong interactions between pH and the soil microbiota determine the development of oxamyl accelerated biodegradation. In particular, they found that three annual applications of oxamyl to a sandy soil with pH 7.3 led to enhanced degradation of oxamyl compared with the untreated soils while ten annual applications of oxamyl to a sandy soil with pH 5.6 show a slight increase in the degradation rates of oxamyl compared with the untreated soil. Enhanced biodegradation, in its extreme, can cause an appreciable decrease of nematode control as observed in two potato fields in the Netherlands (Smelt et al. 1987). It has also been demonstrated that soil samples previously treated with the oximino carbamate aldicarb or the aryl-carbamate carbofuran can lead to cross-enhancement of oxamyl biodegradation (Harris et al. 1984; Smelt et al. 1987).

The isolation of oxamyl-degrading microorganisms from soil exhibiting enhanced biodegradation of the nematicides further support the involvement of the soil microbiota in its rapid degradation in soil. In particular, Osborn et al. (2010) first reported the isolation of 27 oxamyl-degrading bacteria from seven different agricultural soils that had previously demonstrated enhanced oxamyl degradation. The bacteria were isolated by the enrichment culture method and were able to utilize oxamyl as the sole carbon source. Partial sequencing and alignment of the 16S rRNA showed that 26 of the 27 isolates demonstrated similarity to the genus *Aminobacter* and one isolate was identified as *Mesorhizobium* sp. However, no details on the metabolic pathway and the genes involved in the degradation of oxamyl were provided. These were the sole oxamyl-degrading bacteria that have been isolated until this thesis.

1.8.4 Toxicity

The oral toxicity of oxamyl is high, with an oral LD_{50} in rats of 2.5 mg/kg. The toxicity during inhalation in rats is also high, LC_{50} (4-h, nose-only) 0.05 mg/L. The

dermal toxicity in rabbits on the other hand was found to be low LD_{50}>2000 mg/kg (FAO/WHO 2002). Exposure to oxamyl will cause similar effects induced by other carbamate compounds. However, as with other carbamates, the cholinesterase-inhibiting effects are short-term and reversible (Baron, 1991) and include increased and irregular respiration, lacrimation, urination, nausea, vomiting and ataxia. Oxamyl did not affect developmental indices in toxicity studies with rats and rabbits. It did not cause mutations, induce chromosomal aberrations, or affect DNA in genetic toxicity assays. In studies with rats, mice, and dogs after 1-2 years of exposure, oxamyl did not cause any increase in tumor incidence (EPA, 2004).

1.9 CARBOFURAN

1.9.1 Physicochemical Properties and Uses

Carbofuran is the ISO common name for (2,2-dimethyl-3H-1-benzofuran-7-yl) N-methylcarbamate (IUPAC). Its structure is shown in Figure 1.8. Carbofuran has the empirical formula $C_{12}H_{15}NO_3$ and a molecular weight of 221.256 g/mol. The pure material is a white, odorless crystalline solid with a melting point of 153-154℃. Carbofuran has a vapor pressure of 2×10^{-5} mm Hg at 33℃ and 1.1×10^{-4} mm Hg at 50℃. The solubility of carbofuran (w/w at 25℃) is 0.07% in water, 4% in benzene of ethanol, 9% in cyclohexanone, 14% in acetonitrile, 15% in acetone, 25% in dımethyl sulfoxide, 27% in dimethylformamide, and 30% in N-methyl-2-pyrrolidone. Its solubility is less than 1% in xylene, petroleum ether, and kerosene. Carbofuran is unstable in alkaline media and degrades at temperatures above 130℃. (Baron, 1991).

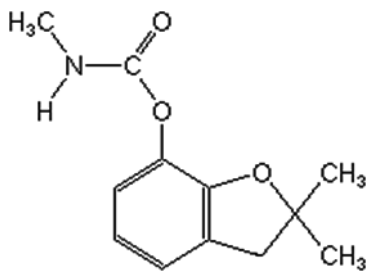


Figure 1.8. Chemical structure of carbofuran

Carbofuran is a broad-spectrum carbamate pesticide that kills insects, mites, and nematodes upon contact or after ingestion. It is used against soil and foliar pests of fruit, vegetable and forest crops. It was first introduced in 1967 by the FMC Corporation. Trade names have been Brifur®, Carbodan®, Crisfuran ®, Cureterr®, FMC10242®, Furadan®, Pillarfuran ®, and Yaltox® (Pohanish 2015). Carbofuran is not approved for use in EU countries since December 13th 2008 by Commission decision 2007/416/EEC. In May 2009, the EPA issued a final order that revoked all tolerances (residue limits) of carbofuran by December 31, 2009.

1.9.2 Environmental Fate

Carbofuran is a relatively unstable compound that breaks down in the environment within weeks or months depending on the environmental conditions. The main cause of dissipation of carbofuran in soil is the abiotic and biotic degradation. Soil pH is the major factor that determines the rate of carbofuran degradation in soil. Carbofuran half-life in soil under laboratory conditions varied form 3 weeks to more than 50 weeks and the degradation rate was found to be 7-10 times faster in alkaline soils (pH 7.9) than in acidic or neutral soils (pH 4.3-6.8) (Getzin 1973). Caro et al. (1973) reported half-lives from 46 day to 117 days in field experiments with increased persistence of carbofuran in more acid soils and in soils with low temperature. Microbial degradation is an important route of carbofuran degradation in soil. Several studies have demonstrated the involvement of microorganisms in the degradation of carbofuran in soil and the ability of microbial isolates to use carbofuran and its degradation products, as sources of carbon and energy. Breakdown products of carbofuran in soil include carbofuran phenol, 3-hydroxycarbofuran, and 3-ketocarbofuran, all of which are relatively non-persistent (Evert 2002). Although carbofuran is not stable in the environment, its relatively high water solubility and low sorption affinity result in the contamination of surface water and groundwater systems (Vryzas et al. 2009; Albanis et al. 1998). Volatilization from moist soil surfaces is not expected to be an important fate process given an estimated Henry's Law constant of 4.5 X 10^{-10} atm- m^3/mol neither from dry soils based upon its vapor pressure 5.4 10^{-7} mm Hg (Hazardous Substances Data Bank, Carbofuran).

Hydrolysis of carbofuran is the major carbofuran degradation process in both water and sediment (Evert 2002). The effect of pH on hydrolysis rates in water is similar to that observed in soils, where carbofuran degradation rates are increased under alkaline conditions. The hydrolysis half-lives in sterile water-ethanol (99:1) were found to be 690, 8.2 and 1.0 week at pH levels of 6.0, 7.0 and 8.0, respectively at 25℃ (Hazardous Substances Data Bank, Carbofuran). The biological degradation of carbofuran has generally focused on the terrestrial environment but the same biodegradation processes should also apply to the aquatic environment. Carbofuran-degrading strains of the genus *Pseudomonas* isolated from soil also degraded carbofuran in fortified oligotrophic lake water, groundwater, and trickling filter sewage effluent (Trotter et al. 1991). Photolysis of carbofuran appears somewhat less important than hydrolysis as a degradation pathway, though it does occur (Evert, 2002).

Because of its high water solubility, low K_{ow} and rapid degradation and biotransformation, carbofuran is not expected to bioaccumulate in the environment. Hydroxylation and hydrolysis, along with polar conjugations, comprise the major metabolic transformations of carbofuran in mammals, forming esters or ester cleavage products (Evert, 2002). In plants carbofuran is rapidly taken up through the roots from soil and water and is translocated mainly into the leaves. The main metabolite in plants has been identified as 3-hydroxycarbofuran (WHO 2004)

1.9.3 Biodegradation

Microbial degradation is an important route of carbofuran degradation. Several studies have reported that carbofuran was relatively more persistent in sterile soils than in non-sterile soils indicating the involvement of microorganisms in carbofuran degradation (Miles et al. 1981; Getzin, 1973). A number of microorganisms able to degrade carbofuran have been isolated from diverse geographical origins. The reported carbofuran-degrading microorganisms belong to the bacterial genera *Achromobacter* (Karns et al. 1986), *Pseudomonas*, *Flavobacterium* (Chaudhry and Ali 1988; Karpouzas et al. 2000), *Arthrobacter* (Ramanand et al. 1988), *Sphingomonas* (Feng et al. 1997), *Novosphingobium* (Yan et al. 2007) and to the fungal genera *Aspergillus* and *Fusarium* (Salama 1998). From the known carbamate

hydrolases, Mcd, CfdJ and McbA have been reported to be able to transform carbofuran, while CehA was able to recognize carbofuran as a substrate, albeit with low catabolic efficiency. These carbamate hydrolases are involved in the first hydrolytic step in the transformation pathway of carbofuran.

Microorganisms can degrade carbofuran via hydrolysis or oxidation. Some microorganisms can degrade carbofuran by hydrolysis of the labile methyl-carbamate linkage, yielding carbofuran-7-phenol and methylamine (Chapalamadugu and Chaudhry 1992). Methylamine is used by microorganisms as a source of carbon and/or nitrogen (Chaudhry and Ali 1988). Further cleavage and mineralization of carbofuran phenol has been reported for some isolates (Feng et al. 1997; Nguyen et al. 2014, Yan et al. 2007). Carbofuran can also be degraded to 3-hydroxycarbofuran, 4-hydroxycarbofuran, 5-hydroxycarbofuran via oxidation of the furanyl ring or benzene ring (Salama 1998; Chaudhry et al. 2002; Yan 2007).

Accelerated degradation of carbofuran has been reported in agricultural soils after repeated applications in the same field site and microorganisms were shown to be responsible for the rapid breakdown of carbofuran (Felsot et al. 1981; Read 1983). Enhanced biodegradation of carbofuran has been proven even when the last carbofuran application in the field had occurred 4 (Karpouzas et al. 1999) or 5 years earlier (Suett et al. 1993). It has also been demonstrated that a single field application of the recommended dose of carbofuran can compromise the biological performance of a fresh carbofuran application even five years later (Suett et al. 1993). Furthermore, there are evidence of cross enhanced biodegradation between carbofuran and other carbamates. For example, soil in which accelerated degradation of carbofuran has been induced by previous treatment is known to accelerate the degradation of other carbamates such as bendiocarb, carbaryl, isoprocarb and propoxur and *vice-versa* (Arbeli and Fuentes 2007).

1.9.4 Toxicity

Like other N-methyl carbamate pesticides, the primary toxic effect seen following carbofuran exposure is neurotoxicity resulting from inhibition of the enzyme AChE.

When AChE is inhibited, Ach is not degraded resulting in prolonged stimulation of nerves and muscles (Hazardous Substances Data Bank, Carbofuran). Physical signs and symptoms of carbofuran poisoning include headache, nausea, dizziness, blurred vision, excessive perspiration, salivation, lacrimation, vomiting, diarrhea, aching muscles, and a general feeling of severe malaise. Due to the reversibility of the carbamate-AChE bond, recovery is expected when exposures are low. However, higher levels of carbofuran exposure can lead to death by respiratory failure (Evert, 2002). Carbofuran is very toxic by ingestion (LD_{50} 7 mg/kg) and inhalation in rats (LC_{50} 0.08 mg/L), whereas toxicity during dermal exposure in rabbits is moderate (LD_{50} ~4400 mg/kg). (US EPA 2007). No evidence for oncogenicity was evident in the chronic toxicity studies. However, there were epidemiologic evidence for an association between carbofuran exposure and lung cancer incidence in pesticide applicators (Evert 2002).

1.10 AIMS OF THE THESIS

Considering the documented importance of biodegradation in the environmental efficacy of soil-applied carbamate insecticides and the limited knowledge about bacteria degrading the carbamate oxamyl, the genes involved in its transformation and the ecology and evolution of these genes, the current thesis aims

(a) To isolate and identify bacteria able to degrade the soil-applied insecticide/nematicide oxamyl from soils exhibiting enhanced biodegradation of oxamyl

(b) To characterize their transformation capacities, the metabolic pathway and the genes involved in the transformation of oxamyl

(c) To explore the function and role of oxamyl-degrading genes in soil

(d) To provide insights into the ecology and evolution of the relevant oxamyl-degrading genes detected in bacteria and soils exhibiting enhanced biodegradation of this carbamate

To achieve these aims we employed enrichment culture and a series of laboratory incubation experiments coupled with q-PCR, RT-q-PCR and analytical methods described in the following two chapters of this thesis.

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CHAPTER 2

Isolation of oxamyl-degrading bacteria and identification of *cehA* as a novel oxamyl hydrolase gene

The work presented in Chapter 2 is included in the following article:

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2.1 INTRODUCTION

Oxamyl [*N,N-dimethyl-2-methylcarbamoyloxyimino-2-(methylthio)acetamide*] is an oximino carbamate nematicide used for the control of nematodes in protected crops and potato cultivation. The recent withdrawal or restricted use of several nematicides like aldicarb, cadusafos, and fenamiphos, made oxamyl one of the leading synthetic non-fumigant nematicides in the global market. Oxamyl is a rather water soluble molecule (280 g L^{-1}), which is only weakly sorbed by soil (K_{om} 21.9 ml g^{-1}) and so is readily mobile for leaching to groundwater (Gerstl, 1984). It is characterized by high acute toxicity to mammals ($LD50_{oral\_rat}$ = 2.5 mg kg^{-1}) and aquatic organisms (EC_{50} 48 h *Daphnia magna* = 0.319 mg L^{-1}; European Food Safety Authority [EFSA], 2005).

Oxamyl is not considered a persistent chemical in soil. This has been illustrated by previous laboratory and field dissipation studies which reported half-life values of 8–60 days (Smelt et al. 1983; Ou and Rao 1986) and 7–28 days (Ambrose et al. 2000), respectively. Microorganisms and pH are the two factors controlling the degradation of oxamyl in soils. It is well documented that the soil degradation of oxamyl is pH-dependent being accelerated in neutral to alkaline soils (Bromilow et al. 1980; Smelt et al. 1983). This is the result of the vulnerability of oxamyl to abiotic hydrolysis (Harvey and Han 1978) and the higher bacterial activities commonly observed under alkaline soil conditions (Smelt et al. 1987). In its extreme biodegradation could lead to the rapid dissipation of oxamyl in soils repeatedly treated with the nematicide and eventually to loss of its biological efficacy (Smelt et al. 1987). This phenomenon has been named enhanced microbial degradation and it has been attributed to the adaptation of a fraction of the soil microbial community to rapidly degrade oxamyl (Osborn et al. 2010a).

Soils exhibiting enhanced biodegradation of carbamates have been used as a source for the isolation of carbamate-degrading bacteria. The majority of earlier studies have focused on the isolation of bacteria degrading aryl methyl-carbamates like carbofuran and carbaryl (Karns et al. 1986; Swetha and Phale 2005; Bano and Musarrat, 2006; Yan et al. 2007; Trivedi et al. 2016). On the contrary, only a few bacterial strains able to degrade the oximino carbamates oxamyl, aldicarb, and methomyl have been isolated to date. Two methomyl-degrading bacteria were previously isolated from activated sludge (Xu et al. 2009) and water samples

(Mohamed, 2009). Similar studies reported the isolation of two aldicarb-degrading bacterial strains which were identified as *Stenotrophomonas maltophilia (*Karayilanoglu et al. 2008) and *Methylosinus* sp. (Kok et al. 1999). Osborn et al. (2010b) first reported the isolation of 27 oxamyl-degrading bacteria identified as *Aminobacter* and *Mesorhizobium* spp. However, no details on the transformation pathway and the genes involved in the degradation of oxamyl were provided and the genetic mechanism driving the microbial hydrolysis of oximino carbamates remains unknown.

Previous studies with carbamate-degrading bacteria have identified the genes involved in the hydrolysis of methyl carbamates. Tomasek and Karns (1989) first isolated a plasmid-encoded carbofuran hydrolase gene *mcd* from an *Achromobacter* strain, which appeared to be widely spread in agricultural soils where carbofuran was applied (Parekh et al. 1996). Later studies by Hashimoto et al. (2002) reported the isolation of a plasmid-encoded esterase gene *cehA* from a *Rhizobium* strain AC100 (*cehA*$_{AC100}$), which was able to hydrolyze the methyl-carbamate carbaryl. Subsequent studies by the same group reported the isolation of an amidase-encoding gene *cahA* from an *Arthrobacter* strain which was able to degrade carbaryl (Hashimoto et al. 2006). Further studies by Ozturk et al. (2016) resulted in the isolation of a *cehA* ortholog from a carbofuran-degrading *Novosphingobium* strain KN65.2 (*cehA*$_{KN65.2}$) which differed by only four amino acids with the *cehA*$_{AC100}$. In parallel Trivedi et al. (2016) reported the isolation of another carbamate hydrolase encoding gene *mcbA* from a carbaryl-degrading *Pseudomonas* strain which displayed only 24% identity at the amino acid level with CehA$_{AC100}$. No such information is available for oximino carbamates like oxamyl and the genes/enzymes involved in their microbial degradation are still unknown.

The main aims of this study were (i) to isolate and characterize oxamyl-degrading soil bacteria, (ii) to explore the microbial transformation pathway of oxamyl, and (iii) to identify the genes involved in the hydrolysis of oxamyl.

2.2 MATERIALS AND METHODS

2.2.1 Pesticides and Media

Analytical grade oxamyl (99.6%, Fluka, Switzerland), oxamyl oxime (100%, DuPont, USA) carbofuran, carbaryl (99%, ChemService, USA), methomyl, aldicarb, aldicarb sulfoxide, and sulfone (99.9%, Fluka, Switzerland), were used throughout this study. An aqueous solution of oxamyl (500 mg L^{-1} in sterile ddH_2O) was used for the preparation of oxamyl-containing media. Bacteria were isolated using a selective mineral salts medium (MSM; Karpouzas et al. 2000), where oxamyl served as the sole C and N source. Mineral salts agar containing oxamyl (10 mg L^{-1}) was prepared in a similar way to the MSM liquid media except that Bacto Agar (15 g L^{-1}; LAB M, UK) was added. Nutrient Broth (NB) and Luria-Bertani broth (LB) were purchased from LAB M (UK) and they were prepared according to manufacturers' instructions. Nutrient agar (NA) was prepared in a similar way to NB except that Bacto Agar (15 g L^{-1}) was added.

2.2.2 Soil Microbial Degradation of Oxamyl and Oxamyl Oxime

The degradation of oxamyl was investigated in a soil from a commercial banana plantation located in the area of Sitia, northeast Crete, Greece. The plantation had been treated with oxamyl twice a year for the last 5 years. Despite that, the farmer was facing severe infestation by the lesion nematode *Pratylenchus goodeyi* and the spiral nematode *Helicotylencus multicinctus.* Approximately 400 g of sieved soil were divided into two sub-samples of 200 g. The first was sterilized in an autoclave at 121°C for 30 min. Both samples were then treated with an aqueous solution of oxamyl (500 mg L^{-1}), aiming at a soil dose rate of 10 μg g^{-1}. Water was then added to the treated soil samples to adjust their moisture content to 40% of their water holding capacity. The two soil samples were then separated into sub-samples of 20 g, which were placed in aerated plastic bags and incubated in the dark at 25°C. Immediately after pesticide application and at daily intervals thereafter, triplicates from each soil were analyzed for oxamyl and its metabolites via HPLC.

In a follow up experiment, we investigated the involvement of microorganisms in the transformation of oxamyl oxime (hydrolysis product of

oxamyl) in the same soil. Briefly, 120 g of sterilized and non-sterilized soil were treated with 8 mg kg^{-1} of oxamyl oxime and the samples were incubated in the dark at 25°C. Immediately after the addition of oxamyl oxime in soil and 10 days later, the levels of oxamyl oxime were determined by HPLC.

2.2.3 Isolation of Oxamyl-degrading Bacteria

Oxamyl-degrading bacteria were isolated from soil via enrichment cultures as described by Karpouzas et al. (2000). At the point of 50% degradation of oxamyl in the fourth enrichment cycle, a 10-fold dilution series was prepared on triplicate MSM + oxamyl (50 μM) agar plates. After incubation at 25°C for 5 days, 50 single colonies were randomly selected and their degrading ability was assessed in MSM + oxamyl (50 μM) at 25°C by HPLC. Cultures showing more than 50% degradation of oxamyl during the first 7 days were considered positive and they were spread onto MSM + oxamyl (50 μM) and NA plates to test purity.

A follow up enrichment was established to isolate bacteria able to degrade the main hydrolysis product of oxamyl, oxamyl oxime. Triplicate flasks containing MSMN and MSM supplemented with 20 mg L^{-1} of oxamyl oxime (as a sole C or as a sole C and N source, respectively) were inoculated with 0.2 g of soil from the banana plantation which had been previously treated with oxamyl oxime. Duplicate non-inoculated controls were also included. The degradation of oxamyl oxime was followed by HPLC.

2.2.4 Phylogenetic Classification of the Oxamyl-degrading Bacteria

Oxamyl-degrading isolates were grown in MSM + oxamyl (50 μM) agar plates for 4 days at 25°C. Bacterial cells were collected in 0.5 ml of sterile ddH_2O and DNA was extracted using the Nucleospin Tissue kit (Macherey-Nagel, Germany). Multilocus sequence analysis (MLSA) of the housekeeping genes 16S rRNA, *rpoD*, and *gyrB* was used for the phylogenetic classification of the isolated bacteria. MLSA of the housekeeping genes 16S rRNA, *gyrB*, and *rpoD* has been proposed as the most comprehensive method to establish phylogenetic relationships among the species in the genus *Pseudomonas* (Mulet et al. 2010; Gomila et al. 2015). Details on the

primers used for the amplification of the target genes are given in Table 2.1. Amplification was carried out in 25-μl reactions containing 1U of DyNAzyme EXT™ (Finnzymes), 0.2 μM of each primer, 1X buffer (DyNAzyme™ EXT buffer), 1.5 mM of $MgCl_2$, and 200 μM of each dNTPs. The PCR thermal cycling conditions for the amplification of the 16S rRNA gene were 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 10 min. PCR amplification of the *gyrB* and *rpoD* genes was performed as described by Mulet et al. (2009, 2010). The PCR products obtained were purified with the Nucleospin II PCR clean-up kit (Macherey-Nagel, Germany), cloned into plasmid vector pGEM – T easy (Promega, USA), and transformed into *Escherichia coli* (DH5a High Efficiency Competent Cells – Invitrogen, USA) following standard procedures (Sambrook et al. 1989). Plasmid DNA from at least three clones per isolate and gene were extracted using the NucleoSpin Plasmid kit (Macherey-Nagel, Germany) and sequenced (Cemia SA, Larissa, Greece).

For the phylogenetic analysis of the isolates, three alignments, one per housekeeping gene sequenced, were prepared by using clustalo (http://www.ebi.ac.uk/Tools/msa/clustalo/). Subsequently, the individual alignments (16S rRNA gene: 1346 nt; *gyrB* gene: 519 nt; *rpoD* gene: 648 nt) were merged in Mesquite ver. 2.75 (Maddison and Maddison, 2011) forming a unified matrix which was used to construct the concatenated phylogenetic tree. Evolutionary distances were calculated by the Jukes and Cantor (1969) and the phylogenetic trees were generated by the "neighbor-joining" method (Saitou and Nei 1987). TREECON for Windows (Van de Peer and de Wachter 1993) was used for tree construction from distance matrix. Sequences of the 16S rRNA, *gyrB,* and *rpoD* genes of the isolates analyzed were deposited in the European Molecular Biology Laboratory (EMBL) database under the accession numbers FN600408 – FN600411 (16S rRNA gene), KT808454-KT808457 (*gyrB*), and KT808458-KT808461 (*rpoD*).

Table 2.1. The primers used for the amplification of the genes utilized for phylogenetic analysis of the bacterial isolates

Gene	Primer	Sequence (5'- 3')	Strains amplified	Fragment length (bp)	Reference
16S rRNA	8f	AGAGTTTGATCCTGGCTCAG	All	1502	Felske et al. 1997
	1512r	ACGGCTACCTTGTTACGACTT			Felske et al. 1997
gyrB	UP1E	AYGSNGGNGGNARTTYRA	OXA17, OXA18, OXA25	966	Yamamoto et al. 2000
	APrU	GCNGGRTCYTTYTCYTGRCA			Yamamoto et al. 2000
	gBMM1F	GTGTCGGTKGTRAACGCCC	OXA20		Mulet et al. 2010
	gBMM725R	GCYTCRTTSGGRTTYTCCAGCAGG			Mulet et al.2010
rpoD	PsEG30F	ATYGAAATCGCCAARCG	All	760	Mulet et al. 2009
	PsEG790R	CGGTTGATKTCCTTGA			Mulet et al. 2009

2.2.5 Growth Kinetics and Oxamyl Degradation

In all studies bacterial inocula were prepared as described by Karpouzas and Walker (2000a). All cultures were incubated on a shaking platform at 150 rpm at 25°C. Triplicates containing 20 ml of MSM + oxamyl (50 μM) were inoculated with each one of the four isolates. Triplicates (10 ml) were amended with 0.2 ml of MSM without bacteria to serve as non-inoculated controls. The degradation of oxamyl and the formation of oxamyl metabolites were determined immediately after inoculation and at regular intervals thereafter. The initial inoculum density [3×10^6 colony forming units (cfu) ml^{-1}] and the subsequent growth of the oxamyl-degrading bacteria during degradation of oxamyl were determined by serial dilution plating in NA.

2.2.6 Mineralization of ^{14}C-labelled Oxamyl by the Oxamyl-degrading Bacteria

The capacity of the isolated bacteria to mineralize ^{14}C-oxamyl labelled at the carbamoyl moiety (Izotop, Hungary) was also examined. Mineralization experiments were performed in 30 ml-Pyrex tubes containing 10 ml of LB medium inoculated with 10^6 cells ml^{-1} of each bacterium. Labelled and unlabelled oxamyl were combined in methanol solutions and added to the medium. The final concentrations of unlabelled oxamyl in the culture was 20 $mg \cdot L^{-1}$, while the final radioactivity was $2.73 \cdot 10^{-4}$ $\mu Ci \cdot ml^{-1}$. All cultures and negative controls (n=3) were incubated at 26±1°C under agitation (100 rpm) in closed respirometer jars in the dark equipped with alkali traps (5ml of 0.2M NaOH) to capture the $^{14}CO_2$ produced as described by Soulas (1993). The evolution of $^{14}CO_2$ resulting from ^{14}C-oxamyl mineralization was measured for 8 days. On each sampling date, NaOH was removed and mixed with 10 ml of Ultima Gold XR scintillation liquid (Perkin Elmer) to quantify radioactivity using a Tri-CARB 2100TR liquid scintillation analyser (Beckman Coulter LS6500 Multi-Purpose Scintillation Counter, Beckman Coulter Inc). At the end of the incubation, the amount of soluble and cell-bound ^{14}C was determined. Liquid cultures were transferred into sterile falcon tubes and centrifuged at 10 000 χg for 5 min; 5 ml of the supernatant was added to 10 ml scintillation liquid in a 20-ml scintillation vial to determine the amount of radioactivity remaining in solution. The cell pellet was mixed with 5 ml

sterile water, vortexed to homogenize the cell suspension and mixed with 10 ml scintillation liquid to determine the amount of radioactivity that was cell-bound.

2.2.7 Degradation of Other Pesticides by the Oxamyl-degrading Bacterium OXA20

The ability of one of the isolated bacteria, strain OXA20, to degrade other carbamate pesticides was investigated. Aqueous solutions of carbofuran (200 mg L^{-1}), methomyl (500 mg L^{-1}), and aldicarb (500 mg L^{-1}) were prepared in sterile ddH_2O and they were used for the preparation of pesticide-supplemented MSM (10 mg L^{-1}). In contrast, MSM supplemented with the more lipophilic carbaryl was prepared as described by Karpouzas et al. (2000). Triplicates containing MSM plus one of the compounds (10 ml) were inoculated with the degrading isolate. Triplicate non-inoculated controls were also prepared for each compound. Viable cell counts by serial dilution plating in NA gave an initial inoculum density of 2×10^6 cfu ml^{-1}. Degradation of pesticides was determined by regularly removing and analyzing samples by HPLC.

2.2.8 Detection and Isolation of a Carbamate-Hydrolase Gene

Total DNA from the oxamyl-degrading bacterial isolates was screened for the presence of the known carbamate – hydrolase genes *mcd* and *cehA*. We limited our study to these two genes because they appear to be the most commonly detected hydrolase genes in the isolated carbamate-degrading bacteria compared to *cahA* and *mcbA*, which was only recently identified (Trivedi et al. 2016). The primers used for this initial screening are listed in Table 2.2. Thermocycling conditions were as follows: Initial denaturation at 95°C, followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 58°C (*mcd*) or 53°C (*cehA*), 1 min extension at 72°C, followed by a final extension for 5 min at 72°C. The concentrations of the PCR reagents were: 200 μM of each dNTP (HT Biotechnology, UK), 1X polymerase buffer, 1.5 mM $MgCl_2$, 1 U DNA polymerase (Finnzymes, Finland), 0.2 μM of each primer and sterile ddH_2O.

Upon initial detection of the *cehA* gene in the DNA of the oxamyl-degrading isolates, the full length *cehA* gene (2385 bp) was obtained by PCR using primers

cehAFLf – cehAFLr, which were designed based on the full length *cehA* gene sequence of *Rhizobium* sp. AC100 (Accession No. AB069723; Table 2.2). Thermocycling conditions were as follows: initial denaturation at 95°C, followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, 2.5 min extension at 72°C, followed by a final extension step for 10 min at 72°C. The concentrations of the reagents in the PCR reaction were as above with the only exception that 0.4 μM of each primer were utilized. The PCR products obtained were purified, cloned and transformed. Clones identified as positive were further processed for plasmid extraction and sequencing. The sequences of the *cehA* gene obtained were deposited in the EMBL database under the accession numbers FR751310 – FR751313.

Table 2.2. The sequences of the primers used for the detection and quantification of the carbamate hydrolase genes

Gene	Primer	Sequence (5'- 3')	Purpose	Strains amplified	Fragment length(bp)	Reference
cehA	RTcehAf	ACCAACGCTCTACCAAATTACG	RT-q-PCR *cehA*	All	156	This study
	RTcehAr	GCAGTTGAGCAGATGATACCAC				This study
gyrB	RTgyrBf_P	CACCTGGTGGGTTTCCGTTC		OXA17, OXA18, OXA25	179	This study
	RTgyBr_P	CAGCTTGTCCTTGGTCTG				This study
	RTgyrBf_Pjin	CCTTCCACAACATTCATTTCAG		OXA20	169	This study
	RTgyrBr_Pjin	TGTTGGTGTTCAGGTATTCGAC				This study
cehA	cehAf	GATGATCCGTCACATAAG AGG	PCR detection	All	552	This study
	cehAr	GCAGTTGAGCAGAT GATACC				This study
mcd	mcdL1	CAAGAACTCAAATCCATCTACCTTGCC		All	561	Parekh et al. (1996)
	mcdL2	ATCCTTCCCTCGGAATGAATCGTCTCG				
cehA	cehA	TTGGACCAACCATTCAAACCAG	PCR amplification of	All	2385	This study
	cehA	TCACGTTAAGTCGCTTTCGGCGA	full length gene			

2.2.9 Transcription Analysis of the *cehA* Gene in Oxamyl-degrading Bacteria

The involvement of *cehA* gene in the hydrolysis of oxamyl was verified by following its expression pattern during degradation of oxamyl. Thus, bacteria were inoculated in triplicate MSM + oxamyl (230 μM) and MSMN + succinate (0.1%). Triplicate non-inoculated controls were also included to determine the abiotic hydrolysis of oxamyl. Immediately prior to inoculation and at regular intervals thereafter, samples (1–5 ml) were removed from the flasks and used for the determination of oxamyl degradation and for RNA extraction. RNA was extracted with the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturers' instructions. A DNAse treatment step (DNAse I, Amplification Grade, Invitrogen Life Technologies) was essential to remove DNA residues from extracted RNA. The absence of DNA contamination was further confirmed by PCR of the 16S rRNA gene. DNA-free RNA was then reverse-transcribed to obtain cDNA (kit Superscript II, Invitrogen Life Technologies) using random hexamers (Takara, Shiga, Japan).

The expression of the *cehA* gene was measured by RT-q-PCR using *gyrB* as a reference gene. New primers sets for the RT-q-PCR analysis of the *cehA* and *gyrB* genes were designed using the program Primer3 (Table 2.2). A single primer pair was designed for the amplification of the *cehA* gene from all oxamyl-degrading isolates, while two primer pairs were prepared to successfully amplify the *gyrB* gene of the different isolates. The specificity of the primers designed was verified by q-PCR using DNA from the targeted bacterial strains. Real-time PCR reaction mixtures contained 10 μl 2x SYBR Green PCR MasterMix (Kapa, Finland), 20 pmoles of each primer,1x ROX Low, 1 μl template cDNA and sterile distilled water to a total volume of 20 μl. Thermal conditions were 95°C for 3 min followed by 45 cycles of 95°C for 15 s and 63°C for 1 min. For detection of primer dimerisation or other artifacts of amplification, a melting-curve analysis was performed immediately after completion of the real-time PCR (95°C for 15 s, 55°C for 30 s, and then slowly increasing the temperature to 95°C). All reactions were performed in triplicate. Three non-template controls were included for each primer pair. Quantification of *cehA* gene expression was performed according to Pfaffl (2001).

2.2.10 Utilization of Methylamine by Oxamyl-degrading Isolates

Considering that the hydrolysis of most carbamates results in the release of methylamine (Topp et al. 1993), the isolated oxamyl-degrading bacteria were tested for their ability to grow on methylamine. In addition, the oxamyl-degrading bacteria were screened for the presence of the *mauA* gene, which encodes methylamine dehydrogenase. This enzyme is commonly found in gram negative methylotrophic bacteria, which utilize methylamine as a C and N source, while gram positive bacteria (not the case in our study) utilize methylamine oxidase to transform methylamine (Chistoserdova 2011).

Thus, oxamyl-degrading bacteria were grown in MSM + oxamyl to mid-logarithmic phase. The bacterial pellet was harvested by centrifugation, washed three times with sterile ddH_2O and used for the inoculation of duplicate MSM + oxamyl and MSM + methylamine (100 μg mL^{-1}) agar plates. Growth on MSM without any C or N source was also tested to avoid false positives. The bacteria were sub-cultured at least three times in the corresponding media to ensure that their growth was purely on methylamine. The presence of the *mauA* gene in the oxamyl-degrading isolates was investigated via PCR using primers mauAII-232f (AAGTCTTGCGATTACTGGCG) and mauAII-526r (GACCGTGCAATGGTAGGTCA) (Hung et al. 2012). The *mauA* gene sequences obtained from the oxamyl-degrading isolates were deposited in the EMBL database under the accession numbers KT808462-KT808464.

2.2.11 Pesticides Analysis

Oxamyl and its hydrolysis products were extracted from soil (10 g) with methanol (20 ml) via agitation for 1 h in an orbital shaker at 200 rpm. Samples were then centrifuged for 15 min at 2200 rcf for and the supernatant was collected, filtered through syringe filters and used for HPLC analysis. For the determination of oxamyl and its derivatives in liquid cultures, aliquots of 0.8 ml were mixed with 0.2 ml of acetonitrile. The mixture was vortexed briefly and injected in an HPLC-UV system equipped with a GraceSmart RP C18 column (150 mm × 4.6 mm; Grace Davison Discovery Sciences, USA). Oxamyl and oxamyl oxime were detected at 220 nm using a mobile phase of acetonitrile:water (20:80 by volume) at flow rate of 1 ml min^{-1}.

Under these chromatographic conditions oxamyl and oxamyl oxime showed retention times of 3.1 and 2.3 min, respectively.

For the extraction of the other pesticides studied, aliquots of 0.5 ml of the liquid cultures were mixed with 0.5 ml of methanol. The mixture was vortexed briefly and it was directly analyzed by HPLC. For the elution of carbofuran and carbaryl, a mobile phase of acetonitrile:water (40:60 v:v) was used with UV detection at 215 nm. The retention times for carbofuran and carbaryl were 5.3 and 6.1 min, respectively. Methomyl was eluted using a mobile phase of acetonitrile:water (20:80 v:v) with UV detection at 235 nm and a retention time of 3.7 min. Aldicarb and its oxidation products (sulfoxide and sulfone) were analyzed as described by Karpouzas and Walker (2000b).

Quantification of pesticide residues was performed by the external standard method using the calibration curves obtained by the injection of standard solutions of the pesticides studied. Good linearity responses were obtained for all the compounds in the ranges studied (0.02–10 μg ml^{-1}). Analysis of fortified samples was conducted to verify the extraction efficiency of the methods. For the extraction of oxamyl of soil, recovery tests at three fortification levels (0.05, 1 and 10 μg g^{-1}) showed recoveries above 90% in all cases. The limit of quantification for oxamyl was 0.02 μg g^{-1}. Regarding the extraction of oxamyl and of the other pesticides tested from liquid cultures, recovery tests at three fortification levels (0.05, 5 and 20 μg ml^{-1}) showed recoveries of 85-98%, 87-92%, 83-89%, 95-101%, 82-91% and 81-99% for oxamyl, carbofuran, carbaryl, methomyl, aldicarb and fenamiphos respectively. The limit of detection for the different pesticides tested was 0.02 μg ml^{-1}.

2.3 RESULTS

2.3.1 Degradation of Oxamyl and Oxamyl Oxime in Soil and Enrichment Cultures

Degradation of oxamyl in the soil from the banana plantation proceeded rapidly with the concurrent formation of oxamyl oxime. The latter did not accumulate and it was fully degraded by day 5 (Figure 2.1A). The degradation of oxamyl in soil followed

FOK with a DT_{50} of 1.6 days. Oxamyl degradation and oxamyl oxime formation was negligible in the sterilized soil (Figure 2.1A).

Subsequent enrichment cultures inoculated with the soil from the banana plantation showed a rapid degradation of oxamyl, which coincided with the formation of oxamyl oxime. In contrast to soil studies, oxamyl oxime was not further transformed and accumulated in the medium at the end of each enrichment cycle (Figure 2.1B). Negligible degradation of oxamyl was evident in the non-inoculated enrichment cultures.

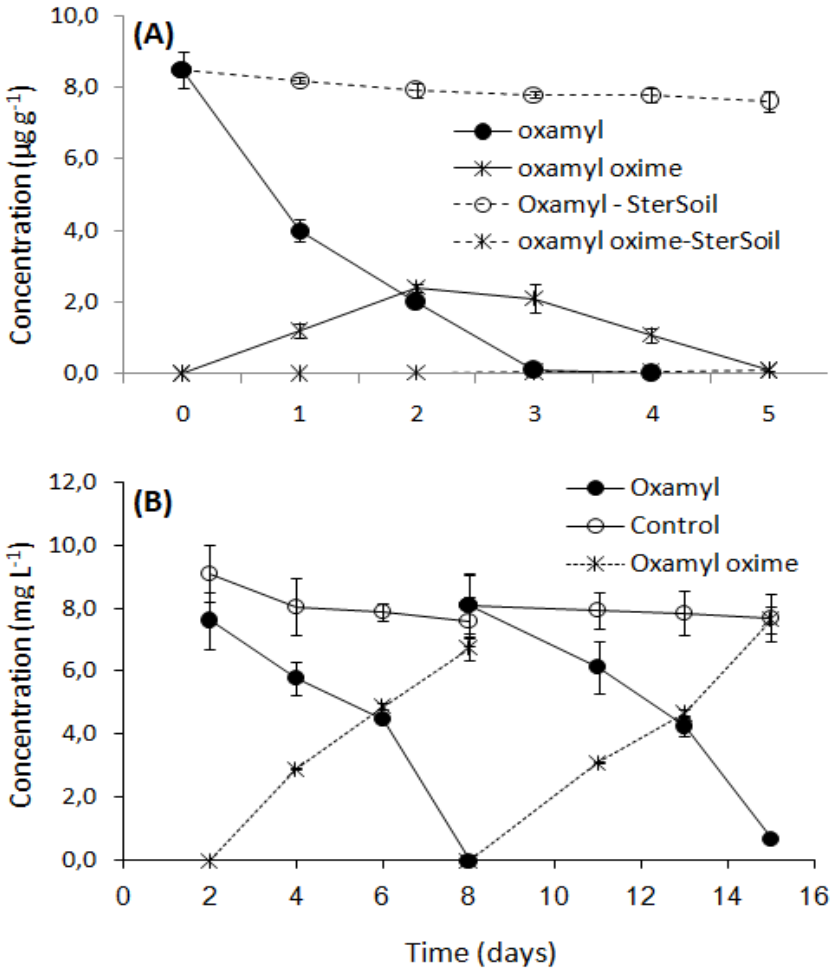


Figure 2.1 (A) The degradation of oxamyl and the formation of oxamyl-oxime in sterilized (dashed lines) and non-sterilized soil (solid lines) collected from a banana plantation in Crete with history of oxamyl treatments. **(B)** The degradation of oxamyl and the formation of oxamyl oxime in soil enrichment cultures in MSM and in non-inoculated controls. For clarity, only the degradation of oxamyl and the formation of oxamyl oxime in the second and third enrichment cycles are presented. Each value is the mean of three replicates ± standard deviation.

Soil sterilization resulted in a complete halting of the degradation of oxamyl oxime, compared to 75% degradation which was observed in the non-sterilized soil in 10 days (Figure 2.2). This degradation pattern was not reflected in the corresponding enrichment cultures in MSM and MSMN, where no appreciable degradation of oxamyl oxime was observed (Figure 2.3). Repeated attempts to re-establish enrichment cultures for oxamyl oxime were not successful.

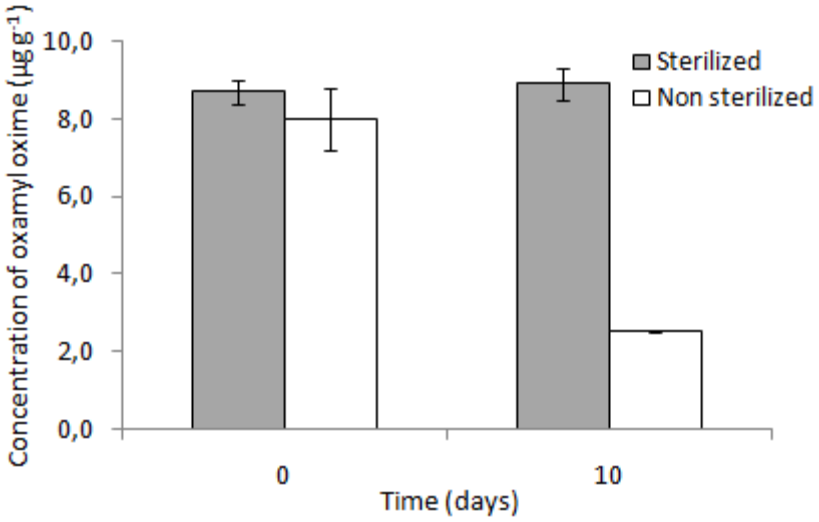


Figure 2.2. The degradation of oxamyl oxime in sterilized and non sterilized soil

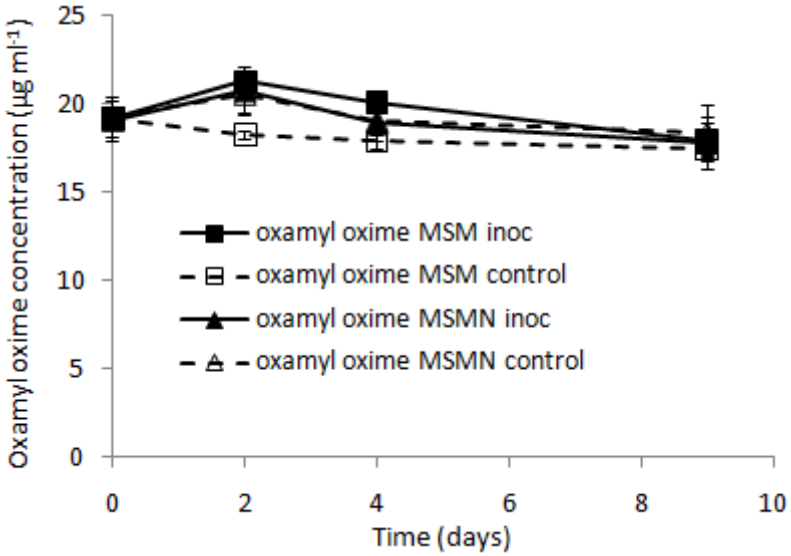


Figure 2.3. Negligible degradation of oxamyl oxime in enrichment cultures (MSM + and MSMN + oxamyl oxime) inoculated with a soil exhibiting enhanced biodegradation of oxamyl which had been repeatedly treated in the laboratory with oxamyl oxime

2.3.2 Isolation and Phylogenetic Classification of the Oxamyl-degrading Isolates

Enrichment cultures resulted in the isolation of four pure bacterial cultures which showed complete degradation of oxamyl within 7 days. MLSA based on partial sequences of the 16S rRNA, *gyrB*, and *rpoD* genes showed that all strains belonged to the genus *Pseudomonas* (Figure 2.4). In particular, strain OXA17 clustered within the *Pseudomonas fluorescens* subgroup and showed closest similarity to *P. extremaustralis* type strain. In contrast, strains OXA18 and OXA25 grouped within the subgroup of *P. putida* and showed closest similarity to *P. monteilii* type strain. Finally strain OXA20 clustered within the *P. aeruginosa* subgroup, and showed closest similarity to *P. jinjuensis* type strain. Based on these results the oxamyl-degrading bacteria were named as *P. extremaustralis* strain OXA17, *P. monteilii* strains OXA18 and OXA25, and *P. jinjuensis* strain OXA20.

2.3.3 Growth Kinetics and Hydrolysis of Oxamyl

Degradation of oxamyl was rapid and coincided with a build-up of bacterial growth, which reached to levels higher than 10^7 cells ml^{-1} at 24 h in the cultures of all the oxamyl-degrading bacteria (Figure 2.5). Degradation of oxamyl by the strains OXA17, OXA20, and OXA25 was completed within 96 h (Figures 2.5 A,C,D), while 7 days were needed for the degradation oxamyl by the strain OXA18 (Figure 2.5 B). Oxamyl degradation coincided with the formation of oxamyl oxime which was accumulated in the liquid cultures. Extending the incubation to 21 days did not result in appreciable degradation of oxamyl oxime. Negligible hydrolysis of oxamyl to oxamyl oxime was observed in the non-inoculated control cultures (Figure 2.5).

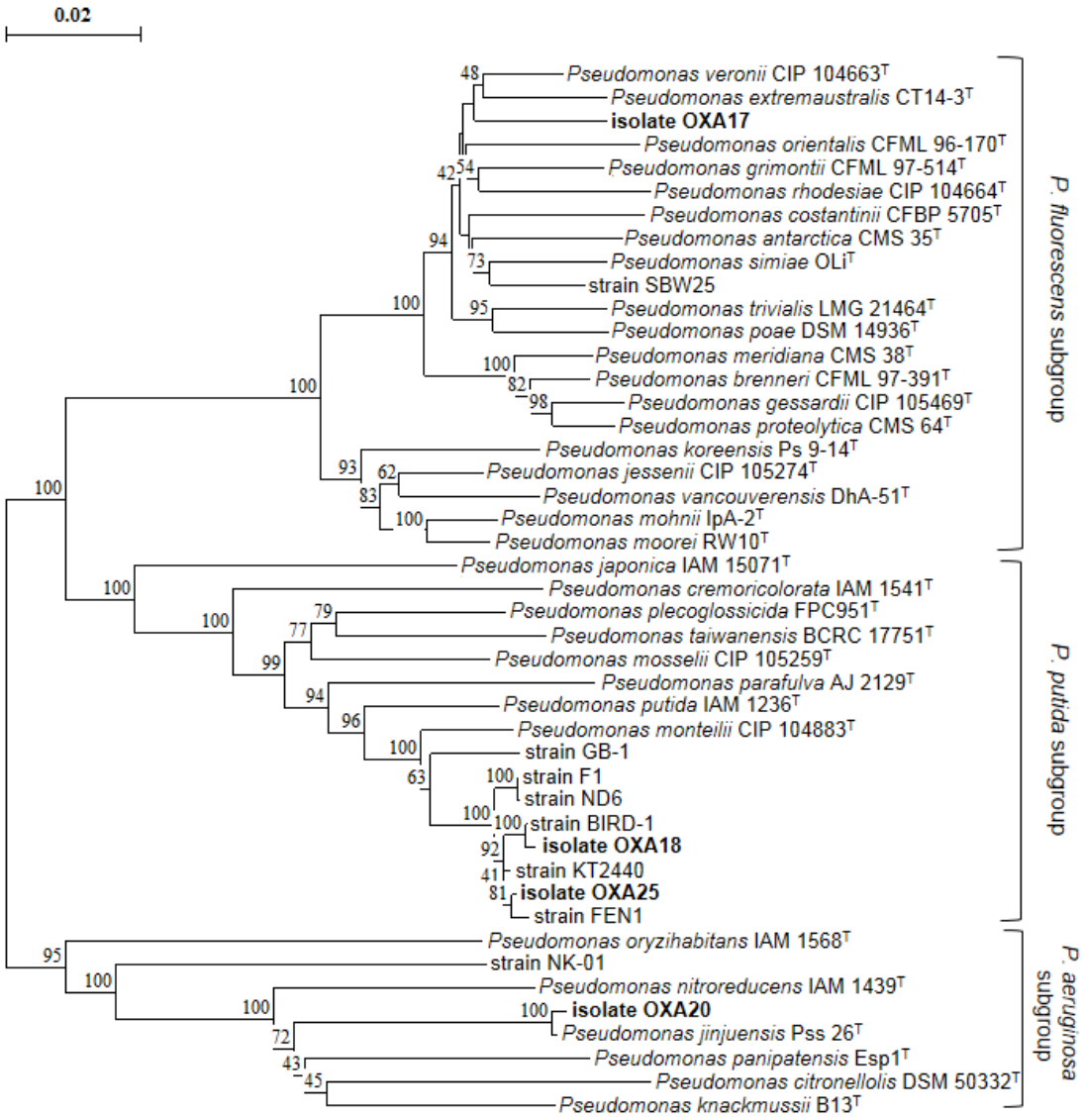


Figure 2. 4. Phylogenetic position of the isolated oxamyl-degrading isolates based on concatenated MLSA of the 16S rRNA, *gyrB*, and *rpoD* gene sequences. Distance matrix was calculated by the Jukes and Cantor (1969) and the dendrogram was constructed by the neighbor-joining method (Saitou and Nei, 1987). Numbers on the nodes denote % bootstrap values based on 1,000 replicates. Scale bar represents 0.02 substitutions per site. *Pseudomonas* genus sub-groups were as defined by Mulet et al. (2010).

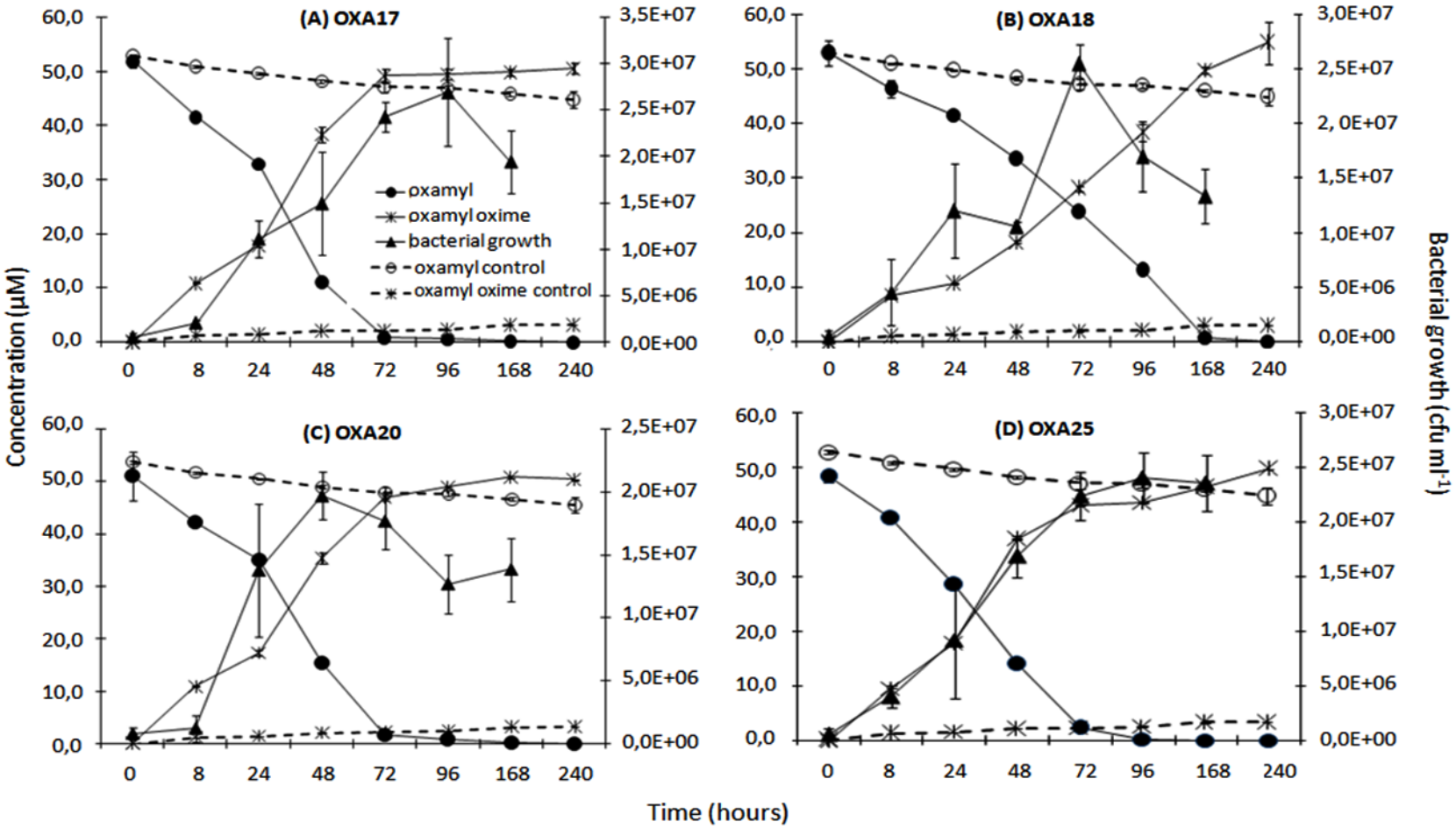


Figure 2.5. The degradation of oxamyl and the formation of oxamyl oxime in MSM inoculated with strains OXA17 (A), OXA18 (B), OXA20 (C), and OXA25 (D). The degradation of oxamyl in non inoculated controls is also presented. The growth of the bacterial strains during degradation of oxamyl is also presented. Each value is the mean of three replicates with error bars showing the standard deviation of the mean.

2.3.4 Mineralization of ^{14}C-labelled Oxamyl by the Isolated Bacteria

The mineralization kinetics of ^{14}C-labelled oxamyl by the four oxamyl-degrading bacteria are presented in Figure 2.6A. All four isolates showed identical mineralization kinetics with the level of cumulative $^{14}CO_2$ produced by the ^{14}C-labelled oxamyl reaching to 80% of the initially applied radioactivity by day 8. Mass balance analysis performed at the end of the mineralization test recovered nearly 90% of the originally applied radioactivity with 5-8% remaining in the liquid culture and recovered through extraction with methanol while negligible amounts of radioactivity (<1%) were recovered by the bacterial pellets (Figure 2.6B)

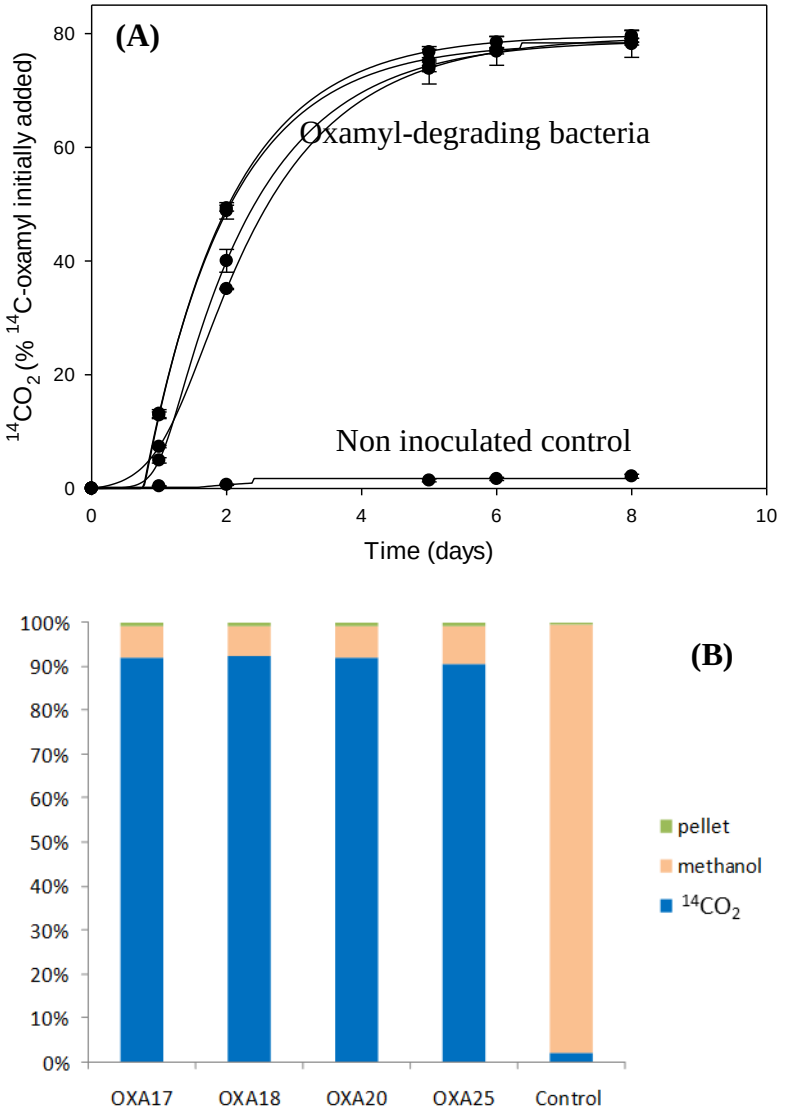
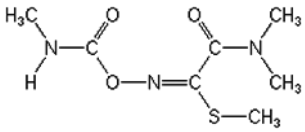
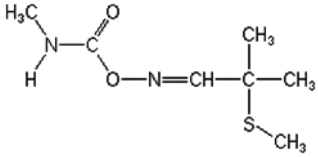
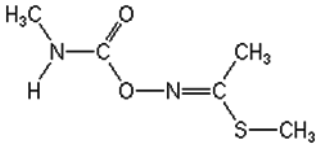
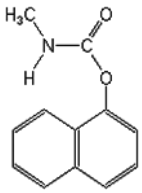
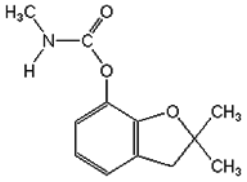


Figure 2.6. (A). Cumulative mineralization of ^{14}C-labelled oxamyl by the oxamyl-degrading bacteria OXA17, 18, 20 and 25 in cultures containing unlabelled oxamyl (20 mg L^{-1}). The mineralization of ^{14}C-labelled oxamyl in non inoculated controls is also shown. Error bars show the standard error of triplicates. **(B)** Mass balance analysis for ^{14}C-labelled oxamyl in the different fractions (pellet: ^{14}C recovered in bacterial biomass; methanol: ^{14}C recovered by the liquid culture via extraction with methanol; $^{14}CO_2$: ^{14}C released as $^{14}CO_2$ and captured in NaOH traps) of liquid cultures of the oxamyl-degrading isolates and in the non inoculated control cultures after 8 days of incubation. Errors bars show the standard error of triplicates.

2.3.5 Degradation of Other Pesticides by the Oxamyl-degrading Strain OXA20

Strain OXA20 was able to completely degrade the oximino carbamates oxamyl and aldicarb, and the aryl-methyl carbamate carbaryl within 4 days, while a period of 7 days was required for the complete degradation of methomyl and carbofuran (Table 2.3). Negligible degradation of all tested pesticides (<10%) was observed in the non-inoculated controls (Table 2.3).

Table 2.3. The degradation (%) of the oximino carbamates oxamyl, aldicarb, and methomyl and of the aryl-methyl carbamates carbaryl and carbofuran in MSMN inoculated with the strain OXA20. The degradation of the pesticides in non-inoculated cultures is also presented. Each value is the mean of three replicates ± standard deviation.

Pesticides		**Time after inoculation (days)**			
		Inoculated		**Non-inoculated**	
		4	**7**	**4**	**7**
Oxamyl		100	-	2.3±0.1	9.1±0.4
Aldicarb		100	-	3.3±0.4	7.1±0.7
Methomyl		73.8±2.1	100	4.3±0.4	8.4±0.3
Carbaryl		100	-	5.6±0.9	6.2±1.2
Carbofuran		77.2±3.1	100	3.4±0.3	8.6±1.0

2.3.6 Detection of a Carbamate-Hydrolase Gene in the Oxamyl-degrading Bacteria

PCR screening of total DNA from the oxamyl-degrading isolates for carbamate hydrolase genes gave positive amplification only for the *cehA* gene (Figure 2.7). PCR amplification and sequencing of the full length *cehA* gene showed that all oxamyl-degrading isolates carried similar *cehA* genes (>99% identity) with a 29-amino acid long signal peptide. Further alignment of the *cehA* genes carried by the oxamyl-degrading isolates ($cehA_{OXA}$) with $cehA_{AC100}$ and $cehA_{KN65.2}$ showed high levels of homology (Figure 2.8). In particular, the *cehA* gene of the strain OXA18 was identical to $cehA_{AC100}$, while the *cehA* genes carried by the other three isolates were identical and differed only in two nucleotide positions (1430 and 1494) with $cehA_{AC100}$. However, only the nucleotide polymorphism at position 1430 resulted in a change in the amino acid sequence of the resulting protein (threonine instead of asparagine). In contrast, the $cehA_{KN65.2}$ gene sequence was more diverse and showed four nucleotide differences with the *cehA* sequence of the strains OXA17, OXA20, and OXA25, and five nucleotide difference with the *cehA* sequence of strain OXA18 (Figure 2.8).

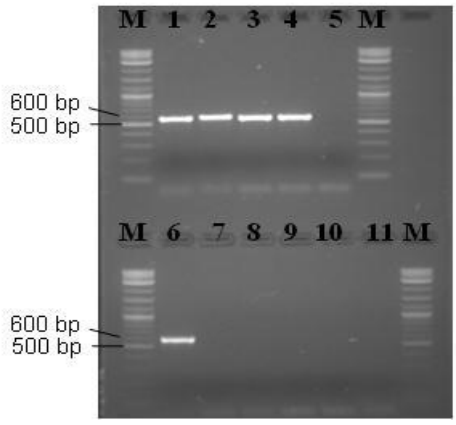


Figure 2.7. Agarose gel electrophoresis showing the PCR product of the *cehA* gene (upper panel lanes) from strains OXA17 (1), OXA18 (2), OXA20 (3), OXA25 (4), Lane 5: negative control, Lane M: molecular marker; and of the *mcd* gene (lower panel lanes) from the isolates OXA17 (7), OXA18 (8), OXA20 (9), OXA25 (10), Lane 6: positive control (total DNA from strain ER2 carrying the *mcd* gene, kindly provided by Prof. Ed Topp) Lane 11: negative control.

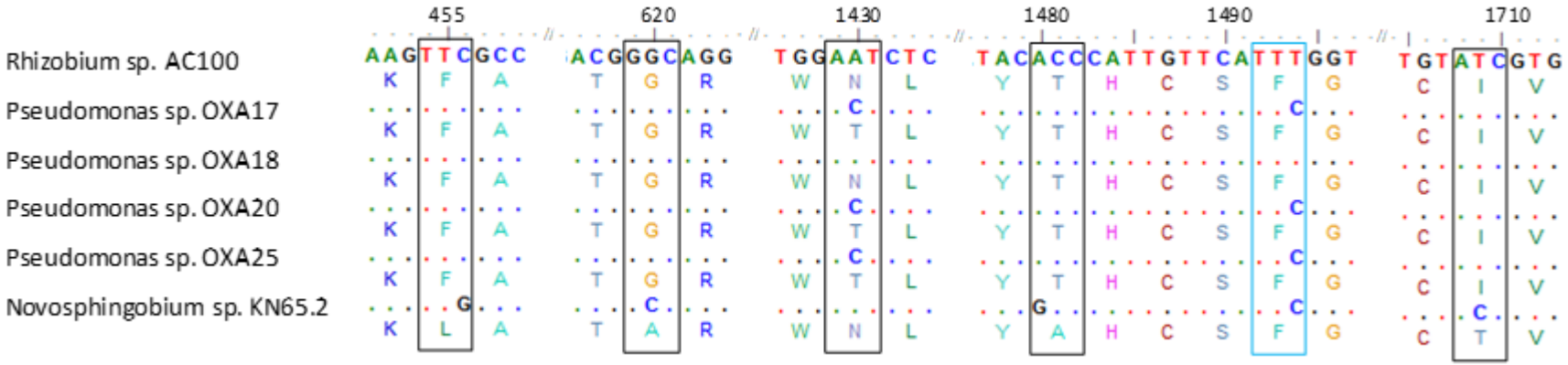


Figure 2.8. Nucleotide (and amino acid) sequence alignment of the *cehA* genes of the oxamyl-degrading strains OXA17, OXA18, OXA20, and OX25 ($cehA_{OXA}$), the carbaryl-degrading strain *Rhizobium*AC100 ($cehA_{AC100}$) (Hashimoto et al. 2002; Accession No. AB069723) and the carbofuran-degrading strain *Novosphingobium* KN65.2 ($cehA_{KN65.2}$) (Nguyen et al. 2014; Accession No. CCBH010000016, CDO34164.1). Only the parts of the alignment where sequence divergence was observed are presented. Polymorphisms in the sequences of the $cehA_{OXA}$ and $cehA_{KN65.2}$ genes compared to the sequence of $cehA_{AC100}$ are shown where blue and black color indicates silent and non-silent nucleotide sequence polymorphisms. Dots represent nucleotide homology between the *cehA* genes compared

2.3.7 Transcription Analysis of the *cehA* Gene in Oxamyl-degrading Bacteria

In order to verify the functional role of the *cehA* gene in the degradation of oxamyl, its transcription during degradation of oxamyl was followed in a time series experiment via RT-q-PCR. The relative expression of the *cehA* gene (ratio of its expression in MSM + oxamyl to its expression in MSMN + succinate) followed the same temporal pattern in all oxamyl-degrading strains (Figure 2.9). The relative expression of *cehA* increased concurrently with the hydrolysis of oxamyl and reached to maximum levels at 71 h (OXA25), 88 h (OXA17, OXA20), or 111 h (OXA 17). For instance, the expression of the *cehA* gene in the strains OXA20 and OXA25 was 140- and 120-times higher in the oxamyl-amended cultures compared to the corresponding succinate-amended cultures (Figures 2.9C and D).

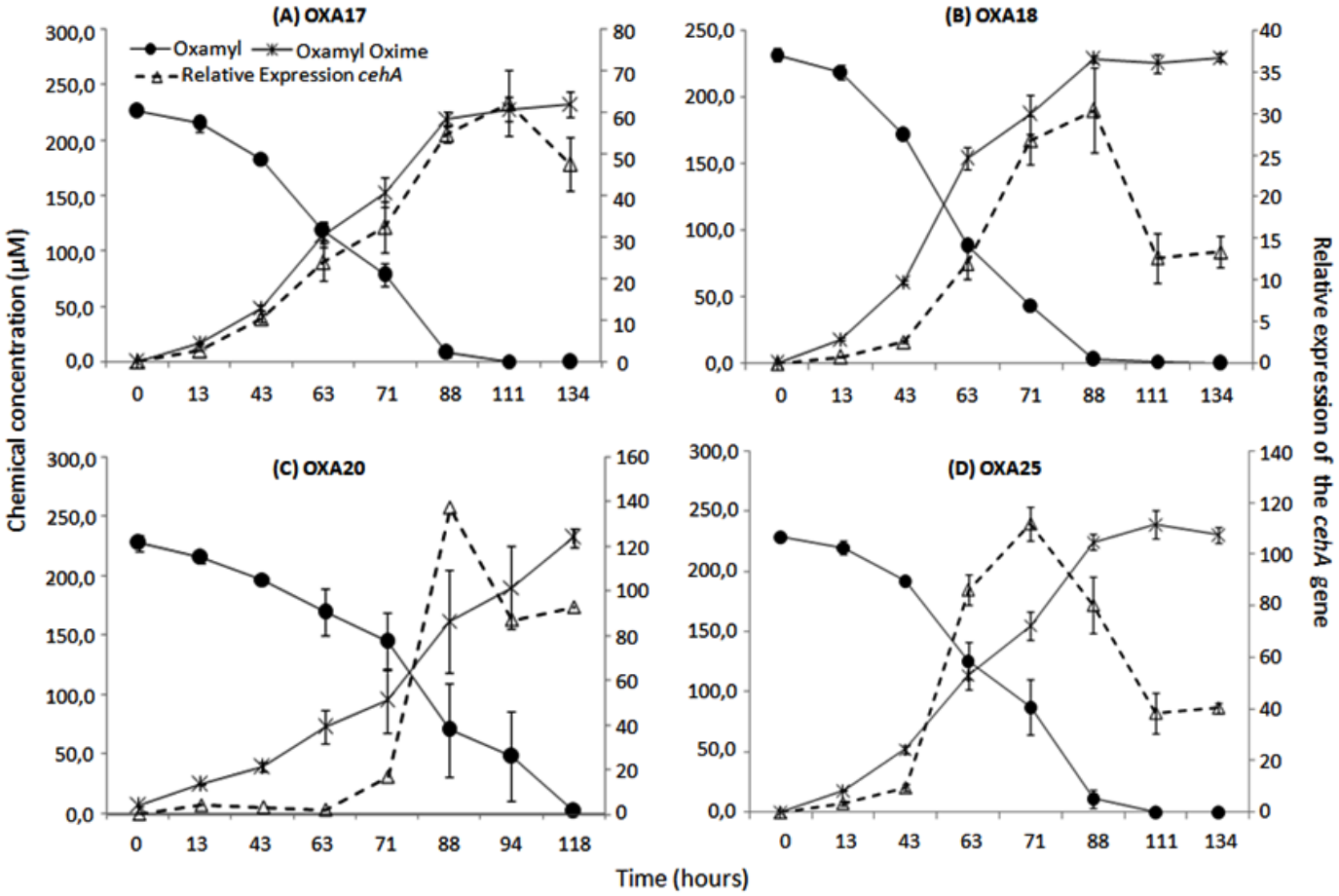


Figure 2.9. The hydrolysis of oxamyl to oxamyl oxime and the relative expression of the *cehA* gene in MSM inoculated with the strains OXA17 (A), OXA18 (B), OXA 20 (C), and OXA25 (D). The relative expression of the *cehA* gene was calculated as a ratio of its expression in MSM + oxamyl to its expression in MSMN + succinate. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

2.3.8 Utilization of Methylamine by Oxamyl-degrading Bacteria

All isolates were able to grow on MSM + methylamine upon numerous transfers. PCR tests showed positive amplification of the *mauA* gene in three of the four oxamyl-degrading strains (OXA18, OXA20, and OXA25; Figure 2.10). Sequencing of the PCR products obtained showed highest sequence match to the methylamine dehydrogenase of *Methylobacterium extorquens* strain AM1 (91% at the DNA level, Accession no. CP001510). Further attempts to amplify the *mauA* gene from the DNA of the strain OXA17 using alternatively primers (Neufeld et al. 2007) and optimization of the PCR conditions did not result in a successful amplification.

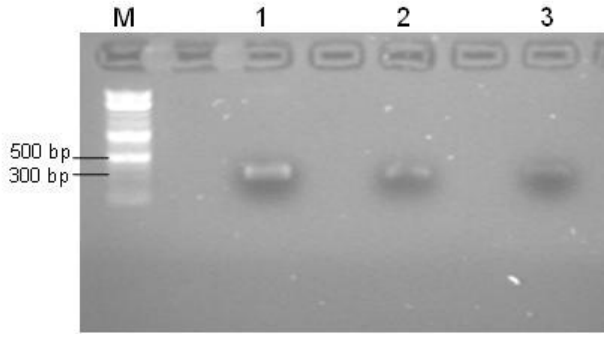


Figure 2.10. Agarose gel electrophoresis showing the PCR products of the *mauA* gene from isolates OXA18 (1), OXA20 (2) and OXA25 (3). Lane M: molecular marker

2.4 DISCUSSION

A rapid microbially driven hydrolysis of oxamyl was observed in a soil with history of previous treatment with oxamyl. This is in line with the reduced biological efficacy of oxamyl in the given field, as reported by the grower. Previous studies have demonstrated the vulnerability of oxamyl to enhanced microbial degradation, which could lead to unacceptable reduction in its biological efficacy (Smelt et al. 1987, 1996).

Enrichment cultures from the adapted soil resulted in the isolation of four bacterial strains, which were all identified, based on MLSA, as *Pseudomonas*. Earlier

studies have reported the isolation of pseudomonads rapidly degrading a range of pesticides including organophosphates (Karpouzas et al. 2000), carbamates (Bano and Musarrat, 2006; Trivedi et al. 2016; Zhu et al. 2019), and pyrethroids (Mallic et al. 2009). Our study constitutes only the second report regarding the isolation of oxamyl-degrading bacteria. Osborn et al. (2010b) isolated several oxamyl-degrading strains from soils exhibiting enhanced biodegradation of oxamyl. Their isolates were phylogenetically assigned to the genera *Aminobacter* and *Mesorhizobium*. However, these authors did not provide any information regarding the transformation pathway of oxamyl. Our isolates were able to transform oxamyl via hydrolysis of its methyl-carbamoyl moiety to form oxamyl oxime, which was not further degraded neither in the soil enrichment cultures nor by the isolated bacteria. Previous metabolism studies in soil have also identified oxamyl oxime as the main metabolite of oxamyl (Ou and Rao, 1986); however, this is the first report for the formation of oxamyl oxime by soil bacteria. The accumulation of oxamyl oxime in enrichment and pure cultures is in contrast to its gradual dissipation in soil (Figure 2.1A). Sterilization of soil halted the degradation of oxamyl oxime suggesting that its transformation was biologically driven. Zhang et al. (2017) isolated a bacterial consortium composed of an *Aminobacter* strain which was able to hydrolyze methomyl (a chemical homologue to oxamyl) to methomyl oxime, this was further degraded, at a slower rate, by the second member of the consortium an *Afipia* strain. In our study further attempts to isolate oxamyl oxime – degrading bacteria following the same enrichment culture method failed, suggesting that its transformation in soil is probably a co-metabolic process performed by non-specialized soil bacteria or fungi. Either way, the hydrolysis of oxamyl is considered a detoxification step since oxamyl oxime is more than an order of magnitude less toxic to mammals, aquatic organisms and earthworms compared to the parent compound (European Food Safety Authority [EFSA], 2005).

The accumulation of oxamyl oxime in the bacterial cultures indicates that the oxamyl-degrading isolates were not able to exploit this metabolic product as an energy source. Thus, it seems probable that the oxamyl-degrading isolates could utilize the methyl-carbamoyl moiety that is released during hydrolysis of oxamyl as a C and N source. This is further supported by the high mineralization levels of the ^{14}C-carbamoyl-labelled oxamyl by all isolates. This pathway is common among

carbamate-hydrolyzing bacteria (Karns et al. 1986; Chaudhry and Ali, 1988; Hashimoto et al. 2006) and leads to the transient formation of carbamic acid, which is unstable and it is rapidly broken down to methylamine and CO_2 (Karns et al. 1986; Feng et al. 1997; Bachman and Patterson 1999). The former product could be utilized as a C and N source through the C1 metabolism of bacteria (Chistoserdova et al. 2009) or solely as N source as it was recently demonstrated with a carbaryl-degrading *Pseudomonas* strain C5pp (Kamini et al. 2018a). These observations are in line with the proven ability of our isolates to utilize oxamyl as C and N source and with their capacity to grow on methylamine. Methylotrophy has been identified as a common feature of carbofuran-degrading bacteria (Chaudhry and Ali 1988; Singh et al. 1993; Topp et al. 1993). Trabue et al. (2001) demonstrated that the use of 2 μg g^{-1} of carbofuran in an adapted soil could lead to the production of adequate amount of methylamine to sustain a significant increase in the carbofuran-degrading microbiota using it as a C and N source. Further evidence for the capacity of our isolates to utilize methylamine was provided by the detection of a methylamine dehydrogenase gene in three of the four oxamyl-degrading strains. Methylamine dehydrogenase is one of the hallmark genes in methylotrophy responsible for the oxidation of methylamine to formaldehyde, which is then assimilated via different paths (Chistoserdova 2011). The non-detection of methylamine dehydrogenase in the strain OXA17 might be a result of the limited coverage of the primers utilized or the presence of an alternative pathway, like the *N*-methylglutamate oxidation pathway of methylamine (Latypova et al. 2010) which was recently showed to be involved in the utilization of methylamine produced during the hydrolysis of carbaryl by the *Pseudomonas* sp. strain C5pp. (Kamini et al. 2018a). Based on all the above a microbial transformation pathway of oxamyl is proposed (Figure 2.10).

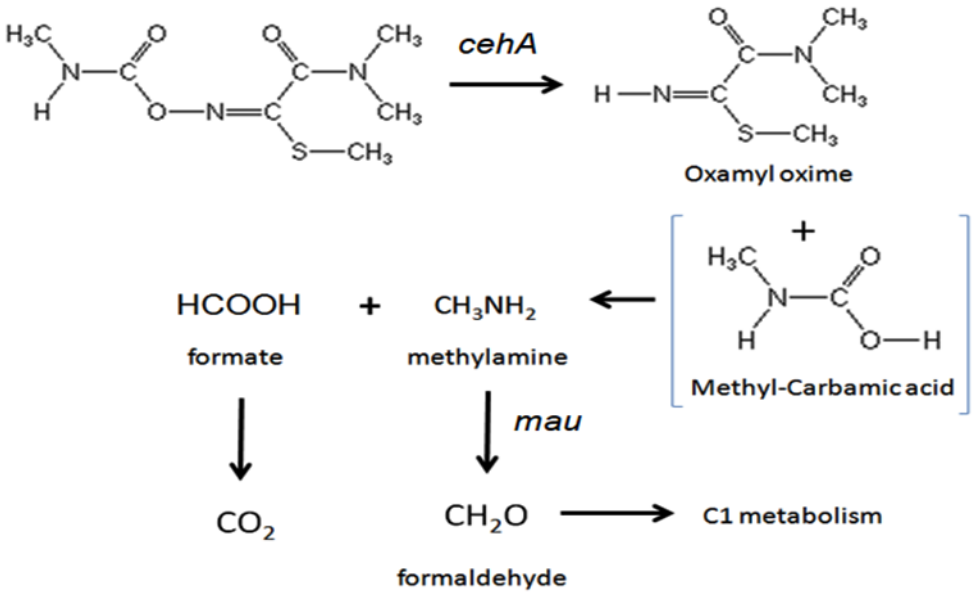


Figure 2.6. The proposed transformation pathway of oxamyl by the isolated bacteria. The steps of the pathway controlled by the genes *cehA* and methylamine dehydrogenase (*mau*) are indicated.

The oxamyl-degrading strains were found to possess the carbamate-hydrolase gene *cehA* detected in a plasmid of the carbaryl-degrading *Rhizobium* strain AC100 (Hashimoto et al. 2002) and in a transposon of the carbofuran-degrading *Novosphingobium* strain KN65.2, (Nguyen et al. 2014). Transcription analysis of the *cehA* gene along the degradation of oxamyl verified its direct involvement in the hydrolysis of oxamyl to oxamyl oxime. Further comparative analysis of the *cehA* genes of different origin showed that the $cehA_{OXA}$ gene sequences were identical (OXA18) or nearly identical (OXA17, OXA20, and OXA25) to $cehA_{AC100}$ (Hashimoto et al. 2002) and shows a single amino acid substitution (Leu instead of Phe) at residue 152 with the translated product of $cehA_{CDS-1}$ found in the carbofuran-degrading strain *Sphingomonas* CDS-1 (Yan et al. 2018). On the other hand a higher sequence divergence was observed with $cehA_{KN65.2}$ of the carbofuran-degrading *Novosphingobium* strain KN65.2 (Nguyen et al. 2014). The environmental relevance of *cehA* for the soil biodegradation of oxamyl is supported by the data presented in Chapter 3 (Rousidou et al. 2017) which observed a significant positive correlation between the degradation rates of oxamyl in 16 agricultural soils from a potato monoculture area in Crete and the abundance of the *cehA* gene.

Strain OXA20, selected as a representative of the oxamyl-degrading isolates available, rapidly degraded both the oximino carbamates aldicarb and methomyl, structurally similar to oxamyl, and the aryl-methyl carbamates carbofuran and carbaryl, which resemble oxamyl only at the methyl-carbamoyl moiety. These results further support our initial suggestion that these bacteria utilize the methyl-carbamoyl moiety produced by the hydrolysis of oxamyl as C and N source. The relaxed substrate specificity exhibited by the strain OXA20 is not common among carbamate-degrading bacteria. Yan et al. (2007) isolated a carbofuran-degrading *Novosphingobium* strain, which was able to degrade carbaryl but not methomyl. Similarly, a methylotrophic carbofuran-degrading strain was able to metabolize carbaryl but not methomyl and aldicarb (Topp et al. 1993). Although the protein encoded by the *cehA* gene of our isolates was not obtained and tested, the capacity of the strain OXA20 to degrade carbofuran and other carbamates is against the initially reported lack of carbofuran degradation activity by the *Rhizobium* strain AC100 (its capacity to degrade oximino carbamates like oxamyl was not tested; Hashimoto et al. 2002). However more recent studies by Yan et al. (2018) demonstrated that $CehA_{AC100}$ was able to recognize carbofuran as a substrate, albeit with low catabolic efficiency.

In line with all the other CehA homologues, $CehA_{OXA}$ contained a Sec or TAT signal peptide which suggests extracytosolic function (Ozturk et al. 2016; Yan et al. 2018). In support of this Kamini et al. (2018b) showed that McbA, a new carbamate hydrolase isolated from a carbaryl-degrading *Pseudomonas* strain, has a transmembrane domain and a signal peptide which facilitates its folding and transportation in the periplasm. The periplasmic and cytosolic localization of McbA and the 1-napthol-transformation enzymes (lower pathway of carbaryl transformation) respectively, was identified by the authors as an elegant strategy for efficient degradation of high concentration of carbamates without cellular toxicity of 1-napthol. A similar periplasmic localization of $CehA_{OXA}$ is possible despite the lower cellular toxicity of oxamyl oxime compared to 1-napthol.

2.5 CONCLUSION

We report the isolation and identification of four oxamyl-degrading *Pseudomonas* strains. The isolated bacteria were capable of hydrolyzing oxamyl to oxamyl oxime, which was not further transformed, and methylamine which was utilized as a C and N source with concurrent mineralization of the carbamoyl moiety. All the strains isolated carried the *cehA* gene, a carbaryl hydrolase gene, which was shown by transcription analysis to be responsible for the hydrolysis of oxamyl.

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CHAPTER 3

Distribution and function of carbamate hydrolase genes *cehA* and *mcd* in soils: the distinct role of soil pH

The work presented in Chapter 3 is included in the following article:

Rousidou, C., Karaiskos, D., Myti, D., Karanasios, E., Karas, P. A., Tourna, M., Tzortzakakis, E.A., Karpouzas, D. G. (2016). Distribution and function of carbamate hydrolase genes *cehA* and *mcd* in soils: the distinct role of soil pH. *FEMS Microbiology Ecology*, *93*(1). doi:10.1093/femsec/fiw219

3.1 INTRODUCTION

Synthetic carbamates constitute one of the major groups of synthetic insecticides on the global market (Casida and Durkin 2013). They are synthetic analogues of the natural carbamate physostigmine, which was detected in the seeds of the poisonous legume *Physostigma venenosum* and acts as a reversible inhibitor of acetylcholinesterase (Zhao et al. 2004). The recent market ban on several carbamates (i.e. aldicarb, carbofuran and pirimicarb) made oxamyl the most important member of this group. Oxamyl is an oximino carbamate used in areas of potato cultivation for the control of potato cyst nematodes (*Globodera* sp.). Oxamyl is not persistent in soil (DT_{50} of 1.1–33 days) (Gerstl 1984; Osborn et al. 2010) and is hydrolysed releasing the carbamoyl moiety and oxamyl oxime (Rousidou et al. 2016). The latter does not persist and is further degraded by soil microorganisms in a co-metabolic process.

Several reports have stressed the key role of the soil microbiota (Smelt et al 1987; Osborn et al. 2010) and pH (Smelt et al. 1983) on the degradation of oxamyl in soil. Smelt et al. (1996) demonstrated that strong interactions between pH and the soil microbiota determine the development of accelerated biodegradation of oxamyl. This phenomenon is common in regions of potato cultivation where the limited number of available soil insecticides/nematicides and the monoculture character of potato cultivation force growers to use the same chemical in the same field for a number of years, favouring the development of accelerated biodegradation (Karpouzas et al. 1999; Karpouzas et al. 2004; Papadopoulou et al. 2016).

Bacteria able to rapidly degrade carbamates like carbofuran (Karpouzas et al. 2000; Desaint et al. 2000; Shin et al. 2012), carbaryl (Hashimoto et al. 2002) and oxamyl (Chapter 2 - Rousidou et al. 2016) have been isolated from soils exhibiting accelerated biodegradation and the genes involved in the hydrolysis of carbamates were identified. Tomasek and Karns (1989) first isolated a plasmid-encoded carbofuran-hydrolase gene *mcd* from *Achromobacter* WM111. Studies that followed detected *mcd* in carbofuran-degrading bacteria isolated from Europe (Parekh et al. 1996) and Canada (Topp et al. 1993). More recent studies reported the isolation of two carbaryl-hydrolase genes, namely *cehA* and *cahA*, from a *Rhizobium* (Hashimoto et al. 2002) and an *Arthrobacter* strain, respectively (Hashimoto et al. 2006).

Homologues of the *cehA* gene with high levels of conservation (99% homology) were then detected in oxamyl-degrading *Pseudomonas* strains from Greece ($cehA_{OXA}$) (Rousidou et al. 2016), in a carbofuran-degrading *Novosphingobium* strain KN65.2 from Vietnam ($cehA_{KN65.2}$) (Nguyen et al. 2014) and recently in a carbofuran-degrading *Sphingomonas* strain CDS-1 ($cehA_{CDS-1}$) (Yan et al. 2018). All we know about these genes comes from studies either with axenic cultures of carbamate-degrading bacteria or with the purified carbamate hydrolases encoded by the *cehA* (Ozturk et al. 2016; Yan et al. 2018) and *mcd* genes (Naqvi et al. 2009). Little is known about the true magnitude of their distribution in soils and the environmental factors controlling their soil dispersal. In addition, their role in the *in situ* biodegradation of carbamates in soil is still not proven. Novel information on these issues will provide a good estimation of the biodegradation potential of soils against carbamates. From an agricultural perspective this will allow the timely implementation of measures for preventing the establishment of accelerated biodegradation and potentially reduced agronomic performance.

The aims of this study were to (i) investigate the role of the known carbamate hydrolase genes in the soil biodegradation of carbamate pesticides, (ii) explore the distribution of the carbamate hydrolase genes, and (iii) identify the factors that drive their occurrence in soils in association with the degradation of carbamates.

3.2 MATERIALS AND METHODS

3.2.1 Pesticides

Analytical grade oxamyl (99.6%), carbofuran (99.9%), iprovalicarb (99%), pirimicarb (99%), prosulfocarb (99%) (PestanalR, Fluka, Switzerland) and oxamyl oxime (100%, DuPont, USA) were used for all soil experiments and analysis. Standard solutions of these chemicals were prepared in methanol (carbofuran, prosulfocarb), acetonitrile (iprovalicarb) and mixtures of acetonitrile– water (20:80 v:v for oxamyl and oxamyl oxime; 50:50 v:v for pirimicarb) and used for chromatographic analysis.

3.2.2 Soils

The agricultural soils used for studying the degradation of oxamyl and other carbamates were collected from the plateau of Lasithi, which constitutes one of the most important potato monoculture areas in Greece characterised by long history of oxamyl use. Soil samples were collected from 16 field sites (Table 3.1, Fig. 3.1) during annual surveys (years 2010 and 2013) for the detection of potato cyst nematodes. Pristine soils were collected in 2015 from forests, grasslands and organic farming field sites from Greece and Chile (Table 3.2). Pristine soils were not exposed to any pesticides for at least the last 20 years.

Table 3.1 The DT_{50} values of oxamyl in the studied soils from the plateau of Lasithi. The coordinates of the field sites from the soils used in the study and the physicochemical properties of the soils are listed. DT_{50} values were calculated after fitting the first order kinetics (FOK) model unless otherwise indicated.

Soils	Coordinates	DT_{50} (days)	pH	Organic C content (%)
L2	35°11'28.2"N, 25°28'33.0"E	3.7[a]	6.93	1.06
L4	35°11'46.8"N, 25°28'33.5"E	18.2	4.85	1.47
L5	35°10'35.1"N, 25°28'11.7"E	4.4[a]	5.48	1.46
L6	35°10'30.2"N, 25°27'42.8"E	8.7	4.81	2.01
L7	35°10'18.3"N, 25°27'48.7"E	21.0	5.07	1.24
L8	35°10'17.8"N, 25°27'43.2"E	17.3	4.06	1.72
L9	35°10'07.3"N, 25°27'15.6"E	7.8	5.20	2.04
L10	35°10'39.2"N, 25°27'19.8"E	9.9[a]	4.25	1.86
L11	35°09'59.3"N, 25°29'11.1"E	10.3	4.52	1.17
L12	35°09'55.5"N, 25°27'39.4"E	26.7	4.14	1.58
L13	35°11'39.7"N, 25°29'22.0"E	4.5[a]	6.57	0.95
L14	35°11'04.7"N, 25°28'40.8"E	3.2[a]	7.69	1.19
L15	35°10'33.6"N, 25°27'52.4"E	3.4[a]	6.19	1.27
L16	35°10'34.6"N, 25°27'44.1"E	3.2[a]	6.85	1.68
L17	35°10'44.2"N, 25°27'19.5"E	11.4	5.30	1.46
L18	35°11'30.4"N, 25°27'47.7"E	2.4	7.87	0.83

[a]DT50 values were calculated by fitting the biphasic hockey-stick kinetic model.

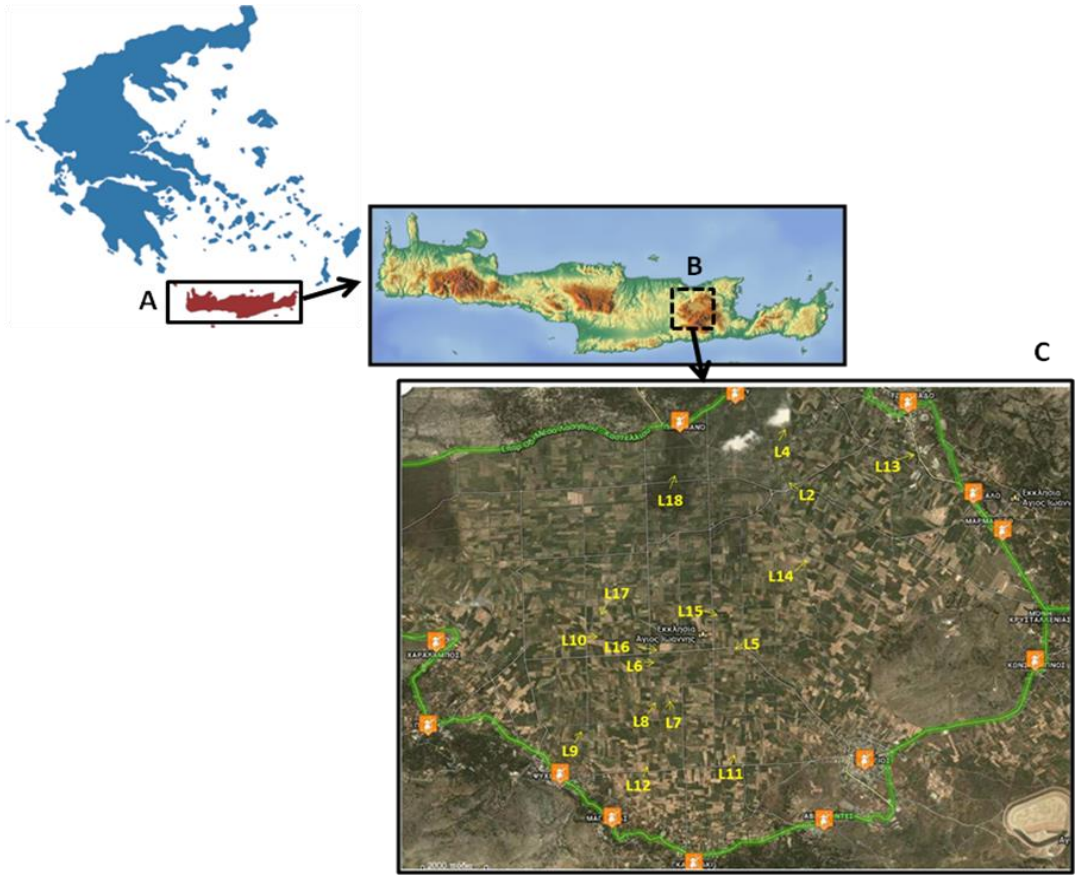

Figure 3.1 A map of the Lasithi Plateau (B) found in Crete (A). The exact position of the field sites from where the studied soils were collected is indicated (C)

From each site (agricultural and pristine), five subsamples were collected from the top 0–20 cm following the W non-systematic pattern of sampling, according to ISO 10381-1 and -2 guidelines (ISO, 2002), and mixed thoroughly to provide a single bulk soil sample per field site. Soils were partially air-dried overnight, if necessary, sieved to pass a 3-mm mesh and stored at 4°C until further processed (storage period never exceeded 30 days). The physicochemical properties of all soils were determined (Tables 3.1 and 3.2). Organic carbon content was measured according to Walkley and Black (1934). Soil pH was measured in mixtures of air-dried soil–deionised water (1:2 w/v). Soil texture was determined using the Bouyoucos hydrometer method (Sheldrick and Wang 1993). Moisture content was determined by oven-drying subsamples at 110◦C for 24 h. Water holding capacity was measured gravimetrically following saturation of the soil (30 g) with distilled water in a funnel with Whatman no. 1 filter paper and allowing it to drain for 24 h.

Table 3.2 The location, uses and physicochemical properties of the pristine soils studied. The copy numbers of the *cehA* gene were determined for all pristine soils 69 days after repeated applications of the carbamate pesticides oxamyl and carbofuran

Soils	Use	Coordinates	pH	Organic C content (%)	Clay (%)	Silt (%)	Sand (%)	*cehA* copies g^{-1} soil
NAGREF	Organic olive tree field	37°06' 51.9" N, 21°58' 92.2" E	8.05	0.91	33.2	50.4	16.4	55.5±11.9[a]
Goritsa	Organic olive tree field	37°1'51.75" N, 21°33' 59.10" E	7.92	0.39	34.2	20.4	45.4	124.4±35.2
Kendrinos Lofos	Forest	40°38' 04.12" N, 22°47' 26.29" E	7.53	3.06	7.2	24.4	68.4	155.5±29.8
Kisavos Forest	Forest	39°48' 45.48" N, 22°47' 47.68" E	7.23	5.13	9.2	30.4	60.4	596.6±233.4
Pyrgetos I	Organic vineyard	39°56' 9.45" N, 22°37' 19.53" E	7.94	1.56	23.2	28.4	48.4	90.7±49.3
Kisavos Walnut	Organic walnut tree field	39°49' 1.92" N, 22°47' 47.68" E	6.45	2.62	8.2	27.4	64.4	bld[b]
Krania	Forest	39°55' 49.74" N, 22°33' 19.66" E	6.22	2.93	11.2	22.4	66.4	bld
Kalamata	Organic olive tree field	37°16' 51.64" N, 21°58' 44.02" E	6.22	1.02	13.2	30.4	56.4	bld
Pyrgetos II	Organic vineyard	39°55' 42.91" N, 22°38' 9.99" E	6.80	0.77	21.2	34.4	44.4	bld
Maquehue	Grassland	38°01' 25.1" S, 72°40' 00" W	5.60	6.25	5.2	24.4	70.4	bld

[a] average ± the standard deviation of the mean
[b] below the limit of detection

3.2.3 Soil Microcosm Experiments

3.2.3.1 Oxamyl degradation and abundance of the carbamate hydrolase genes in agricultural soils

Soil samples (200 g) from each field site received an equal volume of an aqueous solution of oxamyl (240 mg L^{-1}) prepared by its commercial formulation (VYDATE®, 240 g L^{-1}) resulting in an oxamyl soil concentration of 10 μg g^{-1} soil dry weight. Distilled water was added to all soils to adjust the moisture content to 40% of their water holding capacity, and samples were briefly mixed by hand. All samples were then divided into 15 subsamples of 10 g (3 replicates × 5 sampling times), which were placed in aerated plastic bags and incubated in the dark at 25°C. Immediately before incubation and 3, 7, 14 and 30 days post-application triplicate, subsamples were analysed for oxamyl and oxamyl oxime residues via HPLC. The weight of all subsamples was recorded at the beginning of the incubation and used to maintain the soil moisture content during the incubation. Apart from chemical analysis, triplicate samples (5 g) from each soil were removed prior to pesticide application and used for the determination of the abundance of the *cehA*, *mcd* and *cahA* genes by q-PCR.

3.2.3.2 Degradation of other carbamates and the dynamics of cehA and mcd genes in soil

We further investigated the interactions of the *cehA* and *mcd* genes with carbamate pesticides previously or currently used in the study region. Five samples (190 g) from the field site Lasithi 15 (L15), where both the *cehA* and *mcd* genes were initially detected, were treated with aqueous solutions (190 mg L^{-1}) of the carbamate pesticides oxamyl, carbofuran, pirimicarb and methanolic solutions (475 mg L^{-1}) of prosulfocarb and iprovalicarb (insoluble in water). These applications aimed at a final pesticide concentration in soil of 5 μg g^{-1}. A final sample of the same soil received the same amount of methanol and water without pesticide to serve as control. The soil samples treated with aqueous pesticides solutions were also treated with methanol for comparative purposes. All soil samples (pesticide-treated and control) were then divided into 15 subsamples (10 g) (3 replicates × 5 sampling times), which were placed in aerated plastic bags and incubated as described above. Immediately after pesticides application and 3, 7, 14 and 28 days later triplicate subsamples from each

treatment were analysed for pesticide residues or used for DNA extraction and q-PCR determination of the abundance of the *cehA* and *mcd* genes.

3.2.3.3 Expression of the *cehA* and *mcd* in soil treated with oxamyl and carbofuran

To further verify the involvement of the *cehA* and *mcd* genes in the degradation of oxamyl and/or carbofuran, their expression in soil during degradation of a fresh pesticide addition was determined. Three soil samples from the field site L15 (3 × 300 g) were prepared. The first two samples received four successive applications of oxamyl and carbofuran (applied as aqueous solutions of 190 mg L^{-1}) aiming each time at a pesticide soil concentration of 5 μg g^{-1}. In parallel the third sample received the same volume of water without pesticides to serve as untreated control. Successive applications were performed only after the degradation of the previous pesticide addition was completed (as determined by HPLC). Right after the fourth pesticide or water addition, the three samples (oxamyl-treated, carbofuran-treated and water-treated control) were separated into 15 subsamples (10 g) (3 replicates × 5 sampling times), which were placed in aerated plastic bags and incubated as described above. Immediately after the fourth pesticide application (time 0 h) and at regular intervals thereafter triplicate subsamples were removed from the incubator and stored at –20°C for determination of pesticide concentration or at –80°C for DNA/RNA extraction and determination of the abundance and expression of the *cehA* and *mcd* genes.

3.2.3.4 Degradation of oxamyl and carbofuran in pristine soils and occurrence of the *cehA* and *mcd* genes

Based on their widespread occurrence in agricultural soils, we explored the distribution of carbamate hydrolase genes in pristine soils from different regions (Table 3.2). Three replicate samples (100 g) from each pristine soil were treated at 0, 26 and 48 days with a mixture of oxamyl and carbofuran (aqueous solution 200 mg L^{-1}) aiming each time at a final soil concentration of 5 μg g^{-1} for each pesticide. In parallel, triplicate samples from each pristine soil (100 g each) were treated on the

same days with the same volume of water without pesticide to serve as untreated controls. Immediately after pesticide and water application, samples were mixed by hand, placed in aerated plastic bags and incubated as described above. At 0, 14, 26 (before and after pesticide application), 33, 48 (before and after pesticide application) and 69 days, subsamples (10 g) were removed from each replicate sample and analysed for pesticide residues by HPLC. A fraction (0.5 g) of the samples collected at the end of the incubation (69 days) was also processed for DNA extraction to determine the abundance of *cehA* and *mcd* genes.

3.2.4 Pesticides Analysis

Residues of oxamyl and oxamyl oxime were extracted from soil (5 g) by shaking (in an orbital shaker at 150 rpm for 2 h) with 10 mL of an acetonitrile–water mixture (50:50 v:v). Carbofuran, iprovalicarb, pirimicarb and prosulforcarb were extracted from soil (5 g) by shaking with 10 mL of methanol, acetonitrile, a mixture of 50% acetonitrile + 50% H_2O + 0.01 M $CaCl_2$, and 98% methanol + 1% H_2O + 1% H_3PO_4, respectively. Samples were then centrifuged for 10 min at 6606 × g and the clear supernatant was removed and filtered (Whatman no. 3) before analysis.

Pesticide residues were determined in an HPLC-UV system equipped with a GraceSmart RP C18 column (150 mm × 4.6 mm; Grace Davison Discovery Sciences, USA). In all cases the flow rate of the mobile phase was 1 mL min^{-1}. A mobile phase of acetonitrile–water of 20:80 (v:v) and 40:60 (v:v) was used for the elution of oxamyl/oxamyl oxime and carbofuran, respectively. Detection of oxamyl/oxamyl oxime and carbofuran was achieved at 220 and 215 nm, respectively. Pirimicarb was eluted with a mobile phase of 55:45 (v:v) acetonitrile–water and detection was achieved at 245 nm. Prosulfocarb and iprovalicarb were analysed as described by Gennari et al. (2002) and Maity and Mukherjee (2009), respectively.

Pesticide residues were quantified by the external standard method using the calibration curves obtained by injection of standard solutions. Good linearity responses were obtained for all the compounds in the ranges studied (0.05–10 μg mL^{-1}). In order to verify the efficiency of the extraction methods described above,

samples from soil L15 were spiked with each of the studied pesticides at three concentration levels (0.05, 0.5 and 5.0 μg g−1). Triplicates per concentration level were processed. The mean percentage recovery for all pesticides in the three concentration levels tested exceeded 85%.

3.2.5 q-PCR of carbamate hydrolase genes in soil

DNA and RNA were extracted from 0.5 and 2 g of soil using the Power Soil DNA and Power Soil RNA Isolation Kit, respectively (MoBio Laboratories, Inc.). DNA/RNA integrity was checked by electrophoresis and they were quantified using a QubitTM fluorometer (Invitrogen).

q-PCR for the carbamate hydrolase genes *cehA*, *mcd* and *cahA* and for the 16S rRNA gene were performed in 10–20 μl reactions containing 1× KAPA SYBRR FAST qPCR Master Mix (2×) Universal, 0.2 μM of each primer, 50 nM ROX Low, 400 ng μL^{-1} BSA, and 10–50 ng DNA for the amplification of the carbamate hydrolase genes or 0.5 ng DNA for the amplification of the 16S rRNA gene. In particular cases (expression of the *mcd* gene) the SSO Advanced SYBR master mix (2×) was used as described above with the only modification being the concentration of primers, which was adjusted to 0.3 μM. The hydrolase-specific primers utilized were designed based on the sequences of the respective genes (Table 3.3). The specificity of the designed primers was verified in samples from the soil L5 fortified with plasmid DNA (pGEM T-Easy vector) containing the target genes as inserts (no background detection of the target genes was observed in preliminary tests). Amplicons obtained from these tests and from the experiments performed with the agricultural soils and the pristine soils (all soils with positive amplification of *cehA*) were cloned. Overall 15 clones of the *cehA* gene (two and 13 from agricultural and pristine soils, respectively) and six clones of the *mcd* gene were sequenced. The sequences of all *cehA* clones were either identical or showed one to two mismatches with the sequence of the *cehA* gene of *Rhizobium* sp. strain AC100 (Accession No. NG 035559.1). All *mcd* clones sequences showed either 100% match or 1 bp difference with the *mcd* sequence of the strain *Achromobacter* WM111 (Accession No. AF160188). Sequences of the *cehA* and *mcd* genes were deposited in the NCBI

database under the accession numbers KX710264– KX710278 and KX710279–KX710286 respectively. The thermocycling conditions used for the amplification of the carbamate hydrolase genes were as follows: 3 min at 95°C; 45 cycles of 15 s at 95°C, 60 s at 63°C for *cehA*, 20 s at 62°C for *cahA* and 20 s at 63°C for *mcd*, 45 s at 72°C; followed by a melting curve to check the specificity of the products. The 16S rRNA gene was amplified as described by Fierer et al. (2005). The copy numbers of the target genes in soil samples were determined via external standard curves constructed as described before (Rousidou et al. 2013). q-PCR amplification efficiencies ranged from 85.9–104.1% with r^2 values >0.986 for all genes. The limit of detection for the *cehA* and *mcd* genes was *ca*. 53 and 70 copies per gram of soil

The expression of the *cehA* and *mcd* genes in soil L15 was determined via RT-q-PCR. Soil RNA (~267 ng) was treated with DNase I (1 U μL–1) Amplification Grade (Invitrogen). The absence of DNA contamination was further confirmed by PCR of the 16S rRNA gene (Rousidou et al. 2013). DNA-free RNA was then reverse-transcribed to obtain cDNA (kit Superscript II, Invitrogen) using random hexamers (Invitrogen). Amplification of cDNAs for the *cehA* and *mcd* genes was performed as described above for DNA.

Table 3.3 Primers used for the enumeration of carbamate-catabolic genes and/or gene transcripts in a variety of soils used in this study. Primers used for detection and/or enumeration of 16S rRNA gene of total bacteria are also included.

Primers name	Gene target	Sequence (5'-3')	Fragment Size (bp)	Reference
cehAF-rtPCR	*cehA*	ACCAACGCTCTACCAAATTACG	156	Rousidou et al. (2016)
cehAR-rtPCR		GCAGTTGAGCAGATGATACCAC		
mcdF-rtPCR	*mcd*	CACGCACTTCTTCAGAGATCAC	168	This study (AF160188)[a]
mcdR-rtPCR		GCGAAATGATGCCAATAGACCG		
cahAF-rtPCR	*cahA*	CTGGAGAGATCGTTGGACCG	190	This study (AB081302)[a]
cahAR-rtPCR		CACTCGTGTAGGTTCGTTTTGC		
Eub338_F	16S rRNA	ACTCCTACGGGAGGCAGCAG	190	Fierer et al. (2005)
Eub518_R		AATTACCGCGGCTGCTGG		

[a]GeneBank accession number of reference sequences used for the design of primers.

3.2.6 Data analysis

Pesticide degradation data were described by first order kinetics (FOK) or by the biphasic hockey-stick (HS) kinetic model (FOCUS 2006). The formation and decay pattern of the hydrolysis product of oxamyl, oxamyl oxime, were described by a model consisting of two first-order equations, each describing the formation and the decline phase (Bergstrom, Borjesson and Stenstrom 2011) and is given by equation (1):

$$C = \text{int} + \frac{MW_m / MW_p \cdot k_1 \cdot C_0}{k_2 - k}\left(e^{-kt} - e^{-k_2 t}\right) \qquad (1)$$

where C is the concentration of the chemical formed at a rate k_1 and degraded at a rate k_2, MW_m and MW_p are the relative molecular masses of the metabolite and the parent compound, respectively, and k and C_0 are the end points of the parent compound after using the FOK model. In cases where a delay in metabolite formation is observed an intercept (int) can be included.

Correlations between oxamyl DT_{50} values, soil properties and genes copy numbers were determined by the Spearman's correlation coefficient (r). Two-way ANOVA was used to determine the effect of time and pesticide application on the abundance and expression of the *cehA* and *mcd* genes. In cases where significant interactions between the two main factors were found, significant differences between treatments within each time point were determined by Tukey's post hoc test ($P < 0.05$). All statistical analysis was performed by using the SPSS 16.0 statistical program.

3.3 RESULTS

3.3.1 Degradation of oxamyl in agricultural soils

The degradation of oxamyl in the soils from the plateau of Lasithi was adequately described by the FOK model or the HS model ($\chi 2 < 15\%$ in nearly all cases)

(Table 3.1). In most soils the degradation of oxamyl coincided with the formation of oxamyl oxime with its highest concentrations detected in soils L13, 14, 15, 16 and L18 (Fig. 3.2). DT_{50} ranged from 2.4 days in soil L18, having the highest pH, to 26.7 days in soil L12, which had the second lowest pH from the soils studied (Table 3.1).

q-PCR analysis in the agricultural soils detected the *cehA* and *mcd* genes in 10 and 3 soils, respectively (Fig. 3.3). The *cehA* gene was the most abundant with its copies (g^{-1} soil) ranging from 2.3×10^3 to 8.5×10^5 compared with the *mcd* gene, which was detected at lower levels (1.3×10^2 –1.2×10^3 copies g^{-1}). The *cahA* gene was not detected in the soils studied.

The DT_{50} values of oxamyl were negatively correlated with pH (−0.872, $P < 0.01$) and the abundance of the *cehA* gene ($r = -0.717$, $P < 0.01$), whereas the latter was positively correlated ($r = 0.640$, $P < 0.01$) with pH (Table 3.4). The abundance of the *mcd* gene did not correlate with any of the parameters tested.

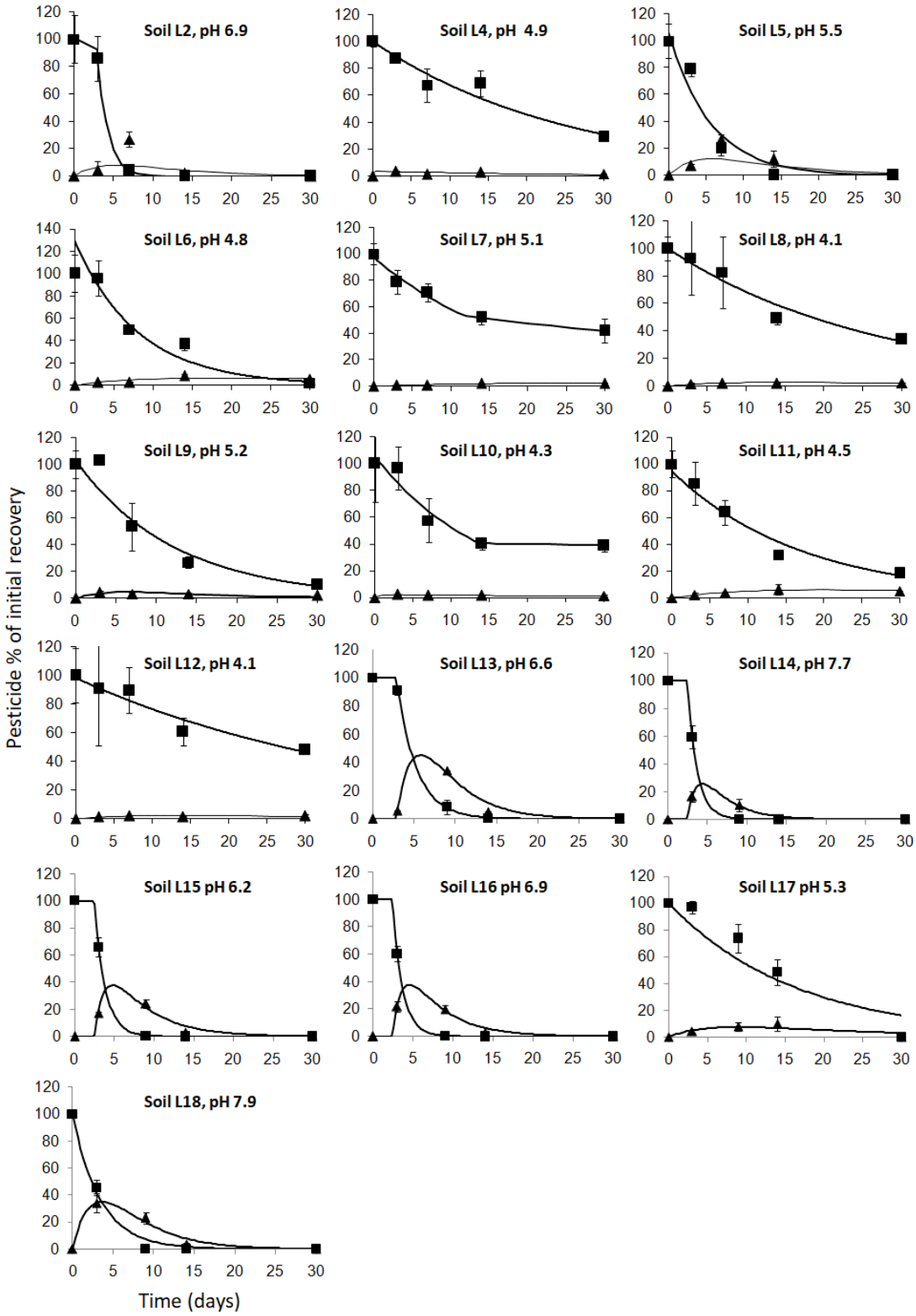

Figure 3.2 The dissipation of oxamyl (squares) and the formation of its hydrolysis product oxamyl oxime (triangles) in soils collected from the Plateau of Lasithi. Each value is the mean of three replicates ± standard deviation. The degradation patterns of oxamyl were fitted to the first-order kinetic (FOK) model or the biphasic hockey-stick (HS) model, whereas the formation and decay patterns of oxamyl oxime were fitted to a model described by Bergstrom et al. (2011).

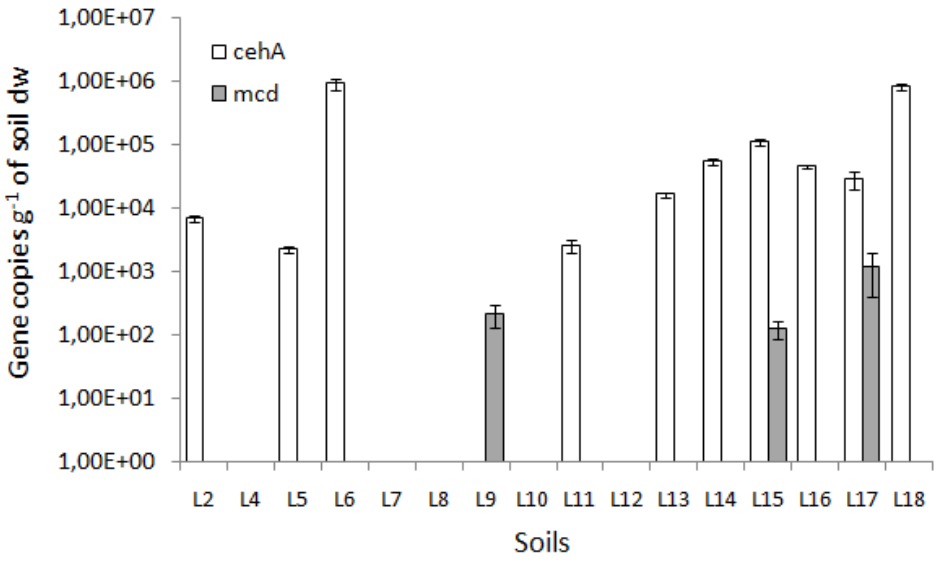

Figure 3.3 The abundance of the *cehA* and *mcd* genes in 16 agricultural soils from the potato monoculture area of the plateau of Lasithi as determined by q-PCR. Each value is the mean of three replicates ± standard deviation.

Table 3.4 The Spearman's correlation coefficient between physicochemical properties, DT_{50} of oxamyl and the abundance of the *cehA* and *mcd* genes in the soils studied. The level of significance is indicated by the asterisks (*, ** signify significant correlations at 0.05 and 0.01 respectively).

	DT_{50}	pH	Organic Carbon Content	*cehA*
pH	-0.872**			
Organic C content	0.352	-0.437		
cehA	-0.712**	0.640**	-0.354	
mcd	-0.017	0.078	0.214	0.164

3.3.2 Degradation of other carbamates and the dynamics of the *cehA* and *mcd* genes in soil

The dynamics of the *cehA* and *mcd* genes upon application of a range of carbamate compounds (oxamyl, carbofuran, pirimicarb, prosulfocarb and iprovalicarb) was investigated (see chemical structures in Fig. 3.4) in soil L15. Prosulforcarb and iprovalicarb are commonly used in the plateau of Lasithi as herbicides and fungicides, respectively, whereas carbofuran and pirimicarb (withdrawn from the market) were regularly used in the region up to 2005 and 2010, respectively. Oxamyl, carbofuran and prosulfocarb were almost fully degraded in 28 days, followed by iprovalicarb (>80% loss) and pirimicarb, which showed a slower degradation (Fig. 3.5A and B). The application of oxamyl and carbofuran induced a significant increase in the relative abundance (relative to the total bacterial abundance) of the *cehA* gene compared with the untreated samples and the samples treated with the other carbamates from day 7 onwards (Fig. 3.5C). The increase in the abundance of the *cehA* gene coincided with the rapid degradation phase of oxamyl and carbofuran. Carbofuran was the sole compound that induced a significant increase in the relative abundance of the *mcd* gene from day 7 onwards (Fig. 3.5D).

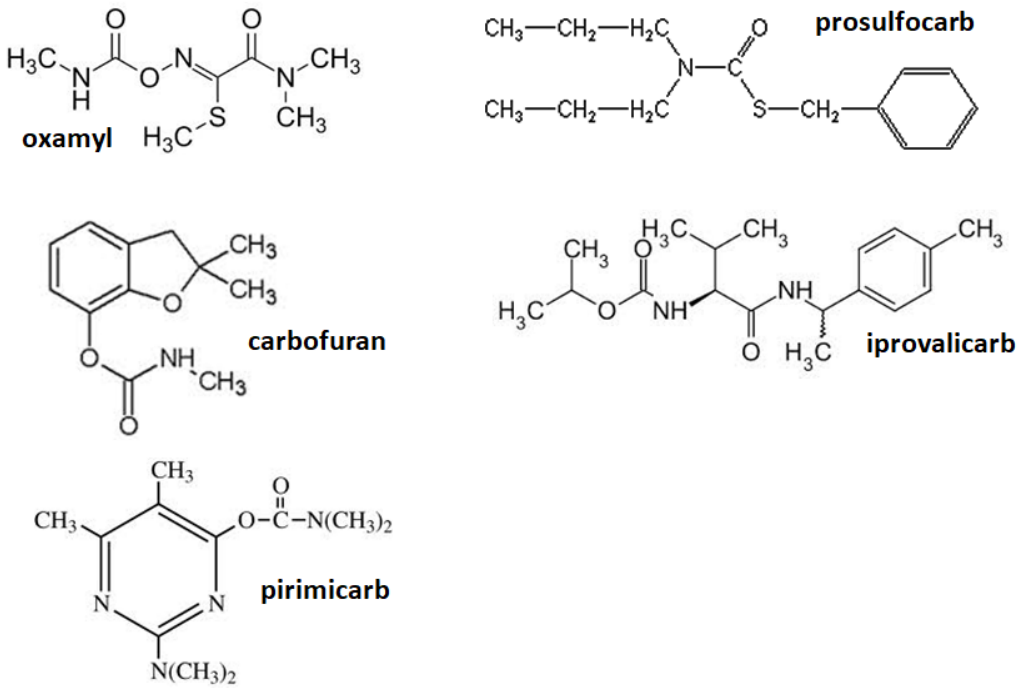

Figure 3.4 The chemical structures of the carbamates used in the current study

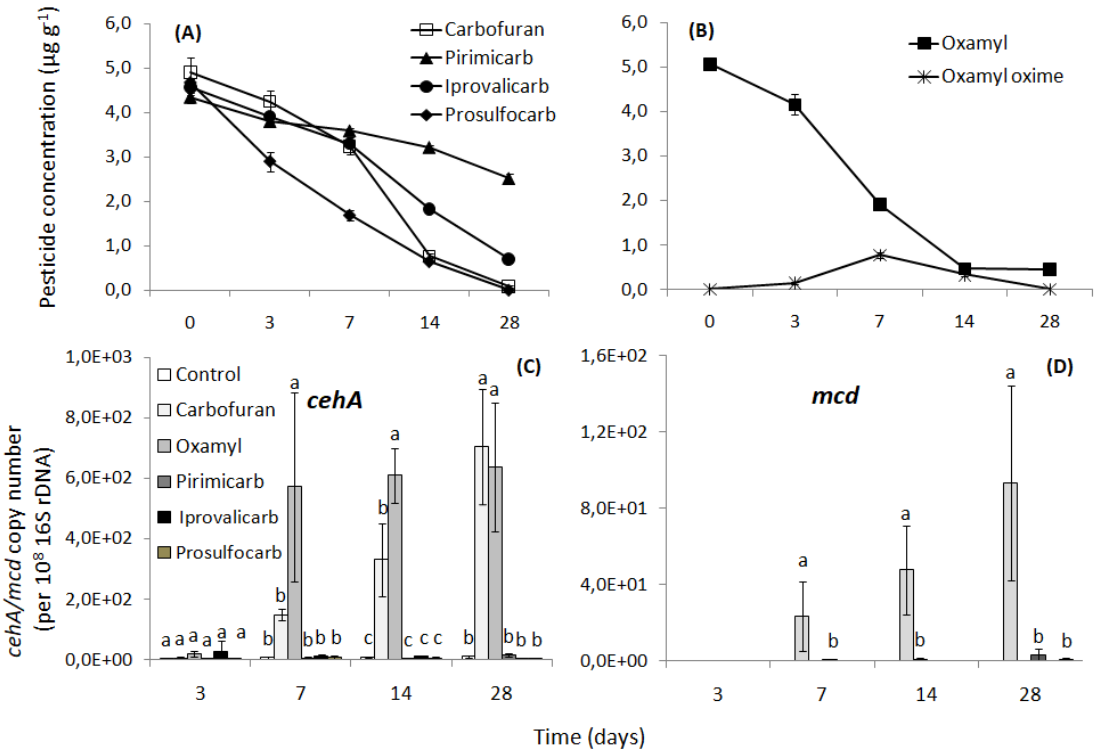

Figure 3.5 (A, B) The degradation of carbofuran, pirimicarb, iprovalicarb, prosulfocarb (A), and oxamyl and the formation of oxamyl oxime (B) in soil L15. **(C, D)** The dynamics of the *cehA* (C) and *mcd* genes (D) in carbamates-treated samples and in untreated samples (control) from soil L15. Each value is the mean of three replicates ± standard deviation. For each sampling time, bars designated by the same letter are not significantly different at the 5% level.

3.3.3 Expression of the *cehA* and *mcd* genes in soil L15 after treatment with oxamyl and carbofuran

Soil L15 was repeatedly treated four times with oxamyl or carbofuran and the degradation of the fourth pesticide addition was determined. Degradation of both compounds was particularly rapid with calculated DT_{50} values (according to the FOK model) of 2.8 and 5.6 h for oxamyl and carbofuran, respectively (Fig. 3.6A and B). q-PCR analysis showed a significantly higher ($P < 0.05$) abundance of the *cehA* and *mcd* genes in the pesticide-treated samples compared with the untreated control from 0 to 74 h post-application (Fig. 3.7). RT-q-PCR analysis in the same samples showed that a fourth fresh application of oxamyl and carbofuran induced a significant increase in the transcript numbers ($P < 0.05$) of the *cehA* gene at 3 and 6 h, respectively (Fig. 3.6C and D). This coincided with the onset of the rapid degradation of the two carbamates. The expression levels of the *cehA* gene in the treated samples further

increased until the degradation of the two carbamates was nearly complete (9 and 12 h) and reverted to the pro-application levels at 74 h when the degradation of oxamyl and carbofuran was completed. The transcript levels of the *cehA* gene in the carbamates-treated samples were significantly higher compared with the untreated control samples at all time points after pesticide application (Fig. 3.6 C and D). Regarding *mcd*, the application of carbofuran resulted in a significant increase ($P < 0.05$) in its transcription in the soil compared with the untreated soil where no transcripts of the *mcd* gene were detected (Fig. 3.6E).

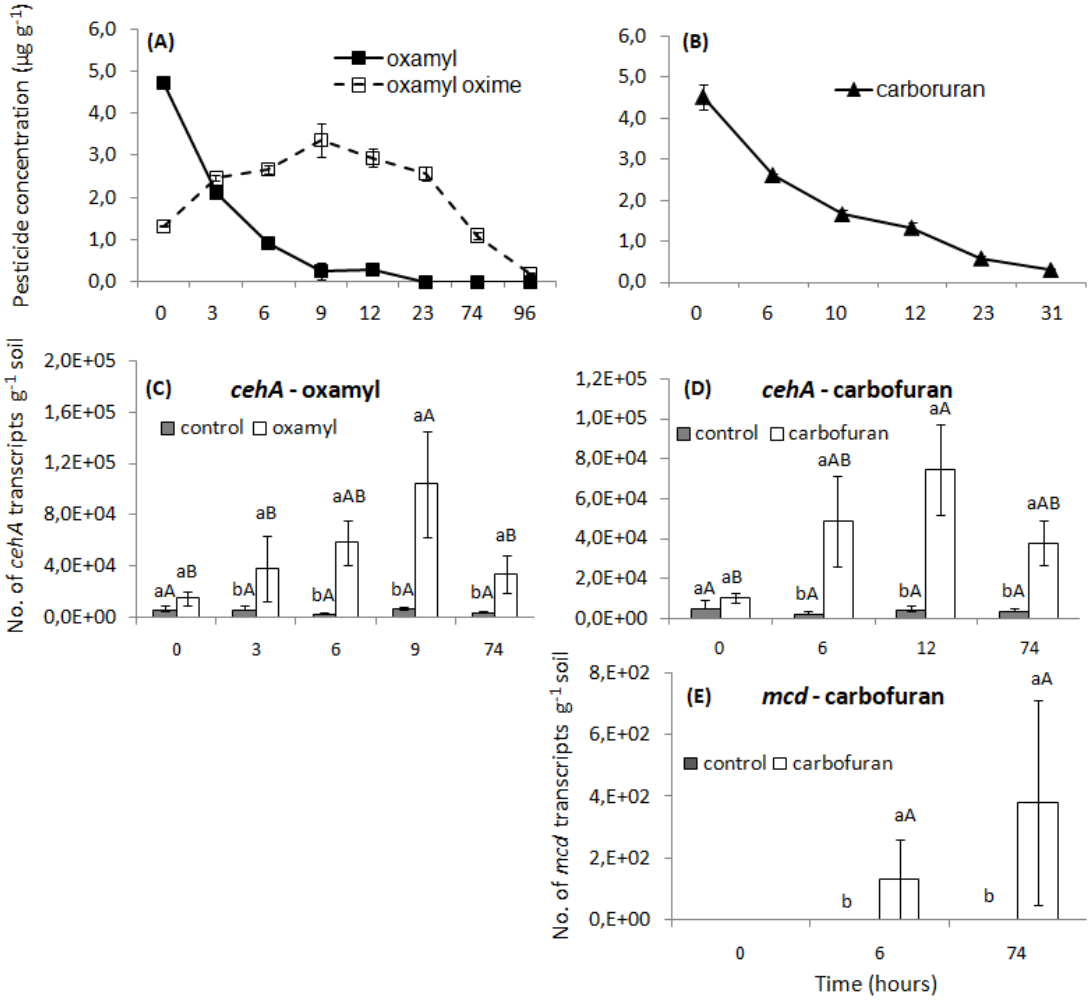

Figure 3.6 The degradation of oxamyl **(A)** and carbofuran **(B)** and the transcription patterns of the *cehA* **(C, D)** and *mcd* genes **(E)** in soil L15 from the plateau of Lasithi upon a fresh, fourth soil addition of oxamyl and carbofuran (white bars). The transcription patterns of the *cehA* and *mcd* genes in samples of the same soil that were not treated with oxamyl or carbofuran are also included in graphs (C, D, E) (grey bars). Each value is the mean of three replicates ± standard deviation. Within each sampling time, bars designated by the same lower-case letter are not significantly different at the 5% level. Within each treatment, bars designated by the same upper-case letter are not significantly different at the 5% level.

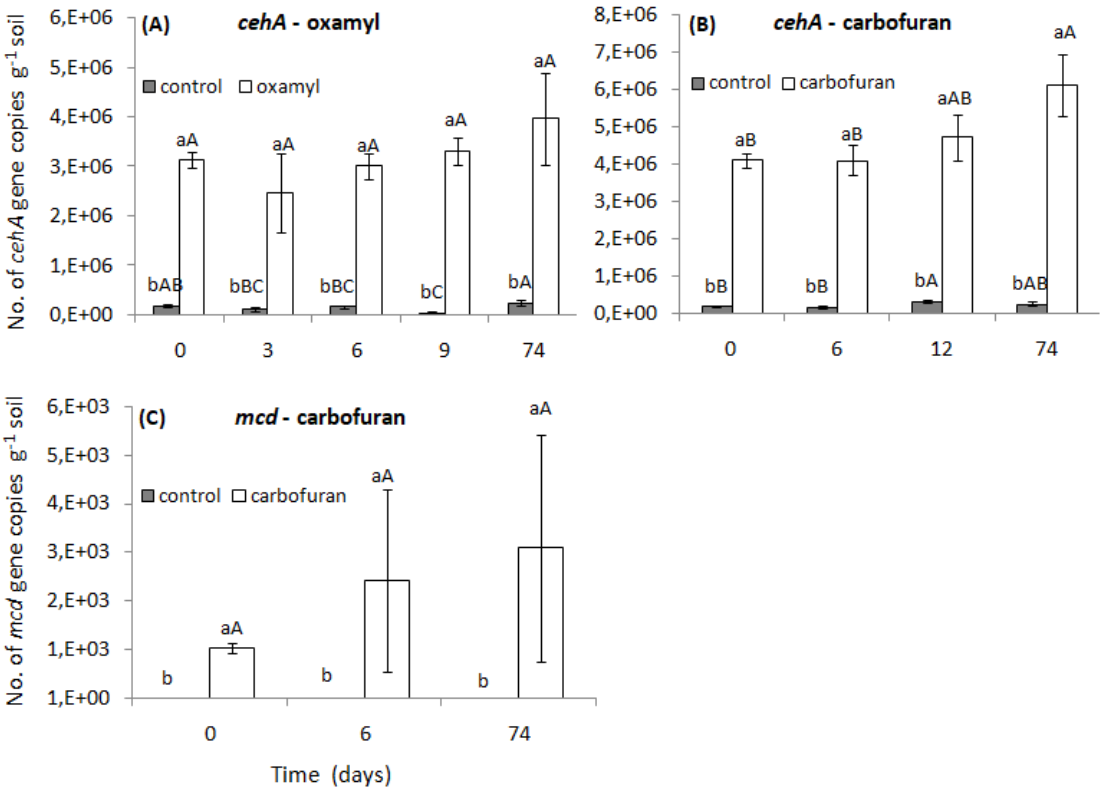

Figure 3.7 The abundance of genes *cehA* **(A, B)** and *mcd* **(C)** in soil L15 from the plateau of Lasithi upon a fresh fourth soil addition of oxamyl and carbofuran (white bars). The abundance of the same two genes in corresponding samples of the same soil which received no addition of oxamyl or carbofuran is also presented (grey bars). Each value is the mean of three replicates ± the standard deviation. Within each sampling time, bars designated by the same lower case letter are not significantly different at 5% level. Within each treatment, bars designated by the same capital letter are not significantly different at 5% level.

3.3.4 Degradation of oxamyl and carbofuran in pristine soils and the abundance of the *cehA* and *mcd* genes

The degradation of oxamyl and carbofuran in pristine soils successively treated (at 0, 26 and 48 days) with a mixture of the two pesticides is presented in Figures 3.8 (oxamyl) and 3.9 (carbofuran). The degradation of oxamyl in pristine soils was generally more rapid compared to carbofuran. This is clearly illustrated by the complete or nearly complete degradation of the third application of oxamyl in 6 of the 10 soils tested (Fig. 3.8) compared with carbofuran whose residues persisted in all soils by the end of the study (Fig. 3.9). The repeated applications of oxamyl resulted in the detection of the *cehA* gene (determined at the end of the incubation period, day

69), in five of the pristine soils tested (Table 3.2). This is in line with the complete degradation of oxamyl in those five soils (Fig. 3.8). Interestingly, these five pristine soils were those with the highest pH (pH ≥ 7.23). In contrast the *cehA* gene copy numbers were below the limit of detection in all pristine soils with pH ≤ 6.8. Regarding untreated pristine soils, the *cehA* gene was only detected in the soil NAGREF (273.3 ± 232.6 copies g^{-1}), which had the highest pH amongst the pristine soils studied (Table 3.2). Statistical analysis indicated a significant positive correlation between pH and the abundance of the *cehA* gene ($r = 0.681$, $P < 0.05$). This correlation was more robust when the untreated samples were included in the analysis ($r = 0.601$, $P < 0.01$). On the other hand, the *mcd* gene was not detected in any of the soils studied.

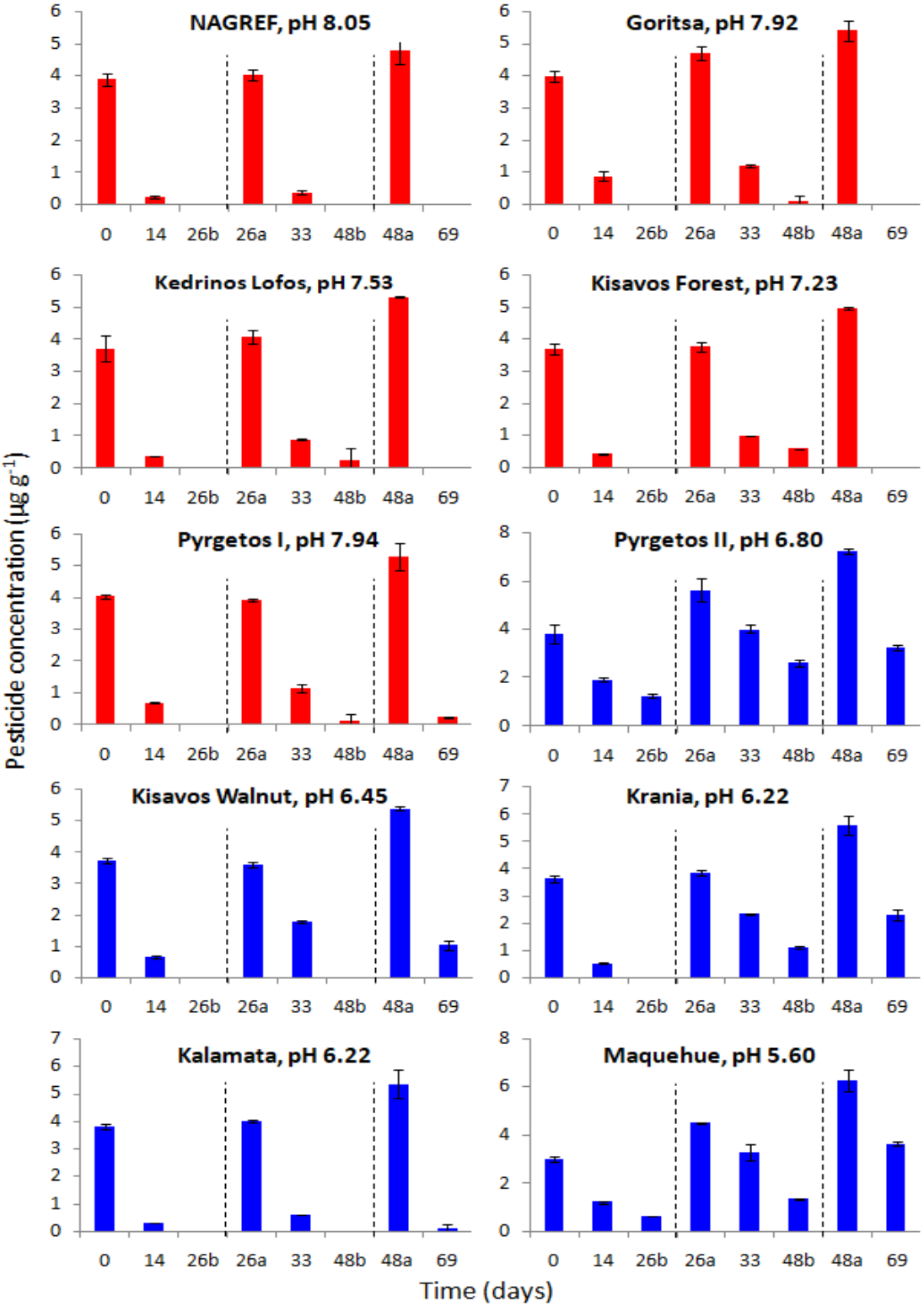

Figure 3.8 The degradation of oxamyl in 10 pristine soils repeatedly treated (day 0, 26 and 48) with oxamyl and carbofuran. The name and the pH of each of the studied soils are indicated. Graphs with red bars indicate soils where the *cehA* gene was detected at 69 days (Table 3.2), whereas graphs with blue bars indicate soils where the copy numbers of the *cehA* gene were below the limit of detection at 69 days. Pesticides concentration were determined before and right after the second (26b and 26a days) and the third (48b and 48a days) pesticide application. Each value is the mean of three replicates ± standard deviation

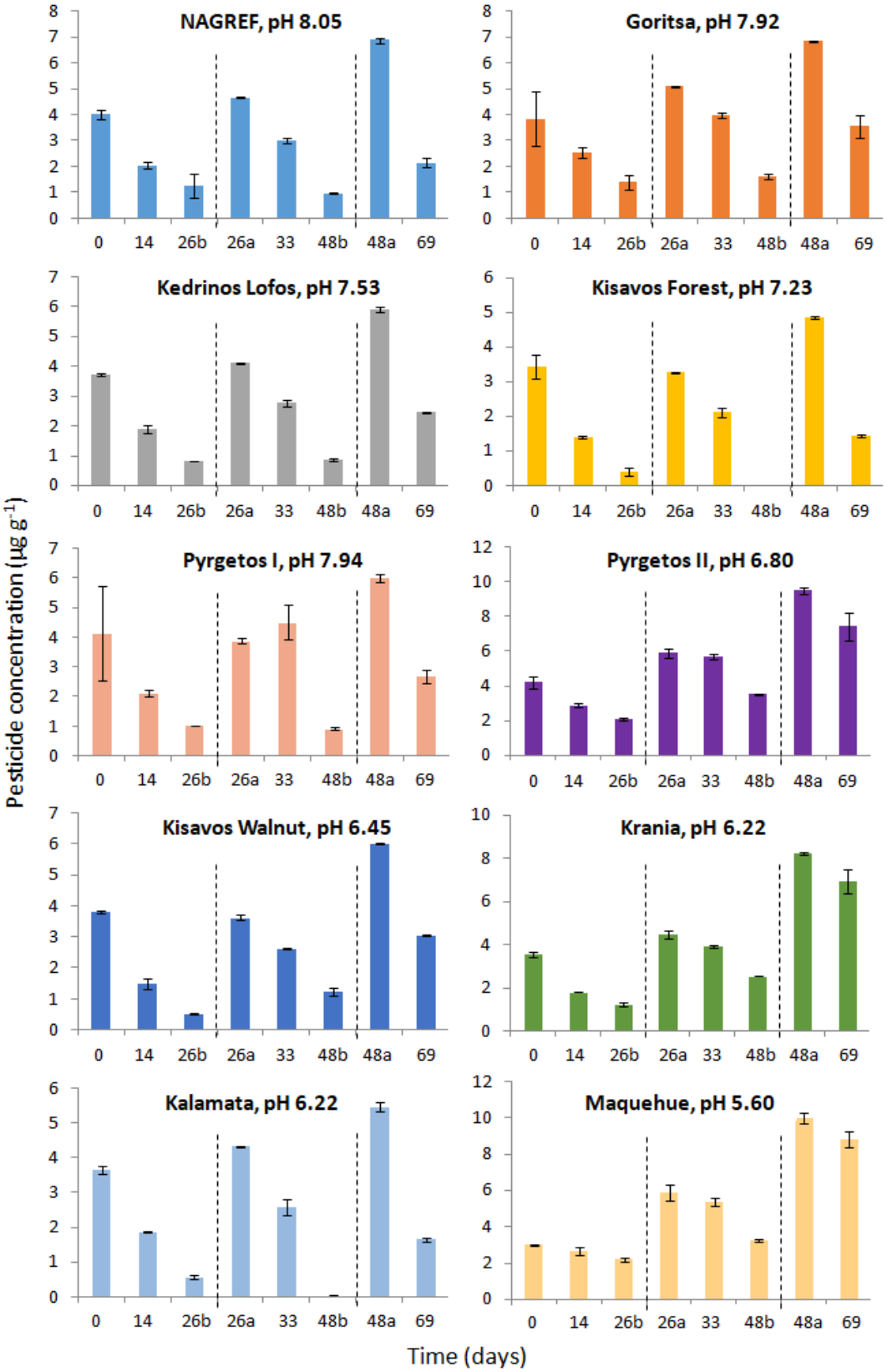

Figure 3.9 The dissipation of carbofuran in 10 pristine soils repeatedly treated with the carbamate. The name and the pH of each of the soils tested are indicated above each graph. The levels of pesticides were measured before and right after the second (26b and 26a days) and the third (48b and 48a days) pesticides applications. Each value is the mean of three replicates ± standard deviation

3.5 DISCUSSION

Degradation studies of oxamyl in 16 agricultural soils collected from a potato monoculture area in Greece verified its short persistence in line with previous studies (Osborn et al. 2010). The degradation of oxamyl in the agricultural soils studied showed a clear pH dependence illustrated by the significant negative correlation between soil pH and DT_{50} of oxamyl. Previous degradation studies of oxamyl were mostly performed in neutral to alkaline soils and failed to establish a correlation between soil pH and oxamyl persistence (Gerstl 1984; Osborn et al. 2010; Haydock et al. 2012). The more rapid degradation of oxamyl in alkaline soils is probably a function of the higher catabolic activity of soil bacteria under alkaline conditions (Smelt et al. 1987) and the vulnerability of oxamyl to alkaline hydrolysis (Harvey and Han 1978).

To further explore the interactions between pH and the fraction of the soil microbiota that drives the degradation of oxamyl we determined the abundance of the three most studied carbamate hydrolase genes, *cehA*, *mcd* and *cahA*, in the studied agricultural soils. The *cehA* gene was detected at the highest frequency and abundance. Its frequent occurrence is probably associated with the regular use of oxamyl in the study region in recent years. Previous studies with oxamyl-degrading bacteria showed the involvement of the *cehA* gene in the hydrolysis of oxamyl (Rousidou et al. 2016). This is further supported by the negative correlation between the abundance of the *cehA* gene and the DT_{50} of oxamyl (Table 3.4). In addition, a positive correlation between the abundance of the *cehA* gene and pH was observed. In general, alkaline soil conditions favour most bacterial taxa (Fierer and Jackson 2006; Rousk, Brookes and Baath 2009) and probably amongst these the fraction of the bacterial community that carry the *cehA* gene. The beneficial effect of soil pH on the activity of pesticide-degrading bacteria has been shown for other pesticides (Karpouzas and Walker 2000; Singh et al. 2003). In line with this Bending et al. (2003) observed that growth-linked degradation of isoproturon by *Sphingomonas* spp., in soil was only possible at pH > 7.0.

The abundance of the *mcd* gene was not correlated with soil parameters and the degradation kinetics of oxamyl. The mere presence of this gene in the studied soils

was surprising considering that carbofuran, which is the only carbamate that has been reported as substrate of the respective enzyme (Topp et al. 1993; Desaint et al. 2000), was banned in 2004 (last used in 2005). The application of other carbamates, regularly used in the study region during the last 10 years, was put forward as a possible explanation for the persistence of the *mcd* gene in the genetic pool of the soil microbial community. Tests in the soil L15 did not verify this hypothesis since none of the other five carbamates tested stimulated an increase in the abundance of the *mcd* gene. Alternatively, in the prolonged absence of its substrate, *mcd* could be maintained by a few members of the soil microbial community that are able to proliferate upon a fresh addition of carbofuran. Similar observations were reported by Cheyns et al. (2012) and Bælum et al. (2006) for atrazine and phenoxy-acid herbicides, respectively. They found that a single soil application of atrazine and 2-methyl-4-chlorophenoxyacetic acid (MCPA), 10–15 years after their last soil addition, triggered the proliferation of bacteria carrying the *atzA/trzN* and class III *tfdA* genes, respectively. These genes were not detectable or they had been detected at low levels before the fresh pesticide addition. The data presented provide novel insights into the ecological role of carbofuran degrading microorganisms and support earlier field studies that have reported a long persistence of the phenomenon of enhanced biodegradation of carbofuran for at least 3–5 years (Suett et al. 1993; Smelt et al. 1996).

The abundance of the *cehA* gene was stimulated by oxamyl and carbofuran. The positive response of *cehA* to the application of carbofuran was not surprising considering recent studies who detected the *cehA* gene in different carbofuran degrading strains (Nguyen et al. 2014; Yan et al. 2018). Studies by Öztürk et al. (2016) demonstrated that the CehA carried by the *Novosphingobium* strain KN65.2 ($CehA_{KN65.2}$) could increase its affinity for carbofuran through a single amino acid transvertion at residue 152 (Phe to Leu). This was further confirmed by Yan et al. (2018) who isolated a CehA homologue from a *Sphingomonas* strain CDS-1 ($CehA_{CDS-1}$) which had the same single amino acid substitution at position 152 and showed high affinity for carbofuran.

Despite the clear correlation between the degradation patterns of oxamyl and carbofuran and the dynamics of the *cehA* and/or *mcd* genes in soil L15, the

transcription profile of these genes constitutes the absolute proof of their direct involvement in the hydrolysis of carbamates in soil. The active transcription of the *cehA* gene in soil L15 was positively correlated with the hydrolysis of a fresh addition of oxamyl and carbofuran. In line with this Rousidou et al. (2016) recently showed that the transcription of *cehA* in oxamyl-degrading bacteria was positively correlated with the hydrolysis of oxamyl. Similar transcription patterns were reported in the past for the *tfdA* and *atz* genes during degradation of fresh soil applications of the herbicides MCPA and atrazine, respectively (Bælum et al. 2008; Monard et al. 2010). Our findings provide the first evidence for the direct involvement of *cehA* in the hydrolysis of oxamyl and carbofuran *in situ* in agricultural soils.

Unfortunately, our analysis could not provide information on the genetic diversity of the *cehA* gene in the studied soils due to the small size amplicons obtained which did not contain the non-conserved region of the *cehA* gene where nucleotide substitutions crucial for the substrate specificity of the translated product are localized. Based on the dual role of *cehA* in the degradation of oxamyl and carbofuran it is tempting to speculate that the carbamate-degrading bacterial community found in the soils of the Plateau of Lasithi carries homologues of the *cehA* with expanded hydrolytic affinity towards carbofuran or oxamyl. Alternatively, the carbamate-degrading bacterial community of these soils is composed of sub-populations of oxamyl- and carbofuran-specific *cehA* homologues which dominate in soil in response to the carbamate applied. Further analysis using high-throughput amplicon sequencing approaches encompassing the non-conserved region of the *cehA* gene will explore this hypothesis.

Regarding the *mcd* gene, application of carbofuran stimulated its expression in soil verifying its specific role in the hydrolysis of carbofuran. The concurrent expression of both carbamate hydrolase genes in the soil L15 during degradation of carbofuran suggests an independent activity of the two enzymes against the same substrate. The co-occurrence of isofunctional catabolic genes in soil was first reported for classes I and III of the *tfdA* genes associated with the biodegradation of phenoxy-acid herbicides (Bælum et al. 2006). Classes I and III of the *tfdA* genes are evolutionary related (78% sequence identity) (Bælum et al. 2010) and showed a remarkable compound-driven response with class I becoming dominant in the soil in

response to 2,4-dichlorophenoxyacetic acid (2,4-D) application and class III taking over after soil treatment with MCPA (Bælum et al. 2008). Horemans et al. (2016) first reported the concurrent presence in an agricultural soil of the evolutionarily unrelated linuron hydrolase genes *libA* and *hylA*, which were equally contributing to the biodegradation of linuron. This is in line with our findings that showed that two evolutionarily unrelated carbamate hydrolase genes *mcd* and *cehA* co-occur in agricultural soils. The concurrent presence and expression of the *cehA* and *mcd* genes in soil L15 after a fresh addition of carbofuran suggests that both genes contribute actively to the soil biodegradation of carbofuran. The substantially higher expression levels of the *cehA* over the *mcd* gene in soil L15 during degradation of carbofuran probably reflects the initial dominance of the *cehA*-carrying bacteria in the specific soil (see Fig. 3.3) and cannot support conclusions about the comparative efficiency of the two hydrolases on carbofuran. Similarly, Nour et al. (2017) found that *hylA* was more responsive than *libA* to linuron additions in an on-farm biopurification system. Overall our findings provide first insights into the functional redundancy of the environmental biodegradation of carbamates.

The occurrence of the *cehA* gene in several of the agricultural soils studied, in addition to its detection in carbamate-degrading bacteria obtained from distant geographical areas, suggests its widespread distribution. This was further tested in pristine soils that had no prior exposure to synthetic carbamates and at the initiation of the experiment showed no detectable levels of the *cehA* and *mcd* genes. Repeated carbamate applications resulted in the detection of the *cehA* gene at low levels only in treated soils with pH > 7.23 and also in one of the untreated soils that had the highest pH (8.05). These results support our hypothesis for the widespread occurrence of the *cehA* gene, which is favoured by alkaline soil conditions and exposure to synthetic carbamates. The widespread distribution of the *cehA* gene in soils could be the result of a parallel evolutionary mechanism from a common ancestor, probably involved in the detoxification of natural carbamate soil compounds. Soil bacteria are exposed to natural bioactive carbamates through root exudation (Sicker et al. 2001) or produce natural carbamate compounds themselves. Maekawa et al. (2010) isolated carbamate-containing fuzanins from the culture of a soil actinobacterium, while Poupot et al. (1995) isolated a novel carbamoylated nodulation factor produced by the symbiotic bacterium *Rhizobium etli*. Phylogenetic analysis of the CehA protein (Fig. 3.10)

showed that all currently characterised CehA proteins are highly conserved and show homology to a group of uncharacterised proteins carried by actinobacteria (*Jiangella* sp. and *Kribbella* sp.) and an acidobacterium (*Terriglobus* sp.) commonly found in soil (Eichorst et al. 2007; Lee 2008) and other pristine environments not previously exposed to anthropogenic organic pollutants (Urzi et al. 2008; Kampfer et al. 2011), in line with their hypothetical role in the transformation of natural carbamate compounds. These hypothetical proteins might constitute the distant ancestor of CehA, a hypothesis which remains to be tested. Based on our results and previous studies which have demonstrated the localization of all *cehA* homologues in transposons flanked by insertion elements (Yan et al. 2018), we speculate that *cehA* has evolved from a common ancestor involved in the degradation of natural carbamates and it is then readily transferred among members of the soil bacterial community via horizontal gene transfer.

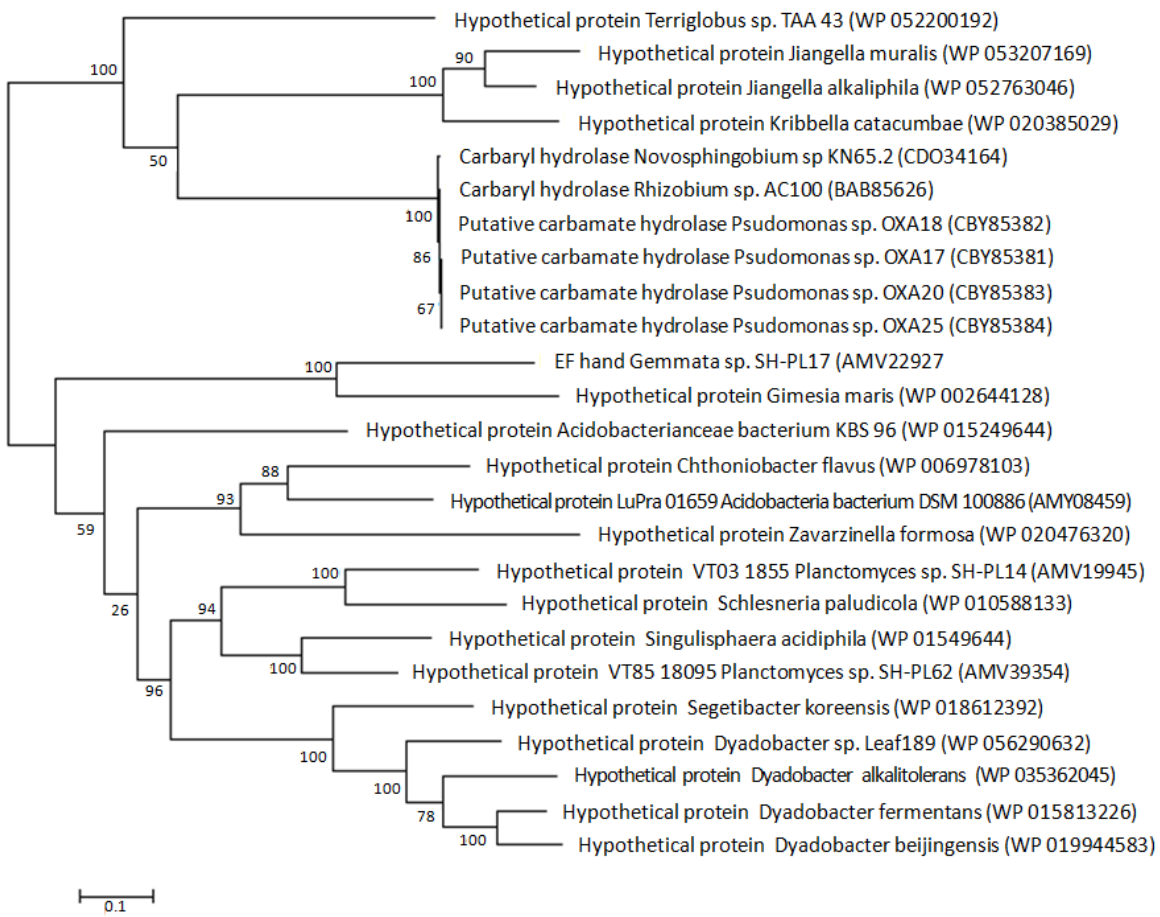

Figure 3.10. Phylogenetic analysis of the 25 CehA amino acid sequences downloaded from NCBI. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model (Jones et al. 1992). Bootstrap support (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 555 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Overall, our findings suggest that the biodegradation of oxamyl in soil is mediated by the *cehA* gene, whereas both the *cehA* and the *mcd* genes are involved in the soil biodegradation of carbofuran, a catabolic functional redundancy which is reported for the first time for carbamates and warrants further analysis. Our findings have clear practical implications: (i) the detection of the *mcd* gene in agricultural soils, despite the long lapse of use of its substrate (carbofuran), suggests that once established enhanced biodegradation of carbamates could be persistent and potentially threatens, under conducive conditions, their long term agronomic performance; and

(ii) the widespread occurrence of the *cehA* gene in the studied potato monoculture region, in its extreme, could be a possible threat for the biological efficacy of oxamyl especially in soils with neutral to alkaline pH. In addition, our findings provide preliminary evidence for the evolution of the *cehA* genes in agricultural and pristine soils from a common ancestor probably involved in the hydrolysis of natural carbamate compounds and stress the significant role of pH as a driver of the distribution of the *cehA* gene in soils.

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CHAPTER 4

General Discussion and Future Perspectives

4.1 GENERAL DISCUSSION

Carbamate insecticides/nematicides are widely used for crop protection and they are of environmental concern because of their high acute toxicity. Microbial degradation constitutes one of the main processes controlling their environmental dissipation. Paradoxically, microbial degradation, which was initially viewed as a desirable process for reducing environmental hazards, has turned into a double-edged sword with the development of the phenomenon of enhanced microbial degradation which can lead to loss of biological efficacy of pesticides. One of the leading synthetic nematicides in the global market is the oximino carbamate oxamyl. Considering the importance of biodegradation in the environmental fate and efficacy of soil-applied carbamate insecticides, we firstly aimed to isolate and identify bacteria degrading the carbamate oxamyl and to characterize the genes involved in its transformation and secondly to explore the ecology, distribution, function and evolution of carbamate hydrolase genes in soils.

As a source for the isolation of oxamyl-degrading bacteria we used a soil from a commercial banana plantation located in the area of Sitia, northeast Crete, in Greece with history of previous treatment with oxamyl. We initially investigated the degradation of oxamyl in the soil and the involvement of microorganisms in the hydrolysis of oxamyl comparing oxamyl degradation in sterilized and non-sterilized soil. We observed a rapid microbially driven hydrolysis of oxamyl in line with previous studies which have also reported the vulnerability of oxamyl to enhanced microbial degradation, threatening its biological efficacy in agricultural soils (Smelt et al. 1987, 1996). The same problem had occurred in the field from which we took soil samples as the farmer was facing severe infestation by nematodes despite the regular use of oxamyl.

Four oxamyl-degrading bacterial strains were isolated from the adapted soil via enrichment cultures and identified, based on MLSA, as *Pseudomonas*. Bacteria belonging to the genus *Pseudomonas* are metabolic champions able to degrade a diverse range of compounds including pesticides like organophosphates (Karpouzas et al. 2000), carbamates (Trivedi et al. 2016; Zhu et al. 2019) and pyrethroids (Mallic et al. 2009). These results constitute only the second report regarding the isolation of

oxamyl-degrading bacteria. Osborn et al. (2010) first reported the isolation of oxamyl-degrading bacteria identified as *Aminobacter* and *Mesorhizobium* spp. However, the degradation pathway of oxamyl was not elucidated in these bacteria. So, our next aim was to explore the transformation pathway of oxamyl by our isolates. We found that our isolates were capable of hydrolyzing oxamyl to oxamyl oxime, which was not further transformed by our strains in contrast to its gradual dissipation in soil. The formation of oxamyl oxime in the studied soil is in line with other soil studies that identified it as the main metabolite (Ou and Rao, 1986). However, this is the first report for the formation of oxamyl oxime by soil bacteria. Based on the gradual dissipation of oxamyl oxime in the studied soil, we assumed that other soil microbes might be involved in its further dissipation. Follow up degradation experiments in sterilized soil samples showed a complete halting of the degradation of oxamyl oxime suggesting the involvement of soil microbes in its degradation. However, our repeated attempts to isolate oxamyl oxime degraders from the same soil via enrichment cultures were not successful. It is possible, therefore, that its transformation in soil is a co-metabolic process performed by non-specialized soil bacteria or fungi. Either way, the hydrolysis of oxamyl, successfully performed by all isolated bacteria, is considered a detoxification step based on the low toxicity of oxamyl oxime compared to its parent compound (European Food Safety Authority [EFSA], 2005)

The capacity of oxamyl-degrading bacteria to grow on oxamyl as C and N source, in spite of the accumulation of oxamyl oxime, strongly suggested that they could utilize the methyl carbamate moiety, also released upon hydrolysis of oxamyl, for their growth. This was further supported by the ability of our isolates to effectively mineralize the ^{14}C-carbamoyl-labelled oxamyl. The formation of carbamic acid is transient and its degradation leads to the formation of methylamine and CO_2 as has been reported for other carbamate-hydrolyzing bacteria (Karns et al. 1986; Feng et al. 1997; Bachman and Patterson, 1999). Subsequently, methylamine could be utilized as a C and N source through the C1 metabolism of bacteria (Chistoserdova et al. 2009). Methylotrophy has been identified as a common feature of other carbamate-degrading bacteria (Chaudhry and Ali, 1988; Singh et al. 1993; Topp et al. 1993). Considering these observations, we tested our isolates for their ability to grow on methylamine and for the presence of methylamine dehydrogenase genes. We observed that all isolates were able to grow on methylamine upon numerous transfers and methylamine

dehydrogenase gene were detected in three of the four isolates. Furthermore the capacity of oxamyl-degrading isolates tested to rapidly degrade both other oximino and, aryl -methyl carbamate compounds which resemble only at the methyl-carbamoyl moiety provided further evidence for the use of the methyl-carbamoyl moiety of oxamyl as C and N source.

All the oxamyl-degrading strains carried the *cehA* gene, encoding a carbaryl hydrolase previously isolated from a *Rhizobium* strain degrading carbaryl (Hashimoto et al. 2002). Transcription analysis of the *cehA* gene verified its key role in the hydrolysis of oxamyl. The $cehA_{OXA}$ gene sequences in the oxamyl-degrading bacterial isolates from our study were identical or nearly identical with other known bacterial *cehA* sequences (Hashimoto et al. 2002; Yan et al. 2018; Nguyen et al. 2014). Also, in line with all the CehA homologues, $CehA_{OXA}$ contained a Sec or TAT signal peptide which suggested extracytosolic function (Ozturk et al. 2016; Yan et al. 2018).

We extended the study of carbamate hydrolase genes from our isolates to their role in the *in situ* biodegradation of carbamates in soils. To date, the distribution and function of carbamate hydrolase genes associated with the soil biodegradation of carbamates is not yet clear. Novel information on this issue will allow accurate predictions regarding the biodegradation potential of agricultural soils against carbamates, precluding reduced efficacy issues. For this purpose, we studied oxamyl degradation in 16 soils from a potato monoculture area in Greece where oxamyl is regularly used. We observed a low soil persistence of oxamyl and a negative correlation between soil pH and DT_{50} of oxamyl. The more rapid degradation of oxamyl in alkaline soils was probably a result of the higher catabolic activity of soil bacteria under alkaline conditions and the vulnerability of oxamyl to alkaline hydrolysis (Smelt et al. 1987, Harvey and Han 1978). We further determined the abundance of the three most studied carbamate hydrolase genes *cehA*, *mcd* and *cahA* in the studied agricultural soils via qPCR. *cehA* was the gene detected at the highest frequency and abundance, the latter was negatively correlated with the DT_{50} of oxamyl. These results provided a further verification of the role of the *cehA* gene in the hydrolysis of oxamyl by the isolated bacteria. In addition, the abundance of the *cehA* gene was positively correlated with pH and this could be a result of the beneficial alkaline conditions on most bacterial taxa (Fierer and Jackson 2006; Rousk, Brookes and Baath 2009)

The *mcd* gene was detected in only three of the 16 agricultural soils studied and its abundance was not correlated with soil parameters and the degradation kinetics of oxamyl. The detection of this gene in the studied soils was a surprising finding since carbofuran, which is the main substrate of the Mcd enzyme, was last used in the studied region in 2005. So, we hypothesized that the unexpected detection of *mcd* gene in the studied soils could be attributed to its stimulation by the application of other carbamates regularly used in the study region during the last 10 years. Contrary to our expectations, subsequent soil degradation tests with other carbamate compounds regularly used in the studied region did not verify this hypothesis. Hence it is probable that the *mcd* gene could be maintained at low abundance in the genetic pool of the soil by a few members of the soil bacterial community that are able to proliferate upon a fresh addition of carbofuran. This observation has been reported for other pesticide groups (Cheyns et al. 2012; Bælum et al. 2006) and is conducive with earlier studies that have reported a long persistence of the phenomenon of enhanced biodegradation of carbofuran once established (Suett et al. 1993; Smelt et al. 1996).

We further investigated the interactions of the *cehA* and *mcd* genes with a range of carbamate compounds previously or currently used in the study region. Amongst the carbamates tested, oxamyl stimulated the abundance of the *cehA* gene, while carbofuran stimulated the abundance of both *cehA* and *mcd*. The stimulation of *cehA* by fresh soil additions of oxamyl and carbofuran was not surprising considering the presence of *cehA* in our oxamyl-degrading isolates but also in carbofuran-degrading strains isolated in previous studies (Nguyen et al. 2014; Yan et al. 2018). The involvement of the *cehA* and *mcd* genes in the degradation of oxamyl and or carbofuran was further verified by the determination of their expression in soil during degradation of a fresh pesticide addition. Indeed application of oxamyl and carbofuran stimulated the expression of *cehA* and *mcd* genes respectively in soil verifying their role in the degradation of these pesticides. This is the first evidence for the direct involvement of *cehA* in the hydrolysis of oxamyl and carbofuran *in situ* in agricultural soils. The concurrent expression of the *cehA* and *mcd* genes in soil during degradation of carbofuran, suggests that both genes contribute actively to the soil biodegradation of carbofuran, in contrast to oxamyl whose degradation is driven solely by the *cehA* gene. This catabolic functional redundancy for the isofunctional genes *cehA* and *mcd* is reported for the first time for carbamates biodegradation and warrants further

analysis. The co-occurrence of isofunctional catabolic genes in soil was first reported for classes I and III of the *tfdA* genes associated with the biodegradation of phenoxy-acid herbicides (Bælum et al. 2006)

The occurrence of *cehA* and *mcd* genes in agricultural soils in Crete and their detection in isolates from distant geographical areas (i.e. Phillipines, Japan, France, UK) suggested their widespread distribution. However, their evolution and dispersal mechanism remained unexplored. In this context we explored the distribution of carbamate hydrolase genes in pristine soils that had no prior exposure to synthetic carbamates. We assumed that *cehA* and *mcd* genes could be also present in pristine soils as a result of a parallel evolution mechanism from a common ancestor, probably involved in the detoxification of natural carbamate soil compounds known to be produced by soil bacteria and plant roots. Indeed, only *cehA* gene was detected in pristine soils upon repeated treatments with oxamyl and carbofuran and only in soils with $pH \geq 7.2$ where the most rapid degradation of oxamyl was observed. The occurrence of *cehA* gene in agricultural and pristine soils verified its widespread distribution and supported our hypothesis for the evolution of these carbamate catabolic genes. Phylogenetic analysis of the CehA proteins from the different carbamate-degrading isolates showed that all currently characterized CehA proteins are highly conserved and show homology to a group of uncharacterized proteins carried by bacteria (*Jiangella* sp, *Kribbella* sp and *Terriglobus* sp) commonly found in soil (Eichorst et al. 2007; Lee 2008) and other pristine environments (Urzi et al. 2008; Kampfer et al. 2011), which might constitute the ancestor of CehA.

Overall, we studied the degradation of oxamyl by oxamyl-degrading strains and its biodegradation in soils. We reported the isolation and identification of four oxamyl-degrading strains and we proposed the microbial metabolic pathway of oxamyl. All the isolated strains carried the *cehA* gene which was shown to be responsible for the hydrolysis of oxamyl to oxamyl oxime and methylamine. Oxamyl oxime was not further transformed, and methylamine was utilized as C and N source with concurrent mineralization of the carbomoyl moiety. In soils we showed that the *cehA* gene is responsible for the degradation of oxamyl, while both *cehA* and *mcd* genes were involved in the degradation of carbofuran suggesting an interesting catabolic functional redundancy first time reported for carbamates. The detection of the *cehA* gene in agricultural and pristine soils (only in soils with high pH), after

oxamyl application suggests its widespread occurrence and stresses the significant role of pH as a driver of the distribution of the *cehA* in soils. These findings have practical implications regarding the agricultural use of carbamates. Specifically, the occurrence of the *cehA* in agricultural soil, in its extreme could be a possible threat for the biological efficacy of oxamyl especially in soils with neutral to alkaline pH. Also, the detection of the *mcd* gene in agricultural soils, despite the long lapse of use of its substrate, suggests that once established enhanced biodegradation of carbamates could be persistent and potentially threatens their long term agronomic performance. Finally, our results provide insights into the evolution of *cehA* gene in agricultural and pristine soils from a common ancestor.

4.2 FUTURE PERSPECTIVES

The results of these thesis provided novel insights in the genetic and evolutionary mechanisms driving the biodegradation of carbamates by soil bacteria while at the same time new scientific questions emerged regarding the evolution, ecological role and function of $cehA_{OXA}$ which would be pursued in the near future. The follow up research steps of this thesis will focus on the:

1. Study of the genetic diversity of *cehA* genes in carbamate-treated field sites in potato cultivation areas using amplicon sequencing analysis targeting the non-conserved gene region containing the variable bases found to control the substrate range of CehA (Ozhturk et al. 2016)

2. Genomic analysis of the oxamyl-degrading bacteria to get insights into the evolution of the carbamate hydrolyzing phenotype in the isolated bacteria

3. Determination of the role of mobilome in the dispersal of carbamate hydrolase encoding genes in soil via LR-PCR targeted metagenomics as recently suggested by Dunon et al. (2018)

4. Determination of the relevance of the isolated oxamyl-degrading bacteria for the *in situ* biodegradation of oxamyl in soil and identification of bacteria actively degrading oxamyl *in situ* via stable isotope probing analysis in soil and

enrichment cultures or using alternative approaches like EPIC-PCR which allow co-amplification of a functional and a housekeeping from the same organism creating hybrid amplicons.

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- IAPP Marie Curie project, Title: *Pesticides – Felicity or curse for the soil microbes* (Acronym LOVE-TO-HATE). Funding Body: European Commission, FP7, Duration: 15/06/2016 - 30/11/2016
- Synthetic Biology: From omics technologies to genomic engineering (OMIC-ENGINE). Funding Body: Co-financed by the European Regional Development Fund of the European Union and Greek national funds. Duration: 25/1/2019 - 15/4/2019
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Environmental Microbiology: Isolation and characterization of bacteria degrading pesticides and of the genetic mechanisms driving this process

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Publications in peer-reviewed journals

1. Karpouzas D. G., **Rousidou C.,** Papadopoulou K. K, Bekris F., Zervakis G., Singh B. K., Ehaliotis C. (2009). Effect of continuous olive mill wastewater

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