University of Thessaly

Department of Biochemistry and Biotechnology

Biological treatment of pesticide-contaminated wastewaters from the fruit packaging industry

A thesis submitted by

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και στον αδερφό μου Στέφανο.

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Extended Summary

Pesticides constitute a significant group of environmental pollutants. The pollution of natural resources by pesticides is attributed to diffuse and point sources. The contribution of the latter in environmental contamination is of outmost importance and measures to diminish their impact have been proposed. On farm improper activities before, during or after spraying could be a significant point source of pollution of natural water resources by pesticides. Biobeds constitute an established, cost-effective and efficient on-farm biodepuration system for the treatment of wastewaters produced by on-farm activities. However post-farm activities involving pesticides application also contribute to the point pollution of natural resources. Such an example are fruit packaging plants where fungicides (thiabendazole (TBZ), imazalil (IMZ), ortho-phenylphenol (OPP)) and antioxidants (diphenylamine (DPA), ethoxyquin(EQ)) are used to protect fruits from fungal infestations and physiological disorders during storage. This results in the formation of large volumes of wastewaters which contain high loads of toxic and persistent pesticides and should be treated *on site.* The environmental risk associated with the postharvest use of pesticides is exemplified in the registration documents of all relevant pesticides which specify the need for treatment of the effluents produced before their environmental release. However no efficient, cheap and sustainable treatment methods are available at the moment in Europe.

In the absence of effective depuration methods, these effluents are discharged in municipal wastewater treatment plants or spread onto agricultural land compromising the quality of water and soil resources. Biobeds modified to cope with the characteristics of these particular effluents (large volumes, seasonality in production, high pesticide loads, low BOD/COD etc) could be an applicable solution for their treatment. Based on all the above, the main aim of this PhD thesis was to assess the potential use of biobeds as treatment methods for the depuration of wastewaters from fruit packaging plants. To achieve this main aim a gradually scalled up experimental approach was implemented which aimed (a) to identify, initially at lab scale level, biobeds packing materials based on Spent Mushroom Substrate (SMS), which exhibit high dissipation and sorption capacity against the pesticides used in the fruit packaging plants (b) to further test, at leaching columns, the capacity of the best performing biobed packing material to retain pesticides under conditions of

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high hydraulic load simulating realistic conditions and (c) to finally test the depuration performance of biobed systems at pilot scale level. Additional points were explored like means to maximize the depuration performance of biobeds against recalcitrant pesticides via bioaugmentation with tailored-made inocula, the response and structure of the microbial community in biobed systems and practical aspects for their implementation like (i) the quality and post-treatment handling of the biobed-treated effluent and (ii) the decontamination of the spent biobed packing material upon the end of the life cycle of biobeds.

In Chapter 2 we studied the dissipation of TBZ, IMZ, OPP, DPA and EQ used by the fruit-packaging industry, in anaerobically digested sewage sludge and in liquid aerobic sewage sludge to test the dissipation capacity of sewage treatment plants. At a second stage we evaluated the dissipation (and the metabolism of EQ) and sorption of all these pesticides to various organic substrates composed of soil, straw and spend mushroom substrate (SMS) in various volumetric ratios to identify the best performing biobed packing material. TBZ and IMZ showed higher persistence especially in the anaerobically digested sewage sludge (DT_{50}=32.3-257.6 d), in contrast to OPP and DPA which were rapidly dissipated especially in liquid aerobic sewage sludge (DT_{50}=1.3-9.3d). EQ was rapidly oxidized mainly to quinone imine (QI), which did not persist, and dimethyl ethoxyquinoline (EQNL, minor metabolite) which persisted for longer. Sterilization of liquid aerobic sewage sludge inhibited pesticides decay verifying the microbial nature of pesticides dissipation in those substrates. Organic substrates rich in SMS showed the highest dissipation capacity with TBZ and IMZ DT_{50s} of *ca*. 28 days compared to DT_{50s} of > 50 days in the other substrates and the general recalcitrance of those compounds in soil (DT_{50} >100 d). TBZ and IMZ showed the highest sorption affinity, whereas OPP and DPA were weakly sorbed. These findings suggested that municipal wastewater treatment plants could not guarantee an efficient removal of the recalcitrant fungicides IMZ and TBZ, whereas SMS-rich biobed organic substrates show much higher dissipation capacity for those chemicals, and also for the less persistent OPP, DPA and EQ.

In Chapter 3 we focused on the citrus fruit-packaging plants which produce large wastewater volumes with high loads of fungicides like OPP and IMZ, two chemicals showing contrasting persistence (OPP is non persistent while IMZ is persistent). In accordance with a gradual scaling up approach we employed a column

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study to assess the capacity of SMS of *Pleurotus ostreatus*, either alone or in mixture with straw and soil plus a mixture of straw /soil to retain and dissipate IMZ and OPP. The role of *P. ostreatus* on fungicides dissipation was also investigated by parallel studying the performance of fresh mushroom substrate of *P. ostreatus* (FMS) and measuring lignolytic enzymatic activity in the leachates. We employed a sequentil treatment scheme which simulated a realistic worst case operation of a citrus fruit packaging plant. All substrates effectively reduced the leaching of OPP and IMZ which corresponded to 0.014-1.1% and 0.120-0.420% of their initial amounts respectively. Mass balance analysis revealed that FMS and SMS/Straw/Soil (50/25/25 by vol) offered the most efficient removal of OPP and IMZ from wastewaters respectively. Regardless of the substrate, OPP was restricted in the top 0-20 cm of the columns and it was bioavailable (extractable with water), compared to IMZ which was less bioavailable (extractable with acetonitrile) but diffused deeper in the columns (20-50 & 50-80 cm) in the SMS- and Straw/Soil-columns. The distribution of the living microbial community was measured via phospholipid fatty acids analysis (PLFAs). Fungal abundance was significantly lower at the top layer of all substrates from where the highest pesticide amounts were recovered suggesting an inhibitory effect of fungicides on total fungi. These results suggested that biobeds packed with SMS-rich substrates could ensure the efficient removal of IMZ and OPP from wastewaters of citrus FPP even under particularly high hydraulic and pesticide loads.

Based on the results of Chapters 2 and 3 and in accordance with the gradual scaling up of the experimentation, we constructed and tested pilot biobed systems under practical conditions of citrus and pome fruit packaging plants. Further aspects tested were (a) the optimization of the depuration capacity of the pilot biobeds through bioaugmentation with tailored-made bacterial inocula, and (b) the composition and functional dynamics of the microbial community in pilot biobeds using molecular approaches (q-PCR). Practical issues were also addressed including the risk associated with the direct environmental disposal of biobed-treated effluents and methods for the decontamination of the spent packing material. Three pilot biobeds of 1 m^3 (non bioaugmented) and 2 pilot biobeds of 0.24 m^3 (bioaugmented) were constructed and treated for a period of 160 days with different combinations of pesticides simulating practical scenarios from citrus and pome fruit packaging plants. Pilot biobeds showed high depuration capacity for the less persistent OPP, DPA

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(>99.9%) but also for the more recalcitrant chemicals IMZ and TBZ (>99.5%). Bioaugmentation maximized the depuration capacity of pilot biobeds for the persistent fungicide TBZ which was fully dissipated by the end of the study. This was followed by a significant increase in the abundance of bacteria, fungi and of catabolic genes *catA* and *pcaH*. Bioaugmentation was the most potent method for the decontamination of the spent packing material, although composting with fresh organic matter and even storage at ambient temperature offer effective alternatives when inocula are not available. Risk assessment based on practical scenarios (pome and citrus fruit-packaging plants), the depuration performance of the pilot biobeds and the currently implemented regulatory framework for pesticides showed that the discharge of the biobed-treated effluents into an 0.1-ha disposal site did not entail an unacceptable risk for aquatic and terrestrial ecosystems, except for TBZ-containing effluents produced by pome fruit packaging plants where a larger disposal area (0.2 ha) or bioaugmentation of biobeds alleviated the risk.

Overall our study provided a comprehensive evaluation of biobeds as a method for the treatment of pesticide contaminated wastewaters produced by the fruit packaging industry. We showed that these systems could be a viable solution for the treatment of these agro-industrial effluents. Further studies will aim explore the application of biobeds for the treatment of the wastewaters produced by other agro-industries (i.e. bulb disinfection, seed-coating).

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Εκτεταμένη Περίληψη

Τα γεωργικά φάρμακα αποτελούν μια σημαντική ομάδα περιβαλλοντικών ρύπων. Η ρύπανση των φυσικών πόρων από τα γεωργικά φάρμακα έχει αποδοθεί σε σημειακές και μη σημειακές πηγές. Η συμβολή των σημειακών πηγρών στη ρύπανση του περιβάλλοντος είναι υψίστης σημασίας και μέτρα για τη μείωση των επιπτώσεων τους έχουν προταθεί. Μη ορθολογικές πρακτικές στις γεωργικές εκμεταλλεύσεις πριν, κατά τη διάρκεια ή μετά τον ψεκασμό αποτελούν τις κύριες πηγές σημειακής ρύπανσης των φυσικών υδάτινων πόρων με γεωργικά φάρμακα. Οι βιοκλίνες έχουν πλέον καθιερωθεί ως οικονομικά, αποδοτικά και αποτελεσματικά συστήματα βιολογικής επεξεργασίας των υγρών αποβλήτων που παράγονται από δραστηριότητες στη γεωργική εκμετάλλευση. Ωστόσο, η εφαρμογή γεωργικών φαρμάκων πέραν του αγρού και ύστερα από την συγκομιδή συμβάλλουν επίσης στη σημειακή ρύπανση των φυσικών πόρων. Τέτοιο παράδειγμα αποτελούν οι βιομηχανίες συσκευασίας φρούτων, όπου μυκητοκτόνα (thiabendazole (TBZ), imazalil (IMZ), *ortho*-phenyphenol (OPP)) και αντιοξειδωτικά (diphenylamine (DPA), ethoxyquin (EQ)) χρησιμοποιούνται για την προστασία των φρούτων από μυκητιακές προσβολές και φυσιολογική υποβάθμιση της ποιότητας τους κατά την αποθήκευση. Η πρακτική αυτή οδηγεί στην παραγωγή μεγάλου όγκου υγρών αποβλήτων επιβαρυμμένων με υψηλές ποσότητες τοξικών και υπολειμματικών γεωργικών φαρμάκων τα οποία χρήζουν άμεσης επεξεργασίας στο σημείο στο οποίο παράγονται. Ο περιβαλλοντικός κίνδυνος που σχετίζεται με τη μετασυλλεκτική χρήση των γεωργικών φαρμάκων επισημαίνεται στις εγκρίσεις όλων των γεωργικών φαρμάκων που χρησιμοποιούνται στα συσκευαστήρια φρούτων, όπου υπογραμμίζεται η ανάγκη για τη διαχείριση των αποβλήτων που παράγονται, πριν από την απελευθέρωσή τους στο περιβάλλον. Ωστόσο σήμερα δεν υπάρχουν αποτελεσματικές, φθηνές και βιώσιμες μέθοδοι διαχείρισης των συγκεκριμέων αποβλήτων στην Ευρώπη.

Εν τη απουσία αποτελεσματικών μεθόδων επεξεργασίας τα συγκεκριμένα υγρά απόβλητα διοχετεύονται είτε στις εγκαταστάσεις επεξεργασίας αστικών λυμάτων είτε απορρίπτονται σε παρακείμενους αγρούς θέτοντας σε κίνδυνο την ποιότητα των υδατικών και εδαφικών πόρων. Τροποποιημένες βιοκλίνες που θα είναι συμβατές με τα ιδιαίτερα χαρακτηριστικά των συγκεκριμένων υγρών απόβλητων (υψηλοί όγκοι, εποχικότητα παραγωγής, υψηλά φορτία γεωργικών φαρμάκων, χαμηλό BOD / COD κλπ), θα μπορούσαν χρησιμοποιηθούν για τη διαχείρισή τους.

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Με βάση όλα τα παραπάνω, ο κύριος στόχος της παρούσας διδακτορικής διατριβής ήταν η πλήρης αξιολόγηση των βιοκλινών ως μέθοδος επεξεργασίας των υγρών αποβλήτων από τις βιομηχανίες συσκευασίας φρούτων. Για την επίτευξη του συγκεκριμένου στόχου, ακολουθήθηκε μια πειραματική προσέγγιση που περιελάμβανε βαθμιαία αύξηση της πολυπλοκότητας (εργαστήριο/στήλες/πιλοτικά συστήματα), η οποία είχε ως κύριους στόχους (α) να προσδιορίσει, αρχικά σε εργαστηριακή κλίμακα, οργανικά βιομίγματα, με βάση το εξαντλημένο υπόστρωμα μανιταριών (SMS), με υψηλή ικανότητα απομάκρυνσης και προσρόφησης των γεωργικών φαρμάκων που χρησιμοποιούνται στα συσκευαστήρια φρούτων (β) να αξιολογήσει περαιτέρω την ικανότητα των αποτελεσματικότερων οργανικών βιομιγμάτων (όπως αυτά προέκυψαν από την εργαστηριακή αξιολόγηση), να κατακρατούν τα γεωργικά φάρμακα υπό συνθήκες υψηλού υδραυλικού φορτίου προσομοιώνοντας έτσι ρεαλιστικές συνθήκες παραγωγής από συσκευαστήρια φρούτων και (γ) να προσδιορήσει την απόδοση των βιοκλινών σε πιλοτικό επίπεδο. Στο πλαίσιο αυτό αξιολογήθηκαν επίσης η προοπτική βελτιστοποίησης της απόδοσης των βιοκλινών έναντι κυρίως υπολειμματικών γεωργικών φαρμάκων μέσω βιοεπλουτισμού (bioaugmentation) με εξειδικευμένα μικροβιακά εμβόλια, η ανταπόκριση της μικροβιακής κοινότητας των βιοκλινών στην συνεχή εφαρμογή γεωργικών φαρμάκων. Παράλληλα μελετήθηκαν και μέτρα προς την κατεύθυνση της πρακτικής εφαρμογής των βιοκλινών που ακόμη και σήμερα παρεμποδίζουν την πλήρη ανάπτυξη τους όπως (α) η ποιότητα και η μετέπειτα διαχείριση των επεξεργασμένων αποβλήτων που προκύπτουν από τις βιοκλίνες και (β) η απορρύπανση του εξαντλημένου βιομίγματος των βιοκλινών μετά το τέλος του κύκλου ζωής τους.

Στο Κεφάλαιο 2 μελετήσαμε την διάσπαση των TBZ, IMZ, OPP, DPA και EQ, που χρησιμοποιούνται στα συσκευαστήρια φρούτων, από λυματολάσπη που έχει υποστεί αναερόβια χώνευση καθώς και από αερόβια υγρή λυματολάσπη ώστε να εκτιμηθεί αρχικά η ικανότητα των μονάδων επεξεργασίας αστικών λυμάτων να απομακρύνουν τα συγκεκριμένα γεωργικά φάρμακα. Σε δεύτερο στάδιο, ελέγξαμε την προσρόφηση και αποδόμηση των παραπάνω γεωργικών φαρμάκων (και το μεταβολισμό του EQ) σε διάφορα οργανικά βιομίγματα που αποτελούνταν από έδαφος, άχυρο και εξαντλημένο υπόστρωμα μανιταριών (SMS) σε διάφορες ογκομετρικές αναλογίες, για να προσδιορίσουμε έτσι το υλικό πλήρωσης των

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βιοκλινών με την καλύτερη απόδοση για μεταγενέστερη χρήση του σε συστήματα βιοκλινών πλήρους κλίμακας. Το TBZ και το IMZ έδειξαν την υψηλότερη υπολειμματικότητα ιδιαίτερα στην λυματολάσπη που είχε υποστεί αναερόβια χώνευση (DT_{50} = 32,3 έως 257,6 ημέρες), σε αντίθεση με τα OPP και DPA που αποδομήθηκαν ταχύτατα κυρίως στην αερόβια υγρή λυματολάσπη (DT_{50} = 1.3-9.3 ημέρες). Το EQ οξειδώθηκε άμεσα προς quinone imine (QI), το οποίο όμως διασπάστηκε περαιτέρω χωρίς να εμφανίζει μεγάλη υπολειμματικότητα, και σε μικρές ποσότητες dimethyl ethoxyquinoline (EQNL) που εμφάνισε υψηλή υπολειμματικότητα σε όλα τα βιομίγματα. Αποστείρωση της αερόβιας υγρής λυματολάσπης ανέστειλε τη διάσπαση των γεωργικών φαρμάκων αποδεικνύοντας το σημαντικό ρόλο των μικροοργανισμών στην διάσπαση των γεωργικών φαρμάκων. Οργανικά υποστρώματα πλούσια σε SMS παρουσίασαν την υψηλότερη ικανότητα αποδόμησης των TBZ και IMZ εμφανίζοντας τιμές DT_{50s} περίπου 28 ημέρων, σε σύγκριση με τα άλλα βιομίγματα που εμφάνισαν τιμές DT_{50s} > 50 ημερών. Τα TBZ και IMZ έδειξαν την υψηλότερη τάση προσρόφησης, ενώ τα OPP και DPA προσροφήθηκαν ασθενώς. Τα παραπάνω ευρήματα υποδεικνύουν ότι οι μονάδες επεξεργασίας αστικών λυμάτων δεν μπορούν να εγγυηθούν αποτελεσματική απομάκρυνση των υπολειμματικών μυκητοκτόνων IMZ και TBZ, ενώ οργανικά βιομίγματα πλούσια σε SMS έδειξαν υψηλή ικανότητα απομάκρυνσης των συγκεκριμένων γεωργικών φαρμάκων, καθώς και των λιγότερο υπολειμματικών OPP, DPA και EQ.

Στο Κεφάλαιο 3, επικεντρωθήκαμε στα συσκευαστήρια φρούτων εσπεριδοειδών που παράγουν υψηλές ποσότητες υγρών αποβλήτων που περιέχουν τα μυκητοκτόνα OPP και IMZ, δυο μυκητοκτόνα με διαφορετική υπολειμματικότητα στο περιβάλλοντ (το OPP είναι μη υπολειμματικό αντίθετα με το IMZ που είναι ιδιαίτερα υπολειμματικό). Έτσι στο πλαίσιο της βαθμαίας αύξησης της πολυπολοκότητας του πειραματισμούς μας μελετήσαμε σε συστήματα στηλών έκπλυσης την δυνατότητα του SMS του μύκητα *Pleurotus ostreatus*, είτε μόνο του είτε σε μείγμα με άχυρο και έδαφος καθώς και ένα μείγμα από άχυρο / έδαφος, να κατακρατούν και να απομακρύνουν από τα υγρά απόβλητα τα OPP και IMZ. Ο ρόλος του μύκητα *P. ostreatus* στη απομάκρυνση των μυκητοκτόνων διερευνήθηκε περαιτέρω διαμέσου μέτρησης της απόδοσης φρέσκου υποστρώματος μανιταριών του *P. ostreatus* (FMS) και μέτρηση της λιγνολυτικής ενζυματικής δραστικότητας στα

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συλλεγόμενα υγρά έκπλυσης. Το σενάριο εφαρμογής των υγρών αποβλήτων στις στήλες που ακολουθήθηκε προσομοιώνει υπό συνθήκες worst-case την παραγωγή αποβλήτων από μια μονάδα συσκευασίας εσπεριδοειδών. Όλα τα υποστρώματα βρέθηκε ότι περιορίζουν σημαντικά την έκπλυση των OPP (0,014-1.1% τη ποσότητας που εφαρμόστηκε στις στήλες) και IMZ, (0,120-0,420%). Ανάλυση ισοζυγίου μάζας έδειξε ότι τα FMS και SMS / Άχυρο / Έδαφο (50/25/25 κατά όγκο) οδήγησαν στην αποτελεσματικότερη απομάκρυνση των OPP και IMZ από τα υγρά απόβλητα αντίστοιχα. Ανεξάρτητα από το υπόστρωμα, τα υπολείμματα του OPP εντοπίστηκαν κυρίως στα 0-20 εκ. των στηλών και ήταν διαθέσιμα (εκχύλιση με νερό), σε σύγκριση με τα υπολείμματα του IMZ που ήταν λιγότερο βιοδιαθέσιμα (εκχύλιση με ακετονιτρίλιο), αλλά εντοπίστηκαν και σε βαθύτερα στρώματα των στηλών (20-50, 50-80 cm) που πακεταρίστηκαν με SMS και Άχυρο/Εδάφος. Η ζωντανή μικροβιακή κοινότητα εντός των στηλών, ποσοτικά και ποιοτικά, προσδιορίστηκε μέσω ανάλυσης των φωσφολιπιδίων των λιπαρών οξέων (Phospholipids Fatty Acids, PLFAs). Η αφθονία των μυκήτων ήταν σημαντικά χαμηλότερη στα ανώτερα στρώματα των στηλών έκπλυσης, από όπου ανακτήθηκαν και τα υψηλότερα ποσοστά των γεωργικών φαρμάκων, υποδηλώνοντας έτσι μια ανασταλτική δράση των μυκητοκτόνων στους μύκητες στα υποστρώματα που δοκιμάστηκαν. Συμπερασματικά, τα παραπάνω αποτελέσματα υποδεικνύουν ότι η χρήση των βιοκλινών και η πλήρωσή τους με υποστρώματα πλούσια σε SMS, θα μπορούσαν να εξασφαλίσουν την αποτελεσματική απομάκρυνση των IMZ και OPP από τα υγρά απόβλητα των συσκευαστηρίων εσπεριδοειδών, ιδιαίτερα ακόμη και υπό υψηλά φορτία όγκου υγρών αποβλήτων και γεωργικών φαρμάκων.

Με βάση τα αποτελέσματα των Κεφαλαίων 2 και 3 κατασκευάσαμε πιλοτικά συστήματα βιοκλινών και εξετάσαμε την αποτελεσματικότητα τους στην απορρύπανση υγρών αποβλήτων που παράγονται υπό πραγματικές συνθήκες από συσκευαστήρια μηλοειδών και εσπεριδοειδών. Άλλες πτυχές που εξετάστηκαν ήταν (α) η βελτιστοποίηση της απόδοσης των πιλοτικών βιοκλινών μέσω βιοεμπλουτισμού τους με εξειδικευμένα βακτηριακά εμβόλια και (β) η σύσταση και λειτουργία της μικροβιακής κοινότητας των βιοκλινών με την χρήση μοριακών προσεγγίσεων (q-PCR). Επίσης, αξιολογήθηκαν πιθανές λύσεις σε πρακτικά ζητήματα που ακόμη παρεμποδίζουν την πλήρη εφαρμογή των βιοκλίνων όπως (i) η εκτίμηση του κινδύνου για το περιβάλλον από την άμεση εναπόθεση των επεξεργασμένων υγρών

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αποβλήτων και (ii) μεθόδων απορρύπανσης του εξαντλημένου υλικού πλήρωσης των βιοκλινών. Για το λόγο αυτό κατασκευάστηκαν τρεις πιλοτικές βιοκλίνες του 1 m^3 (δεν βιοεμπλουτίστηκαν) και δυο πιλοτικές βιοκλίνες των 0,24 m^3 (βιοεμπλουτίστηκαν) και αξιολογήθηκε η απόδοση τους ύστερα από εφαρμογή για διάστημα 160 ημέρων υγρών αποβλήτων που περιείχαν διαφορετικούς συνδυασμούς γεωργικών φαρμάκων προσομοιάζοντας πραγματικά σενάρια συσκευαστηριών μηλοειδών και εσπεριδοειδών. Οι πιλοτικές βιοκλίνες έδειξαν υψηλή ικανότητα απομάκρυνσης τόσο των λιγότερο υπολειμματικών OPP, DPA (> 99,9%) όσο και των πιο υπολειμματικών IMZ και TBZ (> 99,5%). Ο βιοεμπλουτισμός μεγιστοποίησε την απόδοση των βιοκλινών ενάντι του υπολειμματικού μυκητοκτόνου TBZ, το οποίο απομακρύνθηκε πλήρως. Παράλληλα παρατηρήθηκε σημαντική αύξηση της αφθονίας των βακτηρίων, των μυκήτων και των καταβολικών γονιδίων *catA* και *pcaH* που εμπλέκονται στην αποδόμηση αρωματικών οργανικών ενώσεων. Ο βιοεμπλουτισμός αποτέλεσε την πιο αποτελεσματική μέθοδο για την απορρύπανση του εξαντλημένου υλικού πλήρωσης των βιοκλινών. Περάν αυτού η κομποστοποίηση με φρέσκια οργανική ουσία αλλά ακόμα και η απλή αποθήκευση του υλικού σε θερμοκρασία περιβάλλοντος αποτελούν αποτελεσματικές εναλλακτικές λύσεις απουσία μικροβιακών εμβολιών για την εφαρμογή βιοεμπλουτισμού. Αξιολόγηση του περιβαλλοντικού κινδύνου που ενέχει η απευθείας απόρριψη των επεξεργασμένων αποβλήτων (με βάση σενάρια πρακτικής εφαρμογής των γεωργικών φαρμάκων, την απόδοση των πιλοτικών βιοκλινών και τις σχετικές Κοινοτικές Οδηγίες) έδειξαν ότι η απόρριψη των επεξεργασμένων αποβλήτων σε επιφάνεια αγρού 0,1 ha, δεν ενέχει μη αποδεκτό κίνδυνο για τα υδάτινα και χερσαία οικοσυστήματα, με εξαίρεση τα απόβλητα που περιέχουν TBZ και παράγονται από συσκευαστήρια μηλοειδών, όπου ο κίνδυνος ήταν αποδεκτός μόνο όταν τα απόβλητα απορρίπτονται σε μεγαλύτερη επιφάνεια (0,2 ha) ή εφαρμόστηκε βιοεμπλουτισμός στις βιοκλίνες που δέχτηκαν TBZ.

Συνολικά η παρούσα διδακτορική διατριβή παρέχει μια ολοκληρωμένη αξιολόγηση των βιοκλινών ως μέθοδοι επεξεργασίας των υγρών αποβλήτων που παράγονται από συσκευαστήρια φρούτων. Τα ευρύματα της παρούσας διατριβής αποδεικνύουν ότι τα συγκεκριμένα συστήματα θα μπορούσαν να είναι μια βιώσιμη λύση για την επεξεργασία των συγκεκριμένων αγρο-βιομηχανικών υγρών αποβλήτων. Περαιτέρω μελέτες θα διερευνήσουν την εφαρμογή των βιοκλινών για

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την επεξεργασία υγρών αποβλήτων επιβαρυμμένων με γεωργικά φάρμακα που παράγονται και από άλλες αγροτικές βιομηχανίες (π.χ. απολύμανσης βολβών, επικάλυψης σπόρων).

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Chapter 1

General Introduction

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1.1. PESTICIDES: DEFINITION, USES AND HISTORY

Into the environment are discharged a large number of pollutants and wastes and thus is as a result of human activities. Worldwide, more than one billion pounds of toxins are released into the air and water. In addition, approximately $6x10^6$ chemical compounds have been produced and as a result of this, annually 1,000 new products are synthetized and between 60,000 and 95,000 chemicals are commercially used (Shukla et al. 2010). Moreover, intensive industrialisation and large-scale use of synthetic xenobiotic compounds have generated hazardous contaminants including organics, inorganics and heavy metals. These contaminants create numerous environmental problems including harmful effects on biogeochemical cycling, environmental health and toxic effects onto non-target organisms including humans (Singh 2009). Among these, synthetic pesticides constitute a major group of chemicals which are used extensively in agricultural regions to minimize pest infestations, protect crops yields and prevent any reductions in the quality of the agricultural products (Shukla et al. 2010).

Pesticides are by no means a new invention. In fact, the history of intentional pesticide use goes back thousand years when Sumerians, Greeks and Romans were killing pests using sulphur, mercury, arsenic, copper or plant extracts. However, the effectiveness of the pesticide use was frequently underestimated because of the primitive chemistry and the insufficient application methods. A rapid emergence in pesticide use began mainly after World War II with the introduction of DDT, BHC (benzene hexachloride), aldrin, dieldrin, endrin, and 2,4-D (2,4-dichlorophenoxyacetic acid). These chemicals were effective, easy to use, inexpensive, and thus enormously popular. However, under constant chemical usage, some pests became genetically resistant to pesticides, non-target organisms were harmed, and pesticide residues often appeared in unexpected places. With the publication of Carson's book 'Silent Spring' in 1962, public confidence in pesticide use was shaken.

In order to minimize the adverse effects of pesticides on the environment and human health EU implemented Directive 91/414/EC which described all the procedures that should be followed for a pesticide to be granted authorization for use at EU level. This Directive was recently replaced by Regulation 1107/2009 and defines PPPs (also referred to as 'pesticides') as *"products in the form in which they*

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are supplied to the user, consisting of active substances, safeners or synergists, and intended for one of the following uses:

(a) protecting plants or plant products against all harmful organisms or preventing the action of such organisms, unless the main purpose of these products is considered to be for reasons of hygiene rather than for the protection of plants or plant products (e.g. fungicides, insecticides);

(b) influencing the life processes of plants, such as substances influencing their growth, other than as a nutrient (e.g. plant growth regulators, rooting hormones);

(c) preserving plant products, in so far as such substances or products are not subject to special Community provisions on preservatives (e.g. extending the life of cut flowers);

(d) destroying undesired plants or parts of plants, except algae unless the products are applied on soil or water to protect plants (e.g. herbicides/weedkillers to kill actively growing weeds);

(e) checking or preventing undesired growth of plants, except algae unless the products are applied on soil or water to protect plants (e.g. herbicides/weedkillers preventing the growth of weeds).

PPPs contain at least one approved pesticide active substance; these could be not only synthetic chemicals but micro-organisms, pheromones and botanical extracts. Before any pesticide and the associated PPP which contain this pesticide active substance can be placed on the market or used, it must be authorised firstly by the European Commission (upon consultation of the European Food Safety Authority (EFSA)) and secondly by the Member State(s) concerned (Storck et al., 2016).

1.2. PESTICIDES SALES AND CONSUMPTION IN EU

In modern conventional agriculture increased production depends largely on the application of pesticides which protect plants from damage by fungi, bacteria, nematodes and insects. The most well-defined and populated categories of pesticides are the insecticides, herbicides and fungicides (Tortella and Diez 2005; Ζιώγας 2007).

In 2014, the total quantity of pesticide sales in the EU-28 amounted to approximately 400.000 tonnes. Spain (19.9 %), France (19.0 %), Italy (16.2 %), Germany (11.6 %) and Poland (5.9 %) were the Member States in which the highest

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quantities of pesticides were sold, and together they made up 72.7 % of the EU-28's pesticide sales (Table 1.1) (Eurostat, 2016)

Table 1.1: Pesticide sales (in tonnes) by major pesticides groups in 2014 (Eurostats, 2016)

	Total pesticides sales	Fungicides and bactericides	Herbicides, haulm destructors and moss killers	Insecticides and acaricides	Molluscicides	Plant growth regulators	Other plant protection products	Share in the total EU-28 pesticide sales
	(Tonnes)							(%)
EU-28 (*)	395 944.4	173 250.8	131 263.5	20 706.3	1 684.4	12 843.7	56 195.7	100.0
Belgium	7 001.1	3 095.0	2 519.7	555.8	47.7	261.2	521.6	1.8
Bulgaria	1 002.0	186.1	652.4	163.4	:	:	:	0.3
Czech Republic	5 663.4	1 788.3	2 755.3	337.7	15.5	350.3	416.2	1.4
Denmark	1 974.6	530.2	1 242.5	38.3	15.4	114.2	33.9	0.5
Germany	46 078.5	12 739.9	17 876.7	977.2	255.5	2 171.3	12 058.0	11.6
Estonia	596.0	88.2	425.8	25.3	:	56.6	:	0.2
Ireland	2 736.0	635.5	2 039.2	51.4	9.9	:	0.0	0.7
Greece	3 907.1	1 866.4	1 194.6	588.8	1.2	148.5	107.7	1.0
Spain	78 818.3	38 379.7	14 908.0	7 515.1	66.2	156.4	17 793.0	19.9
France	75 287.5	34 430.6	30 965.5	2 610.9	870.2	2 802.9	3 607.5	19.0
Croatia	2 119.1	1 004.8	889.1	143.1	5.4	72.2	4.5	0.5
Italy	64 071.1	37 907.1	7 864.4	2 251.9	75.0	367.4	15 605.2	16.2
Cyprus	1 046.7	698.1	153.4	180.6	1.0	1.2	12.5	0.3
Latvia	1 417.4	224.7	847.5	64.0	0.0	274.5	6.6	0.4
Lithuania	2 545.6	604.8	1 394.2	43.6	0.0	502.9	:	0.6
Luxembourg (²)	176.1	91.0	82.8	:	2.3	:	:	0.0
Hungary	8 959.5	3 634.1	4 011.1	916.5	3.5	203.3	190.9	2.3
Malta	108.4	97.4	7.6	2.9	0.5	0.0	:	0.0
Netherlands	10 665.6	4 869.1	3 266.4	252.0	45.1	452.0	1 780.8	2.7
Austria	3 373.2	1 641.1	1 375.8	240.2	16.2	53.5	46.4	0.9
Poland	23 550.6	7 442.5	12 073.4	1 479.2	35.3	2 128.0	392.3	5.9
Portugal	12 889.2	8 244.4	2 410.8	732.9	35.7	1.4	1 464.0	3.3
Romania	10 021.2	4 131.9	5 025.4	569.0	1.2	270.6	23.1	2.5
Slovenia	1 009.0	723.7	238.5	33.5	2.2	0.6	10.5	0.3
Slovakia	2 198.0	567.2	1 215.1	106.5	:	179.8	129.4	0.6
Finland	3 579.9	198.5	1 305.4	12.8	:	88.6	1 974.5	0.9
Sweden	2 486.7	302.3	2 103.8	34.2	:	29.3	17.1	0.6
United Kingdom	22 662.7	7 128.1	12 418.9	779.4	179.4	2 156.8	:	5.7
Norway	859.8	121.8	692.0	4.8	1.3	39.1	0.7	:
Switzerland	2 240.9	1 002.2	745.4	83.1	55.9	30.7	323.6	:

(*) Confidential data have been removed from the sums of pesticides sales. They represent 0,003% of Total pesticides sales in the EU.
(²) 'Fungicides and bactericides': 2012 data , other data: 2013.

"Fungicides and bactericides" were the most sold group of pesticides with a 44% share of the market, followed by "herbicides, haulm destructors and moss killers" with a 33% share of the market. Together with the group "other plant protection products" (14 %), these three groups added up to 91 % of the pesticides sold in the EU-28 in 2014. Of the other three groups of pesticides, "insecticides and acaricides" had a 5% share, plant growth regulators 3% and molluscicides held the smallest share with less than 1% (Fig. 1.1). The quantities of pesticides that are put on the market yearly can be associated with other statistics directly related to the use of the pesticides (Eurostats, 2015 and 2016).

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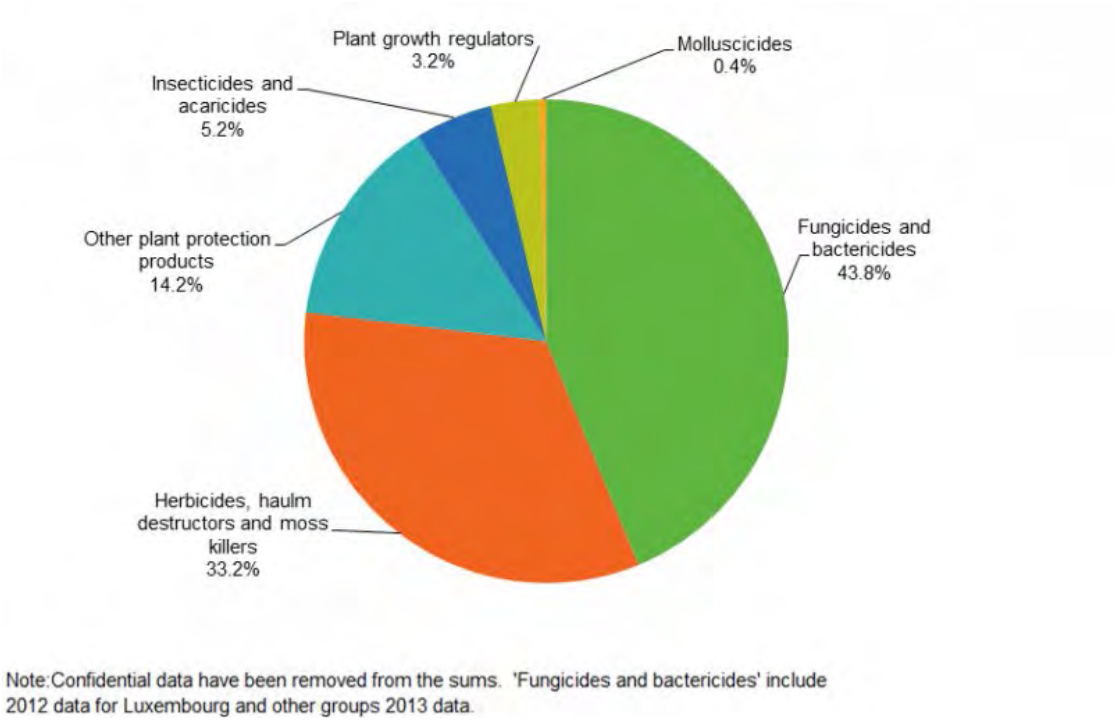

Figure 1.1. Pesticide sales by major groups in EU-28, 2014, (%). (Eurostats, 2016)

1.3. BENEFITS AND RISKS BY PESTICIDES USE

1.3.1. Benefits

The ideally definition of a pesticide must be lethal to the target pests and at the same time have no effects on non-target organisms including especially humans. Unfortunately, this is not always the case, so the controversy of use and abuse of pesticides has been growing. The rampant use of these chemicals, under the adage, “if little is good, a lot more will be better” has played havoc with human and other life forms (Aktar et al. 2009). Pesticides are widely used in most sectors of the agricultural production. The main reason of their use is to prevent or reduce losses by pests and thus can improve yield as well as the quality of the produce. Although sometimes quality is even in terms of cosmetic appeal, which is often important to consumers (Oerke et al. 2004: Cooper and Dobson, 2007). Pesticides can also improve the nutritional value of food and sometimes its safety (Boxall, 2001: Narayanasamy, 2006). There are also many other benefits that may be attributed to pesticides, but these benefits often go unnoticed and there are not so obviously by the general public (Cooper and Dobson, 2007: Dalamas, 2009). Thus, from this point of view, pesticides can be considered as an economic, labor-saving, and efficient tool of pest management with great popularity in most sectors of the agricultural production.

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Worldwide approximately 9.000 species of insects and mites, 50.000 species of plant pathogens, and 8.000 species of weeds damage crops. Different pests such as insects and weeds cause yield losses estimated to approximately 14% and 13% respectively. Pesticides are an indispensable part of agricultural production. Without pesticide application the loss of fruits, vegetables and cereals from pest injury would reach 78%, 54% and 32% respectively. Crop loss from pests declines to 35% - 42% when pesticides are used (Ortiz-Hernandez et al. 2013).

The primary benefits are the consequences of the pesticides effects, the direct gains expected from their use. The secondary benefits are the less immediate or less obvious benefits that result from the primary benefits. They may be subtle, less intuitively obvious, or of longer term. For example, improving productivity is one of the tremendous benefits that have been derived from the use of pesticides in forestry, public health and the domestic sphere and, of course, in agriculture (Aktar et al. 2009). In certain instances pesticides could safeguard public health. Such a case is the application of pesticides to control mosquitoes, which act as vectors of malaria (Ross, 2005). In addition, the consumption of high quality fresh fruits and vegetables ensured by pesticide use (but remaining free of pesticides residues) could reduce the risk of cancer, high blood pressure, heart disease, diabetes, stroke, and other chronic diseases (Dietary guidelines, 2005).

1.3.2. Risks

The use of pesticides, despite their popularity and extensive use, has raised serious concerns about health risks. Some of the most important risks are the exposure of farmers on pesticides when mixing and applying pesticides or working in treated fields and for the general population from residues on food and in drinking water (Damalas and Eleftherohorinos, 2011). On the other hand, food is the basic necessity of life and the use of pesticides can increase the quality of the food, but contaminated food with toxic pesticides is associated with severe effects on the human health. Obviously, exposure to pesticides poses a continuous health hazard, especially in the agricultural working environment. By their very nature and their definition most pesticides show a high degree of toxicity because they are designed to kill certain organisms and thus create some risk of harm. Within this context, pesticide use has raised serious concerns and questions not only of potential effects on human health,

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but also about impacts on wildlife and sensitive ecosystems and microorganisms (Stoate et al. 2001: Power 2010).

The pesticide impact on human health is not an easy and particularly accurate process because of differences in the periods and the levels of application, type of pesticides (regarding toxicity), mixtures or cocktails of commercial pesticide used in the field, and the geographic and meteorological characteristics of the agricultural areas where pesticides are applied. In addition, such multivariate differences are refered mainly to the farmers who prepare the mixtures in the field, the pesticide sprayers, and also the population that lives near the sprayed areas, pesticide storage facilities, greenhouses, or open fields (Bolognesi, 2003: Pastor et al. 2003: Magkos et al. 2006).

Regardless of the difficulties in assessing risks of pesticide use on human health, in the authorization dossier for a pesticide placement in the market at EU level currently requires data of potential negative effects of the pesticide active substances on human health. There are several tests that is used to obtaine those e.g., metabolism patterns, acute toxicity, sub-chronic or sub-acute toxicity, chronic toxicity, carcinogenicity, genotoxicity, teratogenicity, generation study, and also irritancy trials using rat as a model mammal or in some cases dogs and rabbits (Damalas and Eleftherohorinos, 2011).

The US EPA (2009) adopted several toxicity tests for human and animals health risk assessments, in which are: (1) the acute toxicity test, which assesses the effects of short-term exposure to a single dose of pesticide, (2) the sub-chronic toxicity test, which assesses the effects of intermediate repeated exposure over a longer period of time (30–90 days), (3) the chronic toxicity test, which assesses the effects of long-term repeated exposure lasting for most of the test animal’s life span and intended to determine the effects of a pesticide product after prolonged and repeated exposures (e.g., chronic non-cancer and cancer effects), (4) the developmental and reproductive tests, which assess any potential effects in the fetus of an exposed pregnant female (*i.e.*, birth defects) and how pesticide exposure may influence the ability of a test animal to reproduce successfully, (5) the mutagenicity test which assesses the potential of a pesticide to affect the genetic components of the cell, and (6) the hormone disruption test, which measures the pesticide potential to disrupt the endocrine system (consists of a set of glands and the hormones they produce that regulate the development, growth, reproduction, and behavior of animals

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including humans). The acute toxicity experiments are required for the calculation of the lethal dose (LD_{50}), which is the pesticide dose that is required to kill half of the tested animals when entering the body by a particular route. These endpoints are used for US EPA toxicity classifications of pesticides shown in Table 1.2. (US EPA, 2009).

Table 1.2. Acute toxicity of pesticides according to the US EPA classification (US EPA, 2009)

Class	Signal Words	Acute toxicity to rats		
		Oral LD50 (mg/kg)	Dermal LD50 (mg/kg)	Inhalation LC50 (mg/L)
I	DANGER	<50	<200	0.2
II	WARNING	50-500	200-2000	0.2-2.0
III	CAUTION	500-5000	2000-20000	2.0-20
IV	CAUTION (optional)	>5000	>20000	>20

Pesticides, despite to their potential negative effects on human, pose unpropitious effects also to the environment (water, soil and air contamination, toxic effects on non-target organisms) (Rayu et al., 2012). In particular, inappropriate and extensive use of pesticides has been linked with: (1) adverse effects on non-target organisms (e.g., reduction of beneficial species populations), (2) water contamination from mobile pesticides or from pesticide drift, (3) air pollution from volatile pesticides, (4) injury on non-target plants from herbicide drift, (5) injury to rotational crops from herbicide residues remained in the field, (6) crop injury due to high application rates, wrong application timing or unfavorable environmental conditions at and after pesticide application (Damalas and Eleftherohorinos, 2011: Palma et al., 2014).

1.4. ENVIRONMENTAL FATE OF PESTICIDES

Upon their application in the environment pesticides are subject to various environmental processes which determine their long or short range transporation to other environmental compartments (Fig. 1.2.). The main processes that determine the environmental fate of pesticides in soil are (a) sorption, (b) degradation (biotic and abiotic), (c) transportation to surface water (runoff, erosion, spray drift) or groundwater (leaching) and air (Andreu and Pico, 2004).

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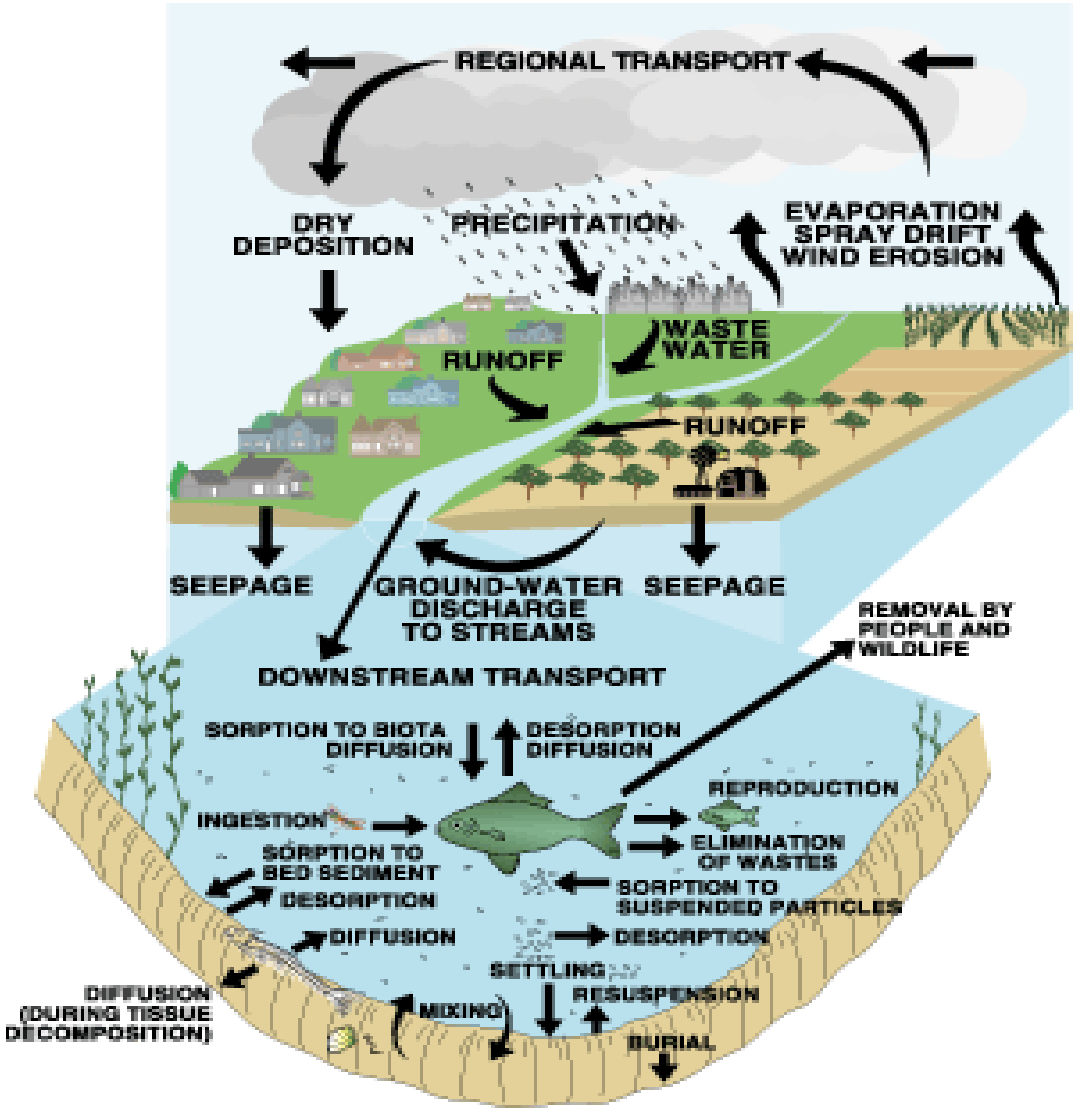

Figure 1.2. A schematic representation of the processes controling the fate of pesticides upon their release in the environment

1.4.1. Pesticides sorption processes

Sorption of pesticides onto soil particules is achieved either through weak or strong chemical bonds or through diffusion of the pesticide molecule in the soil structure. Diffusion of pesticides into the capillaries of soil structure is a physical process, where the compound is still in the liquid phase, but “hanging” in capillaries. Because of the sorption and the diffusion, the measurements of the pesticide concentration in the aqueous phase will be without the contribution from the fraction of the pesticide that is trapped in the soil structure. Diffusion is reversible and the compound will contribute to the equilibration in a desorption study. However, reversible sorption by

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chemical bonding is due to the ionic properties of the pesticide compound. For example, phenols and organic acids will be sorbed to positively charged microsites of the soil colloids, while in contrary positively charged compounds, like quaternary amines, will be sorbed onto negatively charged surfaces of soil colloids. Hydrogen bonds are weakly capable to sorb pesticides, in contrast, pesticide sorption through covalent bonding will be performed by irreversible sorption where the chemical is bonded into the humic acid structure of the soil (Duus Børgensen et al., 2015).

The extent of pesticide sorption onto soil influences the mobility and risk for contamination of the soil and water environment. Compounds showing weak sorption will conservatively follow the water movement while a strongly sorbed compound will be retained depending on the mechanism of sorption. The degree of sorption depends on the properties of the compound and the soil properties (de Wilde et al, 2008). Pesticide sorption in soil is currently determined following international guidelines developed and mutually recognized by OECD (OECD, 2000). This allows the proper comparison of the sorption affinity of different pesticide compounds with safety.

1.4.2. Pesticides transportation processes

Depending on their mobility and persistence, pesticides can migrate within and outside the soil and contaminate water resources and air. The main pesticide transfer processes are a) atmospheric: spray drift, volatilisation, and atmospheric transportation followed by re-deposition, or b) water-driven: drainage, leaching, and surface and sub-surface runoff. The relative importance of each of the processes depends on the pesticide application conditions, the pesticide properties, the climatic conditions, and the soil properties partly governed by agricultural practices (Alletto et al., 2010; Reichenberger, 2007). Most pesticide transfer processes have a diffuse-source nature, but point sources in the form of farmyard runoff, accidental spills, or sewer outflows can cause significant contamination of water bodies with pesticides.

Volatilisation is an important pathway for the loss of pesticides and occurs when pesticide surface residues change from a solid or liquid to a gas or vapor after a pesticide application The extent of volatilization of a pesticide compound is governed by pesticide properties (such as vapour pressure, Henry's law constant, K_{OC}), soil properties (water content, organic carbon content), farming practices (mode of

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pesticides application, presence of a mulch) and the climatic conditions (wind, solar radiation, temperature). (Bedos et al., 2002).

Leaching is the vertical movement of pesticides through soil due to water percolation to rivers, lakes and streams, wells, storm sewers, or into groundwater. Pesticide leaching depends on soil physical-chemical properties such as the hydraulic conductivity and the water solubility of the pesticides. Pesticides with high water solubility are vulnerable to leaching, while important role plays the climatic conditions like the intensity and timing of rainfall events after pesticide application facilitating leaching of pesticides (Alletto et al., 2010).

Runoff is the primary mechanism contributing to pesticide contamination of surface waters. The most important parameter to control a runoff event and pesticide losses is the rainfall. Thus, depending of the rainfall, for example its occurence after pesticide application, its intensity and the interval between two rainfall events, precipitation of the compound can lead to very contradictory results for the same study site, the same molecule or even the same practice (Schulz and Matthies, 2007).

1.4.3. Pesticides degradation processes

Degradation of pesticides refers to the breakdown of pesticides within the environment. The degradation may be occurred by two pathways a) through *abiotic processes* i.e. photodegradation or photolysis and chemical degradation or b) through *biotic processes* i.e. biodegradation (Fig 1.3.) (Topp et al., 1997). In both pathways, in some cases there is a complete mineralization of the pesticides, whereas in other cases only a partial degradation occurs. This may potentially lead to an accumulation of metabolites, which sometimes are more toxic, i.e. more hazardous, than the parent compound (Giacomazzi and Cochet, 2004). In some cases the pesticides are not degraded even though they have proven to be biodegradable and this is may be due to different environmental factors that can cause the effectiveness of the activity of the degrading organisms; essential nutrients may be missing, environmental conditions may be unsuitable, or the concentration of the pesticide may be too high or too low (Amellal et al., 2001; Iranzo et al., 2001). All chemicals are susceptible to photodegradation to some extent. The degree of photodegradation of a chemical compound depends on the intensity of the sunlight and the time of exposure. However many pesticides are more mobile into the soil and are thus no longer exposed to sunlight and therefore not susceptible to photodegradation (Gavrilescu 2005).

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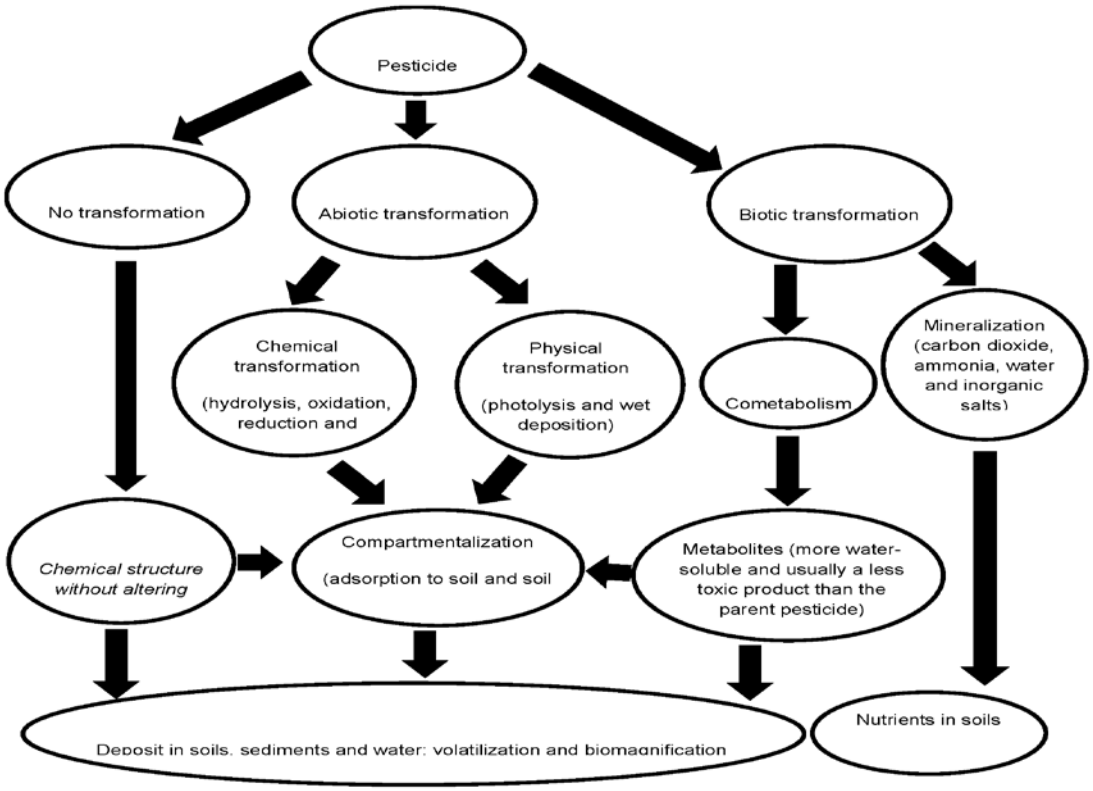

Figure 1.3. Degradation of pesticides within the environment

Chemical degradation is due to reactions of the pollutant with e.g. water, oxygen or other chemicals in addition to the biodegradation witch refers to the degradation of pesticides by organisms, most often microorganisms like bacteria and fungi, but also in some cases plants may be involved as well (Topp et al., 1997). The degradation rates of the pesticides are affected mostly by soil properties and environmental conditions e.g. pH, organic matter content, temperature and moisture. The optimum environmental conditions for the degradation of pesticides are reported in Table 1.3. The effect of pH on soil degradation will depend on the compound being degraded and the organisms responsible for the degradation. Studies by Walker et al. (2001) showed a more rapid degradation of isoproturon, a phenylurea herbicide, in soils with higher pH. Similarly Simon et al., (1992) and Singh et al. (2003) found a positive correlation between the degradation of fenamiphos and chlorpyrifos, both organophosphorus insecticides, and soil pH. The temperature of the soil also influences degradation rates; the rate of most reactions catalyzed by enzymes tends to double for every 10°C increase in temperature (between 10 and 45°C). An increase in soil temperature will thus lead to an increase in degradation rates (MacRae and

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Alexander, 1965). Soil moisture constitutes another important factor affecting the dissipation of pesticides in soil. Increasing soil moisture up to a level which ensures optimum conditions for pesticide solubility and the activity of soil microorganisms is expected to have a positive effect on pesticide degradation. For example Walker (1978) reported an increase in methazole DT_{50s} from 3.5 to 5 and 9.6 days when the soil moisture content was adjusted to 100%, 50% and 25% of field capacity.

Table 1.3. Optimum environmental conditions for the degradation of pesticides

Parameters	**Condition required for microbial activity**	**Optimum value for an oil degradation**
Soil moisture	25–28% of water holding capacity	30–90%
Soil pH	5.5–8.8	6.5–8.0
Oxygen content	Aerobic, minimum air-filled pore space of 10%	10–40%
Nutrient content	N and P for microbial growth	C:N:P = 100:10:1
Temperature (°C)	15–45	20–30
Contaminants	Not too toxic	Hydrocarbon 5–10% of dw of soil
Heavy metals	Total content 2000 ppm	700 ppm
Type of soil	Low clay or silt content	

1.5. REMEDIATION OF PESTICIDE POLLUTED SITES

In the recent decades, a gradual increase in environmental pollution from several xenobiotic compounds, such as pesticides, polycyclic aromatic compounds, chlorinated biphenyls, polychlorinated dibenzo-dioxins has been observed (Singh and Chen, 2008). The frequent detection of high concentrations of organic pollutants, including pesticides, in the environment has created global concern because of the increased likelihood of health problems in humans and animals. These problems stimulated the development of technologies that guarantee pesticides elimination in a safe, efficient, and economical way. Different methods have been developed and implemented to remediate contaminated sites and remove pesticide residues and/or obsolete pesticides. Existing technologies could be categorized to those that utilize physical processes, such as sorption, those that are based on chemical processes, such as advanced oxidation and those which are characterized as “green technologies” such as bioremediation (Ortiz-Hernandez et al., 2013).

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1.5.1 Physical and Chemical remediation techniques

The conventional physicochemical approaches are generally expensive and often incomplete due to the conversion of the parent compound to metabolites which are more persistent and equally or more toxic than the parent compound. The conventional techniques used for remediation include (a) diging up of contaminated soil, removal and landfilling or (b) to cap and contain the contaminated areas of a site. These methods have some drawbacks. The first method simply moves the contamination elsewhere and may create significant risks during excavation, handling, and transportation of the hazardous material. Additionally, it is very difficult and increasingly expensive to find new landfill sites for the final disposal of the material. The 'cap and contain' method is only an interim solution since the contamination remains on site, requiring monitoring and maintenance of the isolation barriers long into the future, with all the associated costs and potential liability (Vidali, 2001).

The physical and chemical remedition approaches can be categorized into:

Solidification/Stabilization (S/S): It is one of the top five source control treatment technologies. “Solidification” refers to a process in which materials are added to the waste to produce an immobile mass. This may or may not involve a chemical bonding between the toxic contaminant and the additive. “Stabilization” refers to converting a waste to a more chemically stable form. This conversion may include solidification, but it almost always includes use of physicochemical reactions to transform the contaminants to a less toxic form (Dadrasnia et al., 2013).

Soil vapour extraction: In cases where the contaminants are volatile, a venting and *ex-situ* gas treatment system can be applied. Soil vapour extraction is a technology that has been proven effective in reducing concentrations of volatile organic compounds (VOC) and certain semi-volatile organic compounds (SVOC). Principally, a vacuum is applied to the soil matrix to create a negative pressure gradient that causes movement of vapors toward extraction wells. Volatile contaminants are readily removed from the subsurface through the extraction wells. The collected vapors are then treated and discharged to the atmosphere or where permitted, re-injected to the subsurface (Dadrasnia et al., 2013).

Soil washing: It uses liquids (usually water, occasionally combined with solvents) and mechanical processes to scrub soils. Solvents are selected on the basis of their ability to solubilize specific contaminants, and on their environmental and health effects. The

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soil washing process separates fine soil (clay and silt) from coarse soil (sand and gravel). Since hydrocarbon contaminants tend to bind and sorb to smaller soil particles (primarily clay and silt), separating the smaller soil particles from the larger ones reduces the volume of contaminated soil (Khan et al., 2004).

Air sparging: It is an *in situ* technology in which air is injected through a contaminated aquifer. Air-sparing stimulates aerobic biodegradation of contaminated groundwater by delivery of oxygen to the subsurface. This is accomplished by injecting air below the water table. This technology is designed primarily to treat groundwater contamination by fuels, non-halogenated VOCs, SVOCs, pesticides, organics, and herbicides. Air sparing has also been demonstrated to be an innovative groundwater remediation technology capable of restoring aquifers that have been polluted by volatile and (or) biodegradable contaminants, such as petroleum hydrocarbons. The process may be applied to halogenated organics, but is less effective (Johnson et al., 2007).

Thermal Desorption: It is an innovative treatment technology where contaminated soil is excavated, screened, and heated to release petroleum from the soil (US EPA, 1995). It involves heating soils to temperatures of 100–600°C so that those contaminants with boiling points in this range will vaporize and separate from the soil. The vaporized contaminants are then collected and treated by other means. There is some confusion about the difference between thermal desorption and incineration: thermal desorption does not aim to destroy the organic but rather to change their form to a more treatable one, while incineration aims to destroy the contaminant. The actual process of thermal desorption involves heating the soil in a chamber where organic contaminants and certain metals can be vaporized. From there, a gas or vacuum system transports the vaporized contaminants to an off-site treatment system (Khan et al., 2004).

1.5.2. Bioremediation techniques

Usually the contaminated sites are treated with traditional physical, chemical and thermal processes. Using these methods, the cost of removal of 1 m^3 of soil from 1-acre contaminated site is estimated to be 0.6–2.5 million US $. Billions of dollars are expected to be used to clean up all sites polluted with polycyclic aromatic hydrocarbon (PAHs) (McIntyre, 2003). Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an

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innocuous state, or to levels below concentration limits established by regulatory authorities (Rayu et al. 2012: Vidali, 2001). By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment (Vidali, 2001).

There are several studies and researchers that have developed and modelled different bioremediation techniques. However, due to the nature and/or the type of pollutant, there is no single bioremediation technique that serves as a 'silver bullet' to restore polluted environments. Indigenous microorganisms present in the polluted environments hold the key to solving most of the challenges associated with biodegradation and bioremediation of pollutants provided that environmental conditions are suitable for their growth and metabolism (Azubuike et al., 2016). The use of the indigenous soil microbial community to remediate a polluted site (achieved through optimization of their growth conditions) is called biostimulation and it is the most commonly utilized bioremediation approach. Alternatively bioremediation frequently involves the addition of microorganisms indigenous or exogenous to the contaminated sites in a process called bioaugmentation. Two factors often limit the use of bioaugmentation in land treatment: (1) non-indigenous microbial inocula rarely compete well enough with an indigenous population to develop and sustain useful population levels and 2) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed (Vidali, 2001).

Most important parameters for bioremediation are the i) nature of the pollutants, ii) soil structure, pH, moisture contents and hydrogeology, iii) nutritional status, iv) microbial diversity and v) temperature and oxidation-reduction conditions in the site (redox- potential). In bioremediation processes, microorganisms use the contaminants as nutrient or energy sources. Bioremediation activity is stimulated by supplementation of nutrients (nitrogen and phosphorus), electron acceptors (oxygen), and substrates (methane, phenol, and toluene), or by introducing microorganisms with the desired catalytic capabilities (Shukla et al., 2010).

Bioremediation approaches can be broadly classified to *ex situ* or *in situ* (Hatzinger et al. 2002). *In situ* techniques are defined as those applied to soil and groundwater at the contaminated site with minimal disturbance. *Ex situ* techniques

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involve excavation or pumping in case of soil and water, respectively, of the contaminated substrate and placement in a contained area where bioremediation is employed.

1.5.2.1. In situ *bioremediation*

These techniques are generally the most desirable options due to their lower cost and less disturbance, since they provide the treatment in place avoiding excavation and transport of the contaminated matrix. *In situ* treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases (Azubuike et al., 2016). The most important *in situ* bioremediation treatments used are:

Bioventing: It involves controlled stimulation of airflow by delivering oxygen to the unsaturated (vadose) zone to increase biodegradation, by increasing the activity of indigenous microbes. In bioventing, amendments are made by adding nutrients and water (to adjust moisture content) to enhance biodegradation with the ultimate goal being to achieve microbial transformation of pollutants to a harmless state (Philp and Atlas, 2005).

Biosparging: It is very similar to bioventing in that air is injected into the soil subsurface to stimulate microbial activities to promote pollutant removal from polluted sites. However, unlike bioventing, air is injected at the saturated zone, which can cause upward movement of volatile organic compounds to the unsaturated zone to promote biodegradation. The effectiveness of biosparging depends on two major factors namely: soil permeability, which determines pollutant bioavailability to microorganisms, and pollutant biodegradability (Philp and Atlas, 2005).

Bioslurping: It combines vacuum-enhanced pumping, soil vapour extraction and bioventing to achieve soil and groundwater remediation by indirect provision of oxygen and stimulation of contaminant biodegradation (Gidarakos and Aivalioti, 2007). The technique is designed for free products recovery such as light non-aqueous phase liquids, thus remediating capillary, unsaturated and saturated zones. It can also be used to remediate soils contaminated with volatile and semi-volatile organic compounds.

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Phytoremediation: It relies on the use of plant interactions (physical, biochemical, biological, chemical and microbiological) in polluted sites to mitigate the toxic effects of pollutants. Depending on the pollutant type (elemental or organic), there are several mechanisms (accumulation or extraction, degradation, filtration, stabilization and volatilization) through which plants achieve removal of the soil pollutants. Elemental pollutants (toxic heavy metals and radionuclides) are mostly removed by extraction, transformation and sequesteration. On the other hand, organic pollutants (hydrocarbons and chlorinated compounds) are predominantly removed by degradation, rhizoremediation, stabilization and volatilization, with mineralization being possible when some plants such as willow and alfalfa are used (Azubuike et al., 2016).

1.5.2.2. Ex situ *bioremediation*

These techniques involve excavation of the polluted matrix and subsequen transportation to another site for treatment. *Ex situ* bioremediation techniques are usually considered based on: the cost of treatment, depth of pollution, type of pollutant, degree of pollution, geographical location and geology of the polluted site. Performance criteria, which also determine the choice of *ex situ* bioremediation techniques, have been described (Philp and Atlas, 2005). The most common *ex situ* bioremediation methods are:

Biopiling: It involves above-ground piling of excavated polluted soil, followed by nutrient amendment, and sometimes aeration to enhance biodegadation by basically increasing microbial activities. The components of this technique are: aeration, irrigation, nutrient and leachate collection systems, and a treatment bed. The populrity of of this particular *ex situ* technique has increased in recent years due to its constructive features including cost effectiveness, which enables effective biodegradation on the condition that nutrient, temperature and aeration are adequately controlled (Whelan et al., 2015).

Windrows: Reilies on the periodic turning of pilled polluted soil to enhance biodegradation by increasing the degradation activities of indigenous and/or transient hydrocarbonoclastic bacteria present in the polluted soil. The periodic turning of polluted soil, together with addition of water speeds up the biodegradation of the target pollutants (Barr 2002).

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Bioreactor: It is a vessel in which raw materials are converted to specific product(s) following a series of biological reactions. There are different operating modes of bioreactor, which include: batch, fed-batch, sequencing batch, continuous and multistage. The choice of operating mode depends mostly on market economy and capital expenditure. Conditions in a bioreactor support natural process of cells by mimicking and maintaining their natural environment to provide optimum growth conditions. Polluted samples can be fed into a bioreactor either as dry matter or slurry; in either case, the use of bioreactor in treating polluted soil has several advantages compared to other *ex situ* bioremediation techniques (Azubuike et al., 2016).

Land farming is the simplest bioremediation technique characterized by low cost and low equipment requirement for implementation. In most cases, it is regarded as *ex situ* bioremediation, while in some cases, it is regarded as *in situ* bioremediation technique. Pollutant depth plays an important role as to whether land farming can be carried out *ex situ* or *in situ*. In land farming, polluted soils are excavated and/or tilled, but the site of treatment apparently determines the type of bioremediation employed. When excavated polluted soil is treated on-site, it can be regarded as *in situ* land farming, whereas in all other cases (transportation and treatment off site) it is considered as *ex situ* bioremediation (Vidali, 2001).

Composting is a biological process which uses naturally occurring microorganisms to convert biodegradable organic matter into a humus-like product. Composting of agricultural waste and municipal solid waste has a long history and is commonly employed to recycle organic matter back into the soil to maintain soil fertility (Sharma et al., 1997). The process destroys pathogens, converts N from unstable ammonia to stable organic forms, reduces the volume of waste and improves the stability of the waste. The effectiveness of the composting process is influenced by factors such as temperature, oxygen supply (i.e. aeration), moisture content (optimum 60-70%), pH, C:N ratio (optimum 25-40:1), particle size and degree of compaction (Imbeah, 1998).

The organic substrates, bulking agents and amendments used in composting are mostly derived from plant material. The main components of the organic matter are carbohydrates (e.g. cellulose), proteins, lipids and lignin. The capacity of microorganisms to assimilate organic matter depends on their ability to produce the enzymes needed for degradation of the substrate. The more complex the substrate, the more extensive and comprehensive is the enzyme system required. Under optimal

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conditions, composting proceeds through three phases: (1) the mesophilic phase, (2) the thermophilic phase, which can last from a few days to several months, and (3) the cooling and maturation phase which lasts for several months (Fig. 1.4). The length of the composting phases depends on the nature of the organic matter being composted and the efficiency of the process, which is determined by the degree of aeration and agitation (Tuomela et al., 2000).

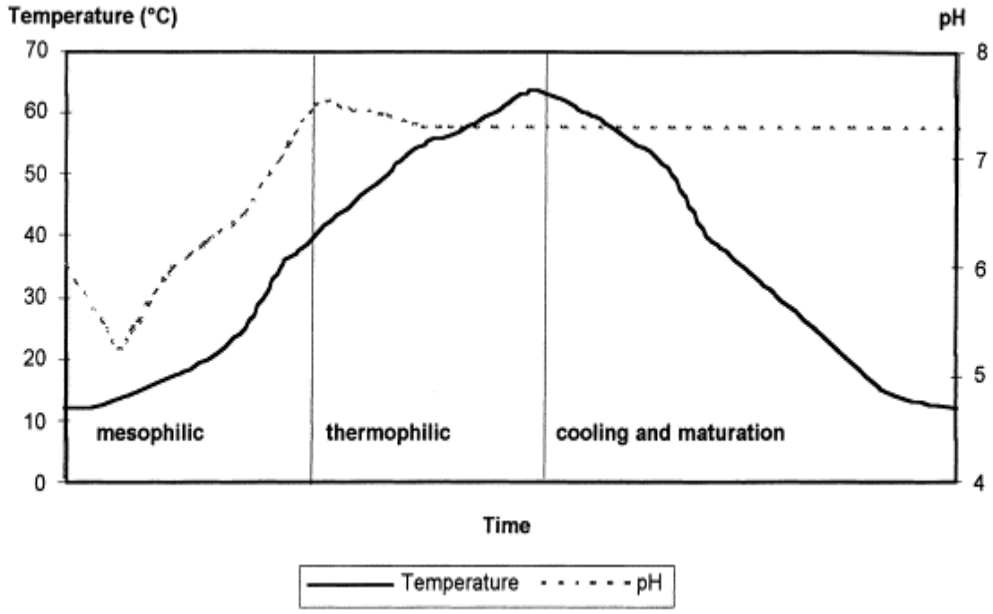

Figure 1.4. Temperature and pH variation during composting. The figure is redrawn from Tuomela et al., (2000)

Composting is a dynamic process carried out by a rapid succession of mixed microbial populations. The main groups of microorganism involved are bacteria, including actinobacteria, and fungi. Although the total number of microorganisms does not significantly change during composting, the microbial diversity can vary during the different phases of composting. The precise nature of succession and the number of microorganims at each composting phase is dependent on the substrate and on the preceding microorganisms in the succession. At the beginning of composting mesophilic bacteria predominate, but after the temperature increases to over 40°C, thermophilic bacteria take over and thermophilic fungi also appear in the compost. When the temperature exceeds 60°C, microbial activity decreases dramatically, but after the compost has cooled mesophilic bacteria and actinomycetes again dominates (Tuomela et al., 2000).

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1.6. ENVIRONMENTAL POLLUTION BY PESTICIDES

The indiscriminate use of pesticides before and after harvest can cause pollution of water resources, including surface and groundwater. This result in possible contamination of drinking water and risk for human health. Several monitoring studies have detected herbicides (i.e. diuron), and insecticides (i.e. chlorpyrifos) used at pre-harvest in surface water and groundwater (Echols et al., 2008: Hu et al., 2009). In addition several studies have reported the presence of fungicides used at post-harvest level, in fruit and vegetable packing plants, like imazalil (IMZ), thiabendazole (TBZ) and *ortho*-phenylphenol (OPP) in surface water systems crossing through fruit producing regions (Belenguer et al. 2014; Castillo et al. 2006; Masia et al. 2013; Ccanccapa et al. 2016)

Pesticides can pollute natural water resources through point and non - point sources (Fig. 1.5.). Non-point source pollution is the diffusion of pesticides to water ecosystems after their agricultural use through transportation processes including runoff, erosion, spray drift and leaching. In contrast, point source pollution originates from non orthodox agricultural practices at on-farm level such as improper activities during filling or emptying the sprayers, washing of spraying equipment or at post-farm level by disposal of pesticide-contaminated effluents produced by agricultural industries (Reichenberger *et al.*, 2007: de Wilde *et al.*, 2007: Candela *et al.*, 2008: Bourton *et al*., 2009). The contribution of point sources in the contamination of natural water resources is considerable and could range from 40-90% depending on several factors (Torstensson and Castillo, 1997; Ramwell et al. 2004). (Fig 1.6). Pollution of surface water and groundwater aquifers by pesticides is a major problem in Europe. For this reason the European Commission has established and defined the maximum permitted pesticide residue limits in water intended for drinking, to 0.1 μg L^{-1} for a single pesticide compound and 0.5 μg L^{-1} for all the pesticide compounds contained in a water sample (de Wilde *et al.*, 2007: Bourton *et al*., 2009).

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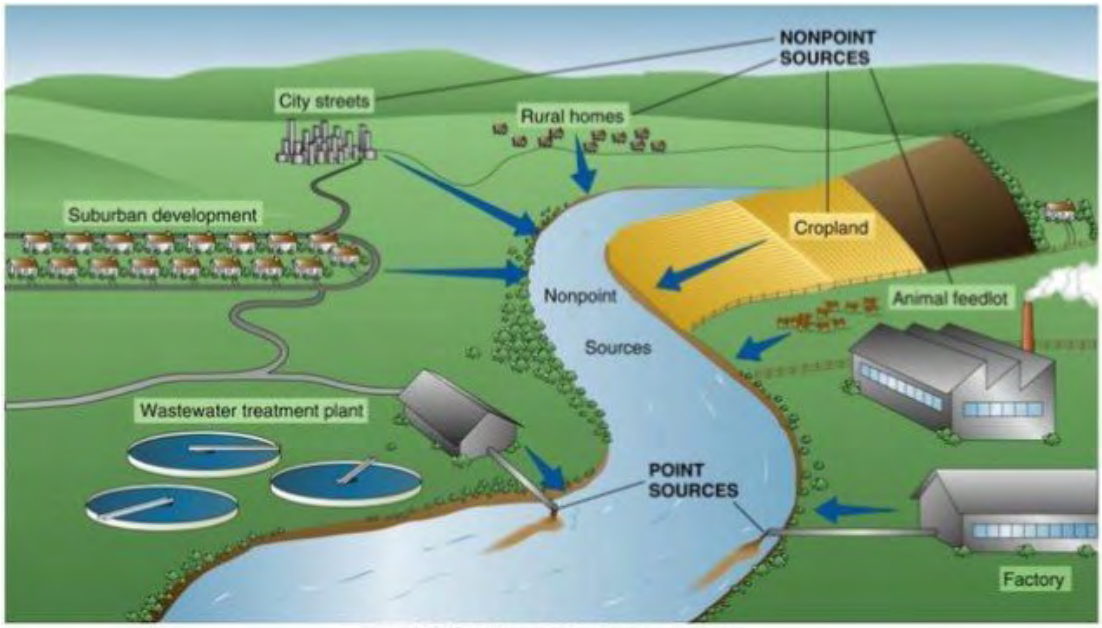

Figure 1.5. Schematic representation of pesticides pollution of water resources through point and non - point sources (http://www.the-macc.org/watershed/newsletters/winter-2015/)

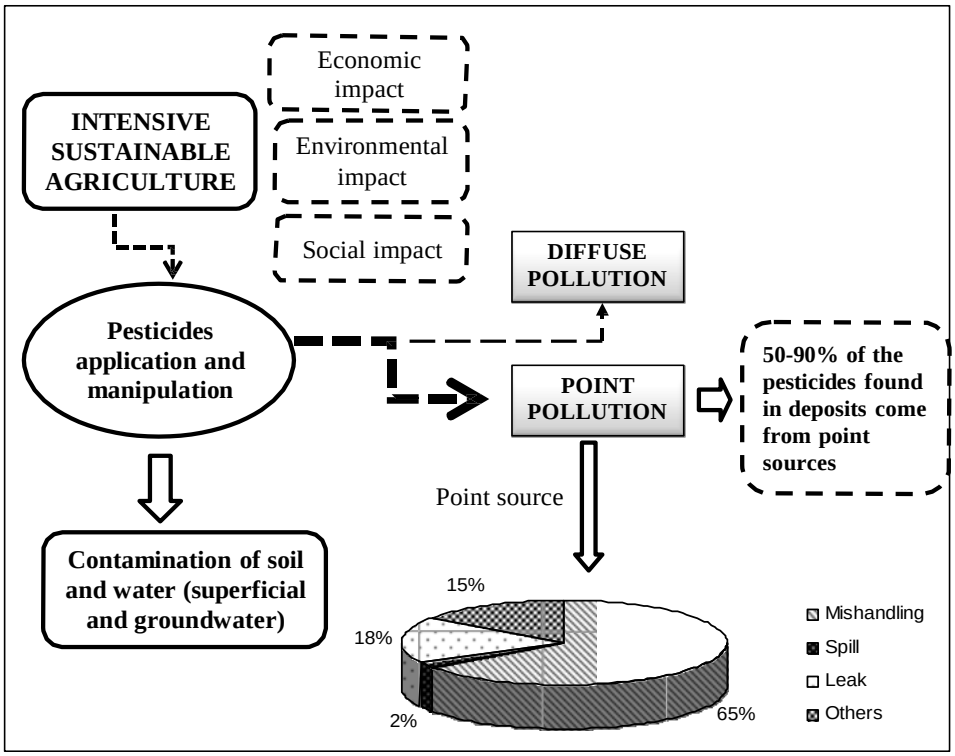

Figure 1.6. A flow chart illustrating the contribution of point and non point sources on the contamination of water resources by pesticides. The individual on-farm activities that contribute to point source contamination are also presented with

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mishandling of the spraying liquids and remnants being the most important point source (65%).

1.7. PESTICIDE-CONTAMINATED WASTEWATERS FROM THE FRUIT-PACKAGING INDUSTRY

The post-harvest treatment of fruit such as apples, pears, citrus and bananas involves the use of fungicides to prevent fungal infestations during storage (Ortelli et al., 2005). In addition, fruit packaging plants handling apples and pears utilize antioxidants to prevent the establishment of the physiological disorder apple scald (Junk and Watkins 2008). Fungicides and antioxidants are applied onto the fruits using variable ways including: 1) spraying of pesticide concentrates at high or low volumes, 2) immersion of fruits in concentrated solutions of fungicides (drenching) and 3) spray misting techniques. The recommended dose rates of the pesticides used in fruit packaging plants range from 600 mg L^{-1} in the aqueous solutions applied onto the fruits to 2000 mg L^{-1} when pesticides application is accompanied by simultaneous fruit waxing (Ritenour et al., 2003: Mari et al., 2003). Consequently, the wastewaters produced from the post-harvest treatment of fruit are contaminated with high concentrations of fungicides and antioxidants and their direct environmental release will have devastating effects for the chemical quality and ecological integrity of natural water resources (Castillo *et al*., 2000). Thus those wastewaters should be treated and detoxified prior to their release in the environment (Rushing *et al*., 1995). This need was identified by the European Commission which has provided authorization to the pesticides used in the fruit packaging industry only under the clause *that appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place* (EC 2009; EC 2010).

1.8. PESTICIDES USED IN FRUIT-PACKAGING PLANTS

In Europe the main fungicides used to prevent postharvest diseases of fruits are TBZ, IMZ, and OPP. Regarding apple scald the only currently registered pesticide for its control is the fumigant 1-methyl cyclopropene (MCP) (EC, 2006), although exemption authorizations for 120 days have been given for diphenylamine (DPA) by

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certain member-states in Europe considering that there are no equally effective alternatives in the market for the control of apple scald. Another antioxidant that was heavily used until recently is ethoxyquin (EQ). The physicochemical characteristics of the pesticides used in the fruit packaging industry are described in Table 1.4.

Table 1.4. The physicochemical characteristics of the pesticides used in the present study

Pesticides	Molecular Mass ($g\ mol^{-1}$)	Water Solubility ($mg\ L^{-1}$)	Vapour Pressure (Pa)	Henry's Law Constrant ($Pa\ m^3\ mol^{-1}$)	$logP_{ow}$
TBZ	201.26	28-30 (25^oC, pH 7)	5.3×10^{-7} (25°C)	3.7×10^{-6}	2.39 (pH 7, 20°C)
OPP	170.20	700 (25^oC)	0.474 (20°C)	0.14	3.18
IMZ	297.20	184 (20°C, pH 7.6)	1.58×10^{-4} (25°C)	1.08×10^{-4}	3.82 (pH 9.2, 25°C)
DPA	169.23	40 (25^oC)	0.033 (20°C)	0.321	3.82 (20°C)
EQ	217.31	170 (25^oC)	3.46×10^{-2} (25°C)	-	2.45 (25°C)

1.8.1. Thiabendazole

TBZ (*2- (4-thiazolyl) -1H-benz-imidazole*, Figure 1.7), belongs to the benzimidazole group of fungicides and it is used at postharvest level to control infestations of fruits by *Penicillium* sp. Also, TBZ is applied pre-emergence in potato seeds, cereals etc. and fruits and vegetables (bananas, citrus fruits, etc.) to combat fungal infestations by *Verticillium* sp., *Penicillium* sp. and *Botrytis* sp. (Hu et al., 2008: Nunes et al., 2001). Typical application rates of TBZ at postharvest level range from 1.2 g L^{-1} (pome fruits) to 2 g L^{-1} (citrus fruits) (EC 2013c).

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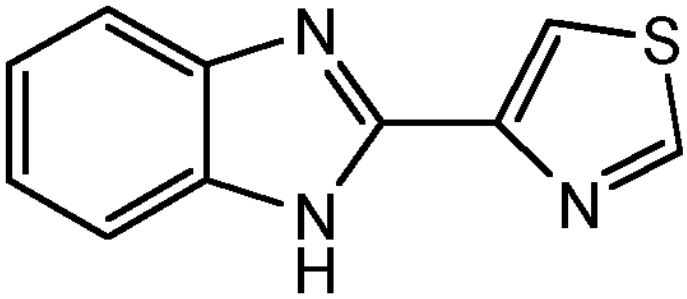

Figure 1.7. The chemical structure of thiabendazole (TBZ)

TBZ acts on target fungi by inhibiting the formation and function of cell spindle microtubules during mitosis (Danaher et al., 2007). In particular it binds to β-tubulin and prevents cytoskeleton-dependent cellular transport processes, including chromosome transport and cell division (Watanabe-Akanuma et al., 2005). It has also been demonstrated that the TBZ inhibits the reductase of fumaric acid which controls the formation of succinic acid and glucose absorption (Bennett and Bryant, 1984).

TBZ shows high toxicity to mammals at doses up to 20 times the recommended (Danaher et al., 2007). It shows low acute and short-term dietary toxicity to rats and birds (LC_{50} rats = 3100 mg kg^{-1} bw; LC_{50} birds acute >2250 mg kg^{-1} bw; LC_{50} short-term birds >5620 mg kg^{-1} diet). It is suspected as carcinogenic at very high concentrations but it is not mutagenic, teratogenic, neurotoxic, genotoxic, and it does not induce reproductive toxicity. It is characterized by high toxicity to aquatic organisms including invertebrates (EC_{50} *Daphnia magna* = 0.81 mg/l, NOEC 21 days *D. magna* = 0.084 mg L^{-1}) fishes (LC_{50} rainbow trout = 0.55 mg L^{-1}) and algae (EC_{50} algae (96h) 9.0 mg L^{-1}) (Cannavan et al., 1998: EC, 2001).

TBZ is characterized by low water solubility and vapour pressure suggesting that volatilization is a minor process in its environmental dissipation (Table 1.4). TBZ is persistent in the soil environment. Extrapolated DT_{50} values ranged from 833- 1100 days in cropped plots and from 1093-1444 days in fallow plots (US EPA, 2002). Laboratory regulatory studies at EU level reported soil DT_{50} > 1 year at 20°C under both aerobic and anaerobic conditions. More recent studies by Omirou et al (2012) reported DT_{50s} = 77.8 days but still TBZ was the most persistent of the pesticides studied (all used in fruit-packaging industries). TBZ is strongly adsorbed onto soil particles as it is suggested by its K_{oc} values ranging from 1104-22467 ml g^{-1} (EC 2001). As expected TBZ is not particularly mobile in the soil environment (Omirou et al., 2012). Little is known regarding the metabolic pathway of TBZ in the

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environmental. Previous studies identified 5-hydroxythiabendazole (5-OH-TBZ) produced after hydroxylation of the 5 'end of the benzimidazole ring as its main metabolic product (Cannavan et al., 1998). Recent studies by Sirtori et al. (2014) showed that TBZ could be degraded in water by the Fenton-oxidation process to various intermediates with thiazole-4-carboxamidine being the major product. In contrast the biodegradation of TBZ is still a black-box. Only recently Perruchon et al. (2016b) reported the isolation of a proteobacteria consortium which was able to rapidly degrade TBZ leading to the production of thiazole-4-carboxamidine, while the benzyl part of the benzimidazole ring was consumed by the bacterial consortium as shown by Stable Isotope Probing analysis.

Evaluation of the use of TBZ at EU level by the Scientific Committee on Plants of the European Union (Scientific Committee on Plants, SCP) concluded that *"the placement of liquid waste generated from the use of the post-harvest fruit treatment, into surface water or the biological cleaning of municipal waste can cause major problems in the operation of these systems and problems of toxicity to non-target organisms*" (EC, 2001). Thus TBZ has been granted authorization for use until 31.12.2021 under the clause that the wastewaters produced by its use should be treated on site before their environmental release.

1.8.2 Imazalil

IMZ (*1- [2- (2,4-dichlorophenyl) -2- (2-propenyloxy) ethyl] -1H-imidazole*, (Figure 1.8), is an imidazole fungicide which acts by inhibiting ergosterol biosynthesis in fungi. It has a broad spectrum activity and is effective against Ascomycetes infesting fruits and vegetables in storage. Its post-harvest use in fruits targets infestations by fungi of the genus *Penicillium, Gloeosporium, Fusarium* etc. (Chu et al., 2007; Kodama et al., 2003; Maruyama et al., 2007; Nunes et al., 2001). EC renewed the authorization of IMZ for post-harvest use in fruits, vegetables and potatoes in Member States until 31.7.2021 only on the condition that *appropriate decontamination system for the treatment of wastewaters produced by its use would be implemented on site* (EC, 2009b). Its recommended dose rates vary with the mode of application onto fruits: the lower dose rates (1 g L^{-1}) are used when applied via drenching and the highest dose rates (2 g L^{-1}) when IMZ is used with spraying or waxing (EC 2009b).

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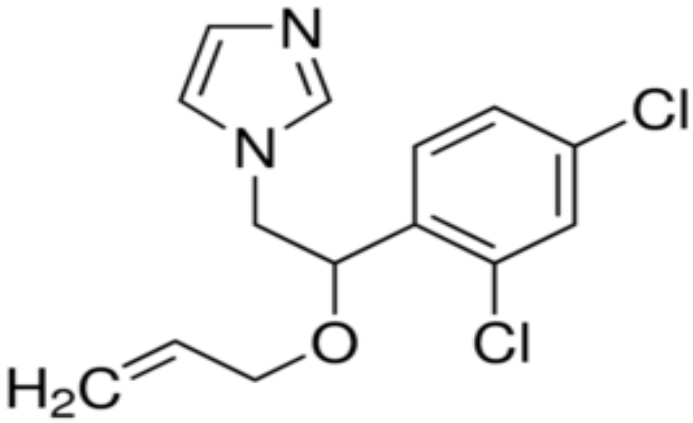

Figure 1.8. The chemical structure of imazalil (IMZ)

IMZ imposes toxic effects to humans and mammals. It has been reported that IMZ showed cytotoxic activity in isolated rat liver cells and it was found to affect the activity of cytochrome P450 (Muto et al., 1997). Other studies showed that IMZ has inhibitory activity against aromatase CYP19, which catalyzes the conversion of androgens to estrogens (Vinggaard et al., 2000). IMZ is classified as "a *likely carcinogen to humans*" according to EPA's July 1999 Draft Guidelines for Carcinogen Assessment. Carcinogenicity studies in rodents indicated that IMZ is carcinogenic to male Swiss albino mice and Wistar rats. In addition, IMZ is placed in Category II, II, and IV for oral, dermal, and inhalation toxicity respectively. It is highly irritating to the eyes (Category I), but it is not a skin irritant (Category IV) or a dermal sensitizer (US EPA, 2003).

Regarding its ecotoxicity, IMZ is considered very toxic to aquatics including fishes (LC_{50} acute *Onchorynchus mykiss* = 1.48 mg/l; NOEC chronic = 0.043 mg/l), invertebrates (EC_{50} *D. magna* 3.5 mg/l), and algae (*Pseudokirch subcapitata* E_bC_{50} = 0.87 mg/l and E_rC_{50} = 1.20 mg/l). Regarding its toxicity to other non-target organisms, IMZ is moderately toxic to birds (LD_{50} acute 510 mg/kg, LD_{50} short term dietary > 5620 mg/kg feed) and moderately to highly toxic to mammals (LD_{50} acute rats = 227 mg/kg and NOEL short term dietary = 2.5 mg/kg diet) (EC 2009b).

IMZ is a racemic mixture of two enantiomers (the R isomer showing higher activity) which do not show any enantiomer-specific degradation activity (Chu et al. 2007). It is stable in pure state, moderately soluble in water and has low vapour pressure suggesting the limited contribution of volatilization in its dissipation in soil (Table 1.4). IMZ degrades slowly in soil under aerobic conditions with DT_{50s} ranging from 44 to 128 days (US EPA, 2003). The long persistence of IMZ was also verified

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by similar regulatory studies at EU level with DT_{50s} mean values of 137 d (aerobic soil laboratory study at 20°C) (EC, 2009). More recent studies by Omirou et al. (2012) and Kreuzig et al. (2010) showed contrasting behaviour of IMZ with DT_{50} values in soil of 29 and 83 days respectively. IMZ showed K_{oc} values > 4000 ml g^{-1} suggesting rather strong sorption and limited mobility in soil (EC 2009, Kreuzig et al. 2010). Biodegradation of IMZ is still a largely unexplored topic. To date no microorganisms able to degrade IMZ have been isolated. The only report on this is by Karas et al. (2011) which showed that the white rot fungi *Trametes versicolor, Phanerochaete chrysosporium* and *Pleurotus ostreatus* effectively degraded 10 mg L^{-1} of IMZ in liquid culture but failed to degrade higher concentrations (50 mg L^{-1}).

IMZ-ethanol constitutes the main metabolite of IMZ in soil. IMZ-ethanol is characterized by lower soil persistence (DT_{50} = 5.1-10.4 days) and medium affinity for soil sorption (K_{oc} = 757-1663 ml g^{-1}) suggesting limited risk for contamination of water resources. Even then IMZ-ethanol is less toxic to aquatics compared to the parent compound (LC_{50} acute fish = 21.26 mg L^{-1}; LC_{50} *D. magna* = 13.6 mg L^{-1}) (EC, 2009).

1.8.3. *Ortho*-phenylphenol

OPP (Figure 1.10), belongs to the chemical group of aromatic hydrocarbons. It is used as a fungicide to treat postharvest rots (*Penicillium digitatum, P. italicum, Botritys cinerea*, etc.), especially on citrus fruit and seed packing. It is also used in the form of fungistatic wax for coating of vegetables to avoid microbial spoilage during storage and transportation. OPP is applied via drenching in citrus fruits at maximum dose rates of 0.6 g L^{-1} (EC 2008). OPP is also used as a general purpose disinfectant in hospital and health care in cleaning and disinfecting machinery (Cnubben et al., 2002; Zamora et al., 2004; Ziogas 2007). Its registration as a pesticide for postharvest use in fruit packaging plants was renewed until 2019 under the clause that all member-states *"should pay particular attention to put in place appropriate waste management practices to handle the waste solution remaining after application, including the cleaning water of the drenching system. Member States permitting the release of those wastewaters into the sewage system shall ensure that a local risk assessment is carried out*" (EC, 2010c).

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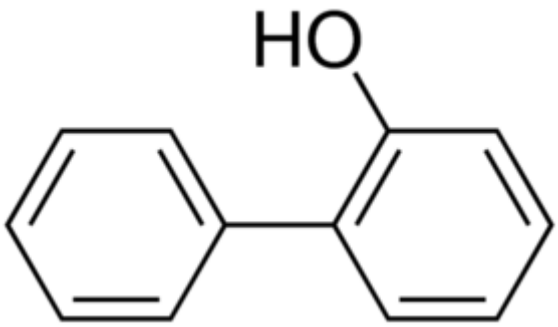

Figure 1.9. The chemical structure of *ortho*-phenylphenol (OPP)

Many studies have been implemented to clarify the mode of action of OPP and many theories have been formulated. However none of these theories adequately explain the primary activity of the fungicide. The prevailing theory suggests that OPP causes lipid peroxidation in the inner mitochondrial and nuclear membrane and endoplasmic reticulum of sensitive fungi exposing the chromosomes to the action of free radicals and lytic enzymes (Ζιώγας 2007).

According to US EPA (2006) OPP showed toxicity to laboratory mice and rabbits only at high concentration levels (*ca*. 1650 mg kg^{-1}). OPP is possibly carcinogenic to humans, but at very high concentrations. Experiments in rats and mice fed with 200 mg/kg/ day of OPP showed tumors in the bladder and the liver respectively. Finally, there are reports showing that the OPP has no mutagenic activity (Tani et al., 2007). Regarding its ecotoxicity, OPP seems to be particularly toxic to aquatic organisms including fishes (LC_{50} *Onchorynchus mykiss* = 4 mg L^{-1}; NOEC 21d = 0.036 mg L^{-1}), invertebrates (*Daphnia magna* EC_{50} = 2.7 mg L^{-1}) and algae (*Pseudokirch subcapitatus* EC_{50} biomass and EC_{50} growth rate 1.35 and 3.57 mg L^{-1} respectively) but shows limited toxicity to birds and mammals (EFSA, 2008).

OPP is generaly stable in abiotic hydrolysis at pH 5-9, it is moderately soluble in water and is characterized by moderate vapour which might suggest some losses via volatilization (EFSA 2008) (Table 1.4.). OPP in soil showed variable persistence, with DT_{50s} of <1 day (EFSA 2008) to 43.3 d (Omirou et al. 2012). OPP shows variable sorption affinity with *Koc* values ranging from 252-393 (EFSA 2008) to 894-1793 (Zheng et al. 2011) suggesting a moderate mobility in the soil profile. In recent column studies Omirou et al (2012) pointed to OPP as the most mobile chemical amongst the ones used by the citrus fruit packaging industry. Although OPP was characterized as 'non readily biodegradable' (EFSA 2008) previous studies have

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showed that OPP is readily degraded by microorganisms isolated from soil. Kohler et al. (1988) first isolated a *Pseudomonas azelaica* strain which was able to utilize OPP as a carbon source and rapidly tranform it through intermediate production of benzoate. Recently, Perruchon et al. (2016a) isolated a *Sphingomonas haloaromaticamans* strain which rapidly metabolized OPP following a pathway similar to the *P. azelaica* strain. Karas et al. (2011) also showed that white rot fungi *P. chrysosporium, T. versicolor* and *P. ostreatus* were able to rapidlly metabolize OPP by activating their ligninolytic enzymes.

1.8.4. Diphenylamine

DPA (chemical structure in Fig. 1.10) is predominantly used as stabilizer for single- or multi-base propellants and nitrocellulose-containing explosives (Drzyzga, 2003). DPA is also used at postharvest level to prevent the appearance of apple scald in pome fruits (Ζιώγας 2007: Drzyzga and Blotevogel, 1997). It is applied via dipping, drenching, and spraying or fogging to apples and pears at concentration levels ranging from 0.4 to 2 g/l (EFSA, 2012). To date DPA is not authorized for use in fruit packaging plants in Europe (EFSA 2012). The decision was based on the lack of information related to the presence, formation and toxicity of metabolites (i.e. nitrosamines) produced in the fruit or in the formulation during processing or storage respectively (EFSA 2012). Despite that several members states like Spain, Italy, Greece and Portugal excemption authorization for 120 days to DPA, while DPA is still heavily used for the treatment of fruits in non EU countries like USA, Canada and Latin American countries

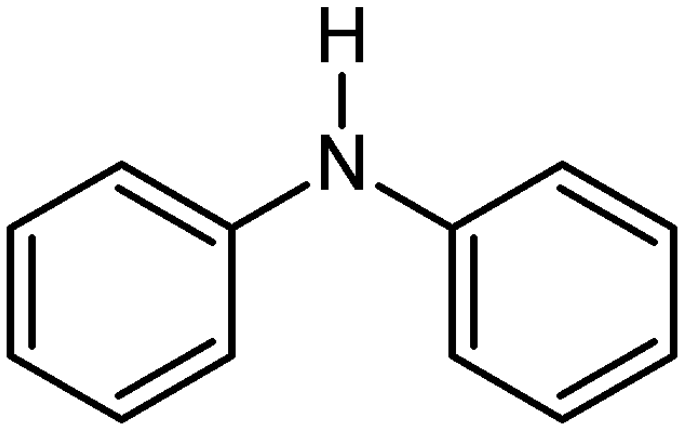

Figure 1.10. The chemical structure of Diphenylamine (DPA)

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The mode of action of DPA is not fully known yet, but it is believed that it acts by preventing the oxidation of naturally occuring terpenes like a-farnesene which when oxidized to trienes induce damages and cell death on the surface of fruits (EC 2007). DPA has been shown to cause skin diseases in the hands of workers who use the formulations and nephrotoxicity in male laboratory rats (Drzyzga et al., 1995). However it is not particularly toxic, acutely and in the short-term to birds and mammals (EFSA 2008). On the contrary DPA is particularly toxic to aquatic organisms like fishes (EC_{50} *Oncorhynchus mykiss* 2.2 mg L^{-1}), algae (*Selenastrum capricornutum* biomass and growth rate EC_{50} 0.18 and 0.30 mg L^{-1} respectively), and invertebrates (*Daphnia magna* EC_{50} 1.2 mg L^{-1}) (Drzyzga, 2003; EFSA, 2012)

DPA shows high reactivity, due to its imine H, and it has low to moderate water solubility. Its Henry's law constant values suggests that losses through volatilization from water are expected (EFSA 2012). Little is known about its persistence and metabolism in the soil environment. However regulatory studies conducted in USA suggested that the molecule is not persistent in soil with DT_{50s} of <1 day. DPA shows relatively high adsorption onto soils (K_{oc} = 1212-6593 ml g^{-1}) which suggests a limited mobility and low leaching potential (US EPA, 1998).

A ready biodegradability study suggested that DPA should be classified as "not readily biodegradable" (EC, 2007b). Experiments with 12 months incubation in aerobic conditions with ^{14}C DPA showed that only 18% was mineralized. On the other hand Drzyzga and Blotevogel, (1997) observed that DPA (50 μM) was co-metabolized by cultures of sulphate-reducing bacteria. After incubating the cultures for 6 weeks a reduction of 60% of the initial amount of DPA with simultaneous detection of the corresponding aniline as its main metabolite. Shin and Spain (2009) first reported the isolation of a *Burkholderia* sp. and a *Ralstonia* sp., which were able to utilize DPA as a C and N source. More recently Perruchon et al. (2015) reported the isolation of *Pseudomonas putida* strain from a soil from a wastewater disposal site which was able to rapidly degrade DPA and use it as a C and N source.

1.8.5. Ethoxyquin

EQ (*1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline*) (Fig. 1.11) is a quinolinic antioxidant which is used to treat pome fruits to prevent the appearance of apple scald in pome fruits. EQ is applied by soaking, spraying or by winding in enriched with EQ

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paper. It acts as a sacrificial antioxidant preventing oxidation of alpha farnesene to trienes which cause necrosis of epidermal cells in apples (Blaszczyk et al. 2013). Since 2012 EQ is not authorized for use in fruit packaging plants. The decision on not granting authorization to EQ by the European Commision was based on several scientific gaps in its dossier which were related (a) to the lack of toxicity risk assessment for EQ, its metabolites and one potentially genotoxic impurity found in the active ingredient, (b) the absence of consumer, operator and worker risk assessment (c) the lack of exposure assessment of the environmental compartments via aerial deposition of potentially volatile metabolites and a genotoxic impurity (EFSA 2010)

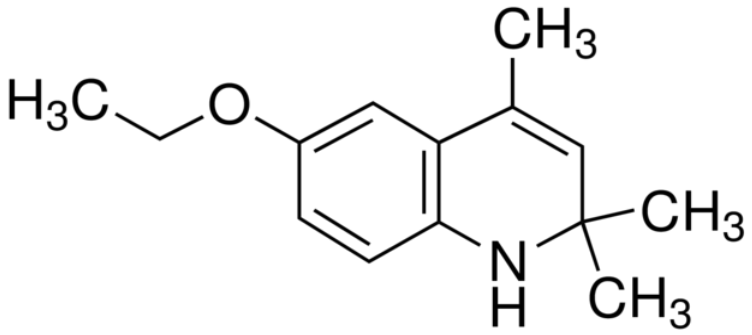

Figure 1.11. The chemical structure of ethoxyquin

EQ is still used as a preservative and antioxidant in fish meal and fish feed (De Koning and Mol. 1989; De Koning 1998). It is applied at levels of 150 mg/kg in complete feed in the EU with the benefit of this use being two fold: i) It preserves fish meal and fish oil from the oxidation of highly unsaturated fatty acids including EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), better known as omega-3 fatty acids, which are known to promote health in both animals and humans; ii) It prevents the spontaneous combustion of fish meal during transport and storage (EFSA, 2010).

Due to its heavy use in animal food an Acceptable Daily Intake of EQ for human consumption in the range 0-0.005 mg kg^{-1} was established based on tests on dogs (Drewhurst 1998). Regarding its ecotoxicity, EQ is not considered toxic to mammals (LD_{50} rats 1726) and birds (*Colinus virginianus* LC_{50}>2417 mg kg^{-1} bw). However EQ is toxic to aquatics including fishes (*Oncorhynchus mykiss* LC50 = 18 mg L^{-1}), algae (*Pseudokirchneriella subcapitata* E_bC_{50} = 6.1 mg L^{-1}) and invertebrates (*Daphnia magna* EC_{50} = 2 mg L^{-1}) (EFSA 2010).

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On the basis of its physicochemical properties EQ may be considered as moderately volatile and it has a moderate solubility in water (Table 1.4.). The estimated photo-chemical half-life in the atmosphere was shorter than 2 days, therefore long- range transport through the atmosphere is not expected for EQ. In a hydrolysis study, under sterile conditions (25ºC), at pH 5, 7 and 9 in the dark, EQ was unstable and underwent relatively rapid hydrolytic degradation, forming four major (> 10% of the applied radioactivity) unidentified transformation products (EC, 2010: EFSA, 2010).

Little is known about the environmental fate of EQ. This is due to its indoor use. Recent studies by Papadopoulou et al., (2016) showed that it is rapidly transformed to quinone imine and dimethyl-ethoxyquinoline. The first constituted the major metabolite and it was rapidly transformed thereafter whereas the latter was formed in small amounts which persisted for long. Overall EQ and its metabolites showed limited persistence in soil but exhibited unacceptable toxicity to ammonia-oxidizing microorganisms, a key microbial group in N cycling.

1.9. TREATMENT OF THE WASTEWATERS FROM THE FRUIT-PACKAGING INDUSTRY

The long persistence of TBZ and IMZ and the high aquatic toxicity of all pesticides used in fruit packaging plants (TBZ, IMZ, DPA, OPP and EQ) led the EC, as mentioned above, to allow their use only under the condition that the wastewaters produced will be treatment *on site*. Despite that no simple, efficient and cost-effective methods are available for the treatment and detoxification of those effluents. The only full scale system currently available for the treatment of those wastewaters is called ControlTec-Eco® (Photograph 1.1), constructed and patented by the Spanish company Technidex (Garcia-Portillo et al. 2004). The system is based on the filtration of effluents through activated carbon and when tested with TBZ-contaminated effluents it achieved a 7000-times reduction in the concentration of TBZ (EC 2000). Despite its high depuration performance ControlTec-Eco® was not widely adopted by the fruit packaging plants in the Mediterannean region due to its high cost and its high engineering needs for operation and maintenance.

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Photograph 1.1. The Control-Tec Eco® system used for the treatment of effluents from fruit packaging plants

Other physicochemical methods have been tested, so far at lab scale level, for the depuration of those effluents with encouraging results. Khodja et al. (2000) first showed that photocatalytic treatment rapidly removed OPP from wastewaters. Subsequent tests with the more persistent fungicides TBZ and IMZ using photocatalysis with TiO_2 showed high removal efficiency although the dissipation of TBZ was affected by the type of water used in the tests (Jimenez et al. 2015). Santiago et al. (2011) evaluated the efficiency of different oxidation methods including TiO_2 catalysis, TiO_2 catalysis combined with activated carbon filtration, Fenton and Photo-Fenton oxidation for the depuration of TBZ and IMZ from agro-industrial effluents. They showed that Photo-Fenton was the most efficient method while TiO_2 catalysis was the least efficient. Subsequent tests by Carra et al., (2014) in a raceway pond reactor using photo-Fenton oxidation showed high removal efficiency of TBZ from wastewaters from the fruit packaging plants. Semi-pilot studies showed that the combination of membrane bioreactor and Fenton oxidation processes could effectively remove TBZ from wastewaters (Sanchez Perez et al., 2014). However there are certain limitations regarding all the above studies: (a) they were performed at concentration levels (0.1 mg L^{-1}) which are multi-fold lower than the levels found in the effluents from fruit-packaging plants treating and storing fruits (b) they were all performed at lab or semi-pilot scale level thus their full-scale implementation and efficiency is still pending and (c) all oxidation methods lead to the production of oxidation intermediates which are of unknown toxicity.

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In the absence of implemented treatment systems for these agro-industrial effluents fruit-packaging plants handle those effluents in various, acceptable or unacceptable ways:

- They dispose of the effluents in municipal wastewater treatment plants. These generic wastewater treatment systems have been shown to be efficient in the removal of OPP (Korner et al., 2000) but failed to remove the recalcitrant fungicides IMZ and TBZ from the wastewater (Campo et al. 2013). Such practices have resulted into point source contamination of surface water systems receiving effluents from municipal wastewater treatment plants with TBZ and IMZ
- They discharge the effluents in nearby fields which results in the build up of particularly high levels of recalcitrant chemicals like TBZ in soil. Recent monitoring in such disposal site showed a gradient of TBZ concentrations in soil ranging from 12 to 12000 mg kg^{-1} (Papadopoulou et al., 2015).
- They deliver their effluents to certified companies for *ex situ* treatment. The particularly high cost of this option (0.7-3 € per litre of waste) constitutes a big economic burden for the viability of fruit packaging plants in the Mediterannean region.

Thus there is an urgent need for an efficient, cost-effective and simple-to-operate method for the depuration of those agro-industrial effluents. The implementation of biological treatment systems was so far prohibited by the general limited biodegradability of the relevant pesticides. However recent studies showed that TBZ, IMZ, OPP and DPA could be biodegraded by specialized soil bacteria and 'generalists' fungi (Perruchon et al. 2015, 2016a, 2016b; Karas et al., 2011). Thus, biological treatment methods could be a viable alternative and biobeds, described in the following section, might be a potential solution for the treatment of these agro-industrial effluents.

1.10. BIOBEDS

Biobeds are simple and cheap constructions intended to collect and degrade spills of pesticides occuring at on farm level (Torstensson, 2000; Torstensson and Castilio, 1997). The original Swedish-type system was a 60 cm deep pit in the ground and consisted of of three major components (Figure 1.12): (i) **a clay layer** at the bottom (10 cm) to prevent percolation of the wastewater to deeper soil layers; (ii) a **packing**

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material or else called "biomixture" composed of straw, peat, and soil (50:25:25 vol %) which was used to pack the remaining of the pit; and (iii) a **grass layer** on the surface which regulates the moisture content of the upper parts of the biobeds through evapotranspiration and accelerates the degradation of pesticides through rhizodeposition, known to promote microbial degradation of pesticides (Campos et al., 2016). These original direct type biobed systems were also equipped with a ramp to allow the sprayer to be driven and parked over the biobed and collect all spillages occurring during preparation of the spraying mixture (Castillo et al., 2008).

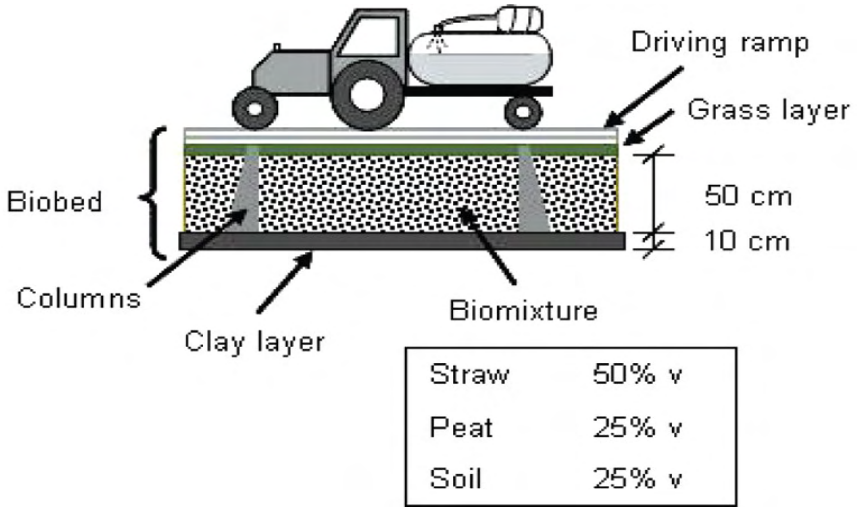

Figure 1.12. A schematic representation of the original Swedish-type biobed system.

Biobed systems were initially categorized according to the insulation of their bottom as unlined or lined systems. *Unlined Biobeds* have no impermeable synthetic layer that isolates them from the soil. The original Swedish-designed biobed belongs to this group. In many cases, a natural clay layer is present at the bottom of the biobed pit. If this is not the case, a clay layer is added. There is no collection of drainage water in this system and they were only used to collect spillages and not water rinsates or washates (Figure 1.13a,b). *Lined Biobeds* resemble the original Swedish biobed but they are lined by a synthetic impermeable layer (plastic, concrete, tarpaulin, etc.) that isolates them from soil. This design allows the collection of drainage water in special wells that are built at the side of the biobed (Figure 1.13c). Drainage layers (gravel, macadam, or sand) are usually placed below the clay. This design was introduced in the United Kingdom (Castillio et al., 2008).

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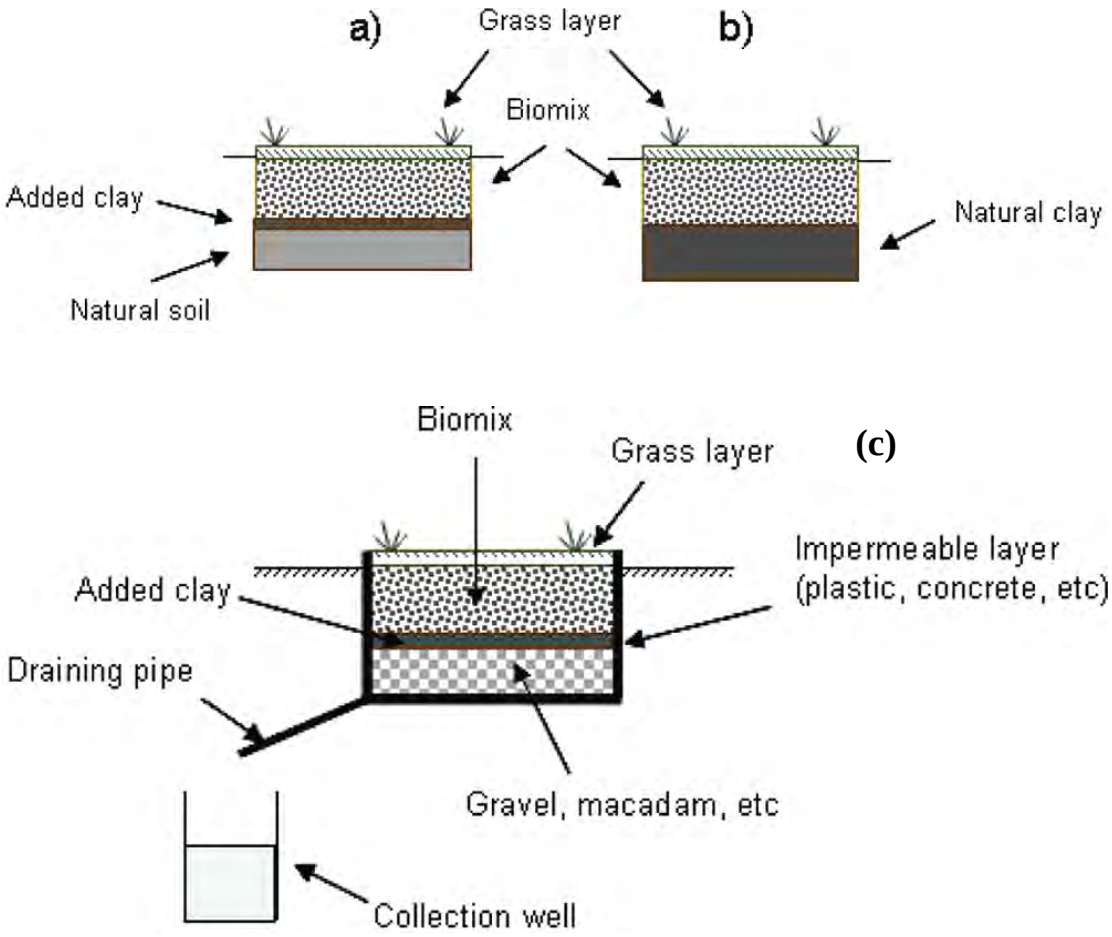

Figure 1.13: Unlined biobeds with (**a**) an added or (**b**) a natural clay layer and (**c**) the lined biobed is isolated with an impermeable layer that allows collection of drainage water in a well.

1.10.1. Application and Uses of Biobeds

1.10.1.1 On farm applications in different countries

Since their initial introduction in Sweden, biobeds generated interest in other countries (e.g., UK, Belgium, Italy, France, Peru and Guatemala), and their implementation led to modifications of the original biobed design to accomodate local needs and practices. These modified systems were given alternative names like Biofilters, Biomassbed, Phytobac / Biobac, and BiomassBed.

Biobeds in UK: The adaptation of biobeds in UK led to two major modifications: (i) insulation of the biobed system by using an impermeable synthetic liner, that is, use of lined biobeds and (ii) modification of the depth of the biobed from 0.6 m in the Swedish design to 1-1.5 m in the UK version to increase the retention time of the pesticides in the bed and allow biobeds to receive larger amounts of water.

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Two systems were outlined, (i) an offset or indirect system where the handling area of the pesticides is separated from the biobed area (requires two collection tanks, one before and one after the biobed) (Fig. 1.14b) and (ii) a drive-over system where the handling area is directly over the biobed area (requires a collection tank after the biobed) (Fig. 1.14a). The liquids collected from the biobeds are drip-irrigated in designated disposal areas. The biobed mixture in use in the UK consists of straw (wheat or barley), soil, and peat-free compost in the proportions 50:25:25 vol %, and the bed is covered with grass to ensure rooting activity and assist moisture management (Castillio et al., 2008: Fogg et al. 2004).

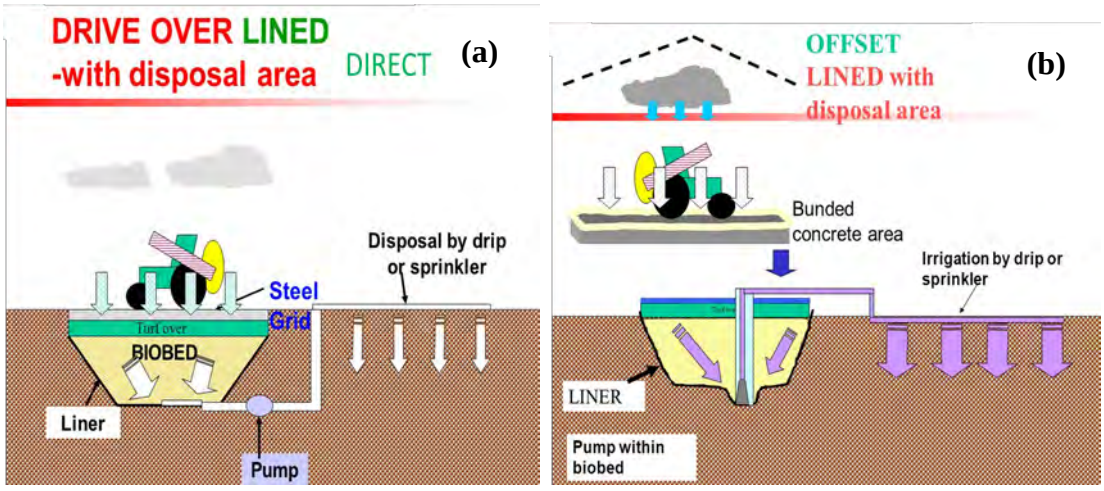

Figure 1.14. Biobeds in the United Kingdom: direct system (**a**) and offset system (**b**) (sourse: http://slideplayer.com/slide/8161925/)

Biobeds in Italy (BiomassBed). The Italian biobed system was called BiomassBed (Figure 1.15a,b),. It is used for the treatment of large volumes of pesticide-contaminated water produced from the filling and washing of spraying equipment. The BiomasBed is packed with a mixture of local organic materials. Because peat is not easily found in Italy and is expensive, other organic materials are being tested as replacements, such as urban and garden composts, peach stones, vine branches, and citrus peels (Coppola et al., 2007: Vischetti et al., 2004).

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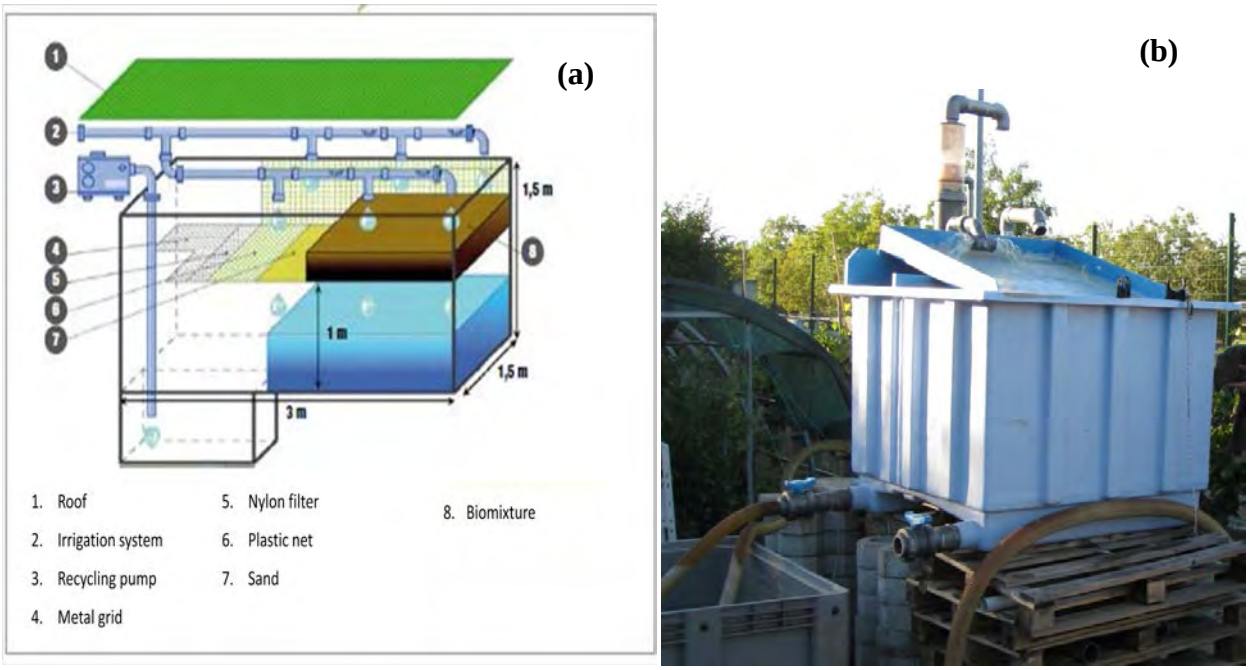

Figure 1.15. Biomassbed in Italy: (**a**) schematic diagram and (**b**) pilot plant installed in Universita Cattolica del Sacro Cuore, Piacenza, Italy. (Sourse: http://lineeguida.iambientale.it/GuideLines_Appendix_2.aspx)

*Biobeds in Belgium (Biofilter***):** In Belgium the biobed takes the form of a Biofilter (Phot 1.2.). The main interest in Belgium was to modify the biobed concept into a more flexible, small system able to treat large volumes of effluents, to recycle them with a pump, and to use different kinds of packing materials. Biofilters consist of two or three units of 1 m^3 plastic containers stacked in a vertical pile and connected with plastic valves and pipes (de Wilde et al., 2007).

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Photograph 1.2. Typical biofilters installed in Belgium (sourse: http://biobeds.net/en/modified-biobed-systems/ , http://wrootwater.com/index.php/water-treatment-2/bio-beds-bio-filters/)

*Biobeds in France (Phytobac & Biobac)***:** Phytobac®, developed by Bayer Crop-Science, was inspired by the Swedish biobed concept. It consists of a 60 cm deep basin made of watertight materials to ensure complete retention of contaminants and effluents (Figure 1.16a,b). The sides of the basin are 30 cm above soil level to avoid flooding from runoff. The substrate consists of topsoil from the farm (70%) and chopped straw (30%). No grass layer is placed on the top and a cover protects the bed from rainfall. The Phytobac is intended to treat all of the contaminated volumes of water coming from tank waste and spillages during mixing/loading, rinsing, and cleaning of sprayers (Guyot and Chenivesse, 2006).

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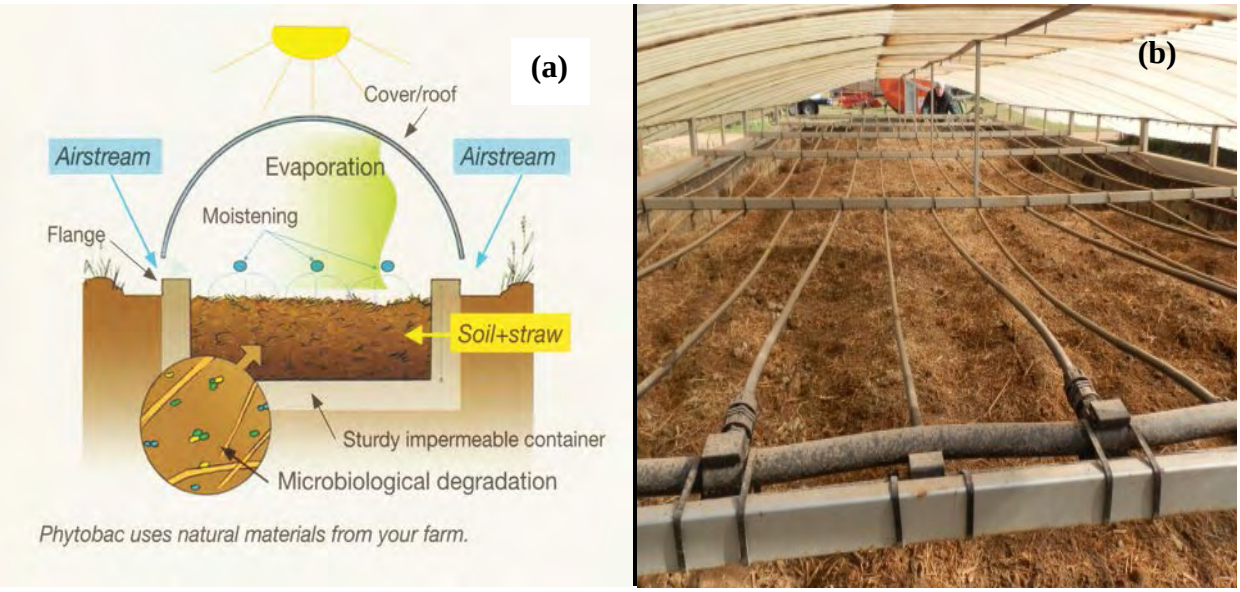

Figure 1.16. Phytobac from France: (**a**) a schematic diagram and (**b**) pilot phytobac installed at a college farm (Sourse: http://www.biobeds.org/5th-workshop-2016 , http://www.bayercropscience.co.uk/news-and-opinion/articles/2014/04/new-phytobac-biobed-system-unveiled/)

The Biobac (Photograph 1.3), developed by researchers at INRA, France, is a system derived from Phytobac. It consists of a tank insulated from the subsoil and filled with a mixture of organic and mineral materials, mainly soil from the farm and chopped straw. The concept behind this system is that farm soil contains microorganisms, which over successive treatments have been adapted to high biodegradation rates for the pesticides used at the farm. This natural detoxifying ability of the soil microflora can be maintained and encouraged in the biobac by the input of a supplementary source of carbon and energy, such as straw. One of the differences in relation to the Phytobac system is that the moisture and aeration levels are controlled (Castillo et al., 2008).

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Photograph 1.3. A typical Biobac system (sourse: http://lineeguida.iambientale.it/GuideLines_Appendix_2.aspx)

Today biobeds are used worldwide to minimize pesticides point source contamination of natural water resources at on farm level. Biobeds have been officially approved in UK, Sweden, Denmark, the Netherlands and France where Phytobac dominates the market (Fig. 1.18). However biobed-type biodepuration systems have been also implemented in other EU countries (Cyprus, Spain, Portugal, Italy, Switzerland, Germany, Belgium, Finland and others) although their implementation is still at pilot-scale level.

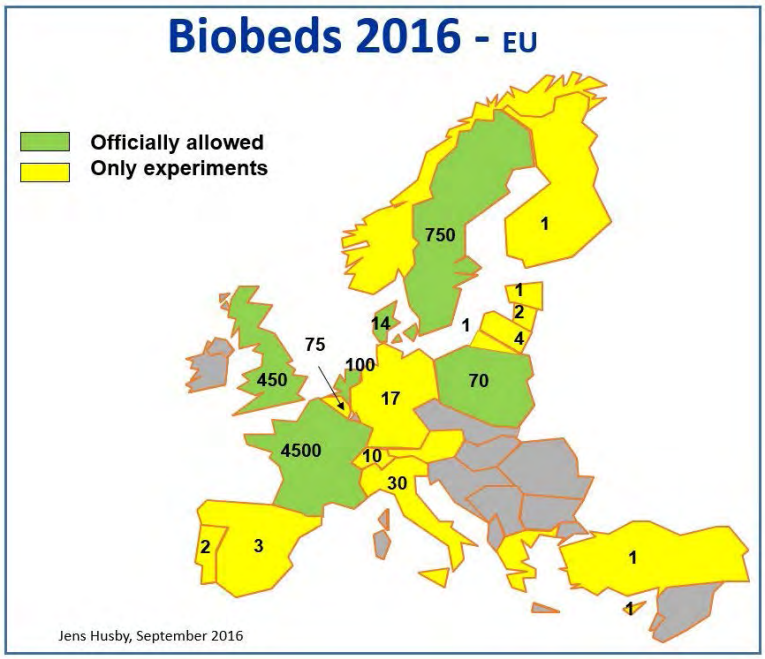

Figure 1.17. The EU map of biobeds distribution (adapted by www.biobeds.org)

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The implementation of biobed systems has spread also to countries in North America (Canada, USA), Latin America (Guatemala, Cost Rica, Columbia, Ecuador, Peru, Chile, Argentina, Uruguay and Brazil), Africa (Maroco, Senegal and Ivory Coast) and Asia (China and Japan) although they are officially approvided only in Guatemala and Ecuador (Jens Husby 5th EU Biobeds Meeting 2016) (Fig. 1.18).

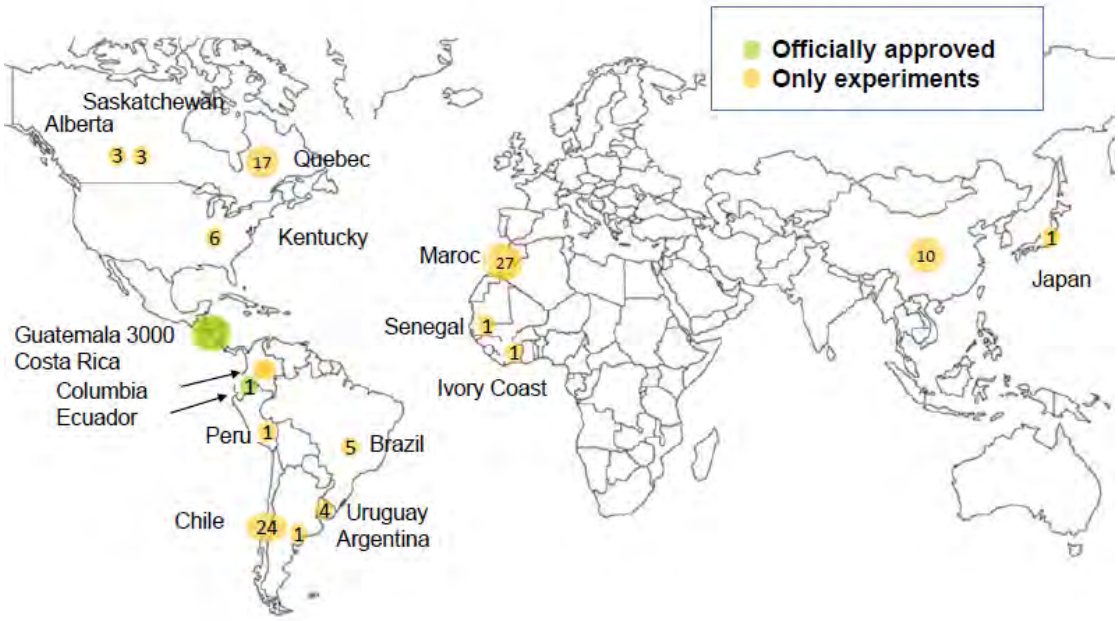

Figure 1.18. The distribution of biobed systems in countries ouside Europe (adapted by www.biobeds.org)

1.10.1.2. Other applications of biobed systems.

Several other agricultural and related industries produce wastewaters with high pesticides loads. The development of systems for the treatment of those agro-industrial pesticide contaminated effluents is still pending and biobeds could be a valuable solution in those cases. Examples of agro-industries which produce effluents destined for treatment by biobeds are (a) seed production (i.e. cotton) industries which perform seed-coating with pesticides and produce *ca*. 2-3 m^3 of effluents per year, (b) ornamental buld dipping treatments with pesticides and (c) fruit and vegetable packaging plants, which depending on the type of fruits treated could produce variable volumes and types of wastewaters. Thus citrus fruit-packaging plants which operate from November to May produce usually two type of effluents: (a) a daily-produced diluted effluent containing low concentrations of OPP (< 5 mg L^{-1}) and (b) a less frequently produced (2-3 times a year) but more dense effluent containing high

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concentrations of OPP or IMZ/TBZ. The volumes of the different effluents vary according to the size of the fruit packaging plant. On the other pome fruits packaging plants which operate from August to October produce effluents containing mainly antioxidants and occassionaly fungicides like IMZ. Again the volume of the final effluent is based on the size of the enterprise.

Omirou et al., (2012) evaluated for the first time the use of biobeds for the treatment of wastewaters produced along the citrus fruit production, at pre- and post-harvest level. Their results showed that biobeds adjusted to the local agricultural practices and conditions were able to remove pesticides from the wastewaters but significant amounts of TBZ and IMZ appear to be retained by the biobed packing material and not degraded. Thus several issues remain uresolved (a) the efficacy of biobeds against pesticides used in pome-fruit packaging plants like DPA and EQ, (b) optimization of biobeds to degrade rather than retain pesticides in its packing material, (c) handling and decontamination of the spent biobed packing material.

1.10.2. The composition of the biobeds packing material (biomixture)

The efficiency of biobed systems relies on the capacity of their organic packing material to retain and degrade the high pesticide amounts discharged on biobeds. The biobeds packing material should ensure primarily high biodegradation and secondly high sorption capacity (Karanasios et al., 2012). Both of these characteristics are a function of the composition, homogeneity, age, moisture, and temperature of the biomixture. Biobeds packing material is commonly composed of a lignocellulosic material (i.e. straw), soil and a humified material (i.e. peat or composts) in various proportions. Each of these components serve a specific purpose.

Straw is the most commonly used lignocellulosic material in biobed packing materials. It offers nutrients, particularly C which is needed for linginolytic white rot fungi to produce phenoloxidases (peroxidases and laccases) and degrade pesticides (Castillo et al., 2008: Karas et al., 2011). The high availability of alternative lignocellulosic materials in different regions, at a reduced or no cost, have led to the replacement of straw by other materials. A list of lignocellulosic materials used as components in biomixtures are shown in Table 1.5 (adapted by Karanasios et al., 2012).

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Table 1.5. Lignocellulosic materials which have been used as components of BPS matrix (adapted by Karanasios et al., 2012).

Substrates	Pesticides	Reference
Straw; leek residues	Atrazine, carbofuran, simazine, diuron, lenacil, bifenthrin, metalaxyl	Spanoghe et al. (2004)
Bagasse	Glyphosate, malathion, lamda-cyhalothrin	de Roffignac et al. (2008)
Coco chips; straw; willow chopping; straw	Linuron, metalaxyl, isoproturon, bentazone	De Wilde et al. (2009b, c)
Coco chips; straw	Linuron, metalaxyl, isoproturon, bentazone, metamitron	De Wilde et al. (2010c, d)
Straw	Azoxystrobin, bentazone, bromoxynil, ioxynil, dimethoate, diuron, fenpropimorph, fluazifop- p-butyl, glyphosate, kresoxim methyl, MCPA, mecoprop-P, pirimicarb, propiconazole, propyzamide, prosulfocarb, metamitron, chloridazon, metribuzin, methabenzthiazuron, isoproturon, terbuthylazine, linuron, metalaxyl, isoproturon, pendimethalin, chlorothalonil, epoxiconazole, chloryprifos, deltamethrin, cypermethrin, ortho-phenylphenol, thiabendazole, imazalil	von Wirén-Lehr et al. (2001), Fogg et al. (2003, 2004a, b), Spliid et al. (2006), Castillo and Torstensson (2007), De Wilde et al. (2010a), Karanasios et al. (2010b), Kravariti et al. (2010), Karanasios et al. (2012a), Omirou et al. (2012), Tortella et al. (2012)
Vine branches, citrus peels	Chloryrifos, metalaxyl, imazamox, bentazone, isoproturon	Vischetti et al. (2004), Coppola et al. (2007), Coppola et al. (2011a)
Corn stovers; corn cobs	Alachlor, acetochlor	Lamar (2001)
Vine braches	Chlorpyrifos, metalaxyl	Vischetti et al. (2008)
Straw; corn cobs; citrus peels; sunflower residues; grape stalks; olive leaves	Chlorpyrifos, indoxacarb, buprofezin, terbuthylazine, metalaxyl, metribuzin, azoxystrobin, iprodione	Karanasios et al. (2010a)
Straw; grape stalks; corn cobs	Chlorpyrifos, terbuthylazine, metribuzin, metalaxyl, iprodione	Karanasios et al. (2012b)

Soil is an important source of pesticide-degrading microorganisms, especially bacteria with the ability to degrade such chemicals (Torstensson, 1996: Bergstrom and Stenstrom, 1997). The enhanced capacity of agricultural soils to degrade specific pesticide groups has been exploited as an indirect bioaugmentation strategy to optimize the biodegradation capacity of biobeds. Sniegowski and Springael (2014) proposed the use of pesticide primed soil (showing enhanced biodegradation capacity

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for certain pesticide groups) in biomixture to accelerate the degradation of pesticides in biobed systems.

Peat was the initial humified material that was used in the preparation of biomixtures. It contributes to the sorption capacity of the biomixture, regulates moisture, and favours the degradation of pesticides by decreasing the pH of the biomixture and thus favouring the growth and activity of white rot fungi (Castillo and Torstensson 2007). In southern European countries, peat is not easily available and it is costly. Studies showed that agricultural composts could be used instead of peat. Compost-based substrates are known to host several microorganisms with different pesticide-degrading activities, and they have also been demonstrated to have good sorption capacity for a wide variety of pesticides (Monaci et al., 2009: Vischetti et al., 2004). One of the materials which was tested as replacement of peat in biomixtures used in South Europe was the spent mushroom substrate (SMS) of the edible fungus *P. ostreatus* (Karanasios et al., 2010). The SMS-biomixture was highly efficient in degrading a pesticide mixture with degradation rates being correlated with the proportion of SMS in the biomixture. Composts and peat differ substantially in physicochemical characteristics, nutrient availability and biological activity (Niklasch and Joergensen 2001). Although the properties of individual composts largely depend on composting practices, they are generally characterized by lower C content, higher levels of macronutrients (N, P, K), neutral to basic pH (Zmora- Nahum et al. 2007) and support a metabolically active microbial community. Peat typically has higher waterholding capacity, significantly lower density, acidic pH and it does not generally support a highly active microbial community. These differences can reflect variability in the overall depuration capacity of the biomixtures.

1.11 AIMS OF THE THESIS

Fruit production constitutes a major agricultural activity at EU level. The production of apples in EU covers the 20% of the global production with all countries of the EU-28 contributing to this. Citrus fruit production is mainly concentrated in the Mediterranean basin covering over 60% of the global exports on edible citrus fruits (USDA 2015). However the high reliance of this agro-industry on pesticides results in the production of high volumes of pesticide-contaminated wastewaters which should be detoxified *on site* (EC Reg. 1109/EC/2009).

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Although detoxification methods for these effluents have been developed (Control Tec Eco®) and others have been tested (photoxidation, Fenton etc) none of them was implemented for reasons explained above. This has forced industries to dispose their effluents directly in municipal wastewater treatment systems which have evidently limited removal capacity (Campo et al. 2013), disharge them in unauthorized soil disposal sites with unacceptable repricautions for soil and environmental quality (Papadopoulou et al., 2015; 2016) or pass them to companies handling toxic wastes at a particularly high cost (0.7-3 € per L). All the above highlight the urgent need for implementation of an economic, efficient and easy-to-operate method for the depuration of these effluents. Biobeds could be a solution for reduction of the environmental footprint of this agro-industrial sector. However further research is required to adjust biobeds configuration to the particular characteristics of the wastewaters produced by the fruit packaging plants (a) high wastewater volumes and (b) high concentrations of persistent and toxic fungicides and antioxidants. Within this frame the main aim of the current thesis **is to evaluate the use of biobeds for the depuration of wastewaters from the fruit-packaging plants**. This main aim would be achieved through a series of research objectives following a gradual experimental scaling up plan:

1. to evaluate, in lab dissipation and sorption tests with the relevant pesticide compounds, different organic materials available by the local agricultural production (i.e. SMS of *P. ostreatus*) as components of an optimized biobed packing material
2. to explore, in a leaching column study, the capacity of the optimized biobed packing material to retain pesticides (a persistent like IMZ and a less persistent but more mobile like OPP) contained in the effluents under high hydraulic loads relevant to the wastewater production scheme commonly found in fruit packaging plants
3. to assess, at pilot scale level, the overall performance of biobeds for the depuration of wastewaters from pome and citrus fruit packaging plants and evaluate methods for the optimization of their performance (i.e. bioaugmentation). The main characteristics of wastewaters from the fruit packaging industry (low BOD/COD (*ca*. 20-500 mg L^{-1}), relatively low concentrations of suspended solids (120-128 mg L^{-1}), low N, sulfates and chlorides and high pesticide levels (Santiago et al., 2011) suggest that

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pesticide loads constitute the main environmental concern prohibiting their environmental release. Thus the quality of the biobed-treated effluent will be determined via risk assessment and potential unacceptable risk for the environment by their disharge will be assessed.

4. to explore methods for the decontamination of the spent biobed packing material in light of the recalcitrance of some of the chemicals contained in the effluents

It is expected that this thesis will provide a comprehensive assessment of the capacity of biobeds to treat wastewaters from the fruit packaging industry, offering a solution for the reduction of the environmental footprint of this important agro-industrial sector.

1.12 REFERENCES

Aktad MW, Sengupta D, Chowdhury A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. Interdisciplinary toxicology, 2(1): 1-12.

Alletto L., Coquet Y., Benoit P., Heddadj D., and Barriuso E. 2010. Tillage management effects on pesticide fate in soils. A review. *Agron.Sustain.Dev*. 30, 367-400.

Amellal N, Portal J, and Berthelin J, (2001). Effect of Soil Structure on the Bioavailability of Polycyclic Aromatic Hydrocarbons within Aggregates of a Contaminated Soil. *Applied Geochemistry* 16, 1611-1619.

Andreu V. and Pico Y, 2004.Determination of pesticides and their degradation products in soil: critical review and comparison of methods, *Trends in Analytical Chemistry*, 23, 10–11, 2004

Azubuike CC, Chikere CB and Okpokwasili G, (2016). Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects, *World J Microbiol Biotechnol* 32:180

Barr D (2002) Biological methods for assessment and remediation of contaminated land: case studies. Construction Industry Research and Information Association, London

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Baszczyk, A.B., Augustyniak, A., Skolimowski, J., 2013. Ethoxyquin: an antioxidant used in animal feed. Int. J. Food Sci. http://dx.doi.org/10.1155/2013/585931 (Article ID 585931).

Bedos C., Cellier P., Calvet R., Barriuso E. and Gabrielle B. (2002) Mass transfer of pesticides into the atmosphere by volatilization from soils and plants: overview, *Agronomie* 22, 21–33.

Behera B and Singh SG. (1999). Studies on Weed Management in Monsoon Season Crop of Tomato. *Indian J Weed Sci* 31(1–2): 67.

Belenguer, V., Martinez-Capel, F., Masiá, A., Picó, Y., (2014). Patterns of presence and concentration of pesticides in fish and waters of the Júcar River (Eastern Spain). J. *Hazard. Mater*. 265, 271–279.

Bennett E.M. and Bryant C. (1984). *Mol. Biochem. Parasitol*., 10, 335

Bergstrom, L. and Stenstrom, J. (1998). Environmental fate of chemicals in soil. *Ambio*, *27*, 16–23.

Bolognesi, C. (2003) Genotoxicity of pesticides: A review of human biomonitoring studies. *Mutat. Res.*, *543*, 251-272.

Boutron O., Gouy V., Touze-Foltz N., Benoit P., Chovelon M.J. and Margouma C. (2009). Geotextile fibres retention properties to prevent surface water nonpoint contamination by pesticides in agricultural areas. *Geotextiles and Geomembranes*, 27, 254–261

Boxall, R.A. (2001). Post-harvest losses to insects—a world overview. Int. Biodeter. Biodegr. *48*, 137-152.

Campo, J., Masia, A., Blasco, C., Pico, Y., (2013). Occurrence and removal efficiency of pesticides in sewage treatment plants of fourMediterranean River Basins. *J. Hazard.Mater.* 263P, 146–157.

Campos M., Perruchon C., Karavasilis D., Diez M.C., Karpouzas D.G., (2016) Optimizing the biodegradation potential of biobed systems against iprodione through rhizosphere-assisted bioaugmentation. *Journal of Environmental Management (in press)* *http://dx.doi.org/10.1016/j.jenvman.2016.11.031*

Candela L., Wallis J.K. and Mateos M.R. (2008). Non-point pollution of groundwater from agricultural activities in Mediterranean Spain: the Balearic Islands case study. *Environmental Geology*, 54, 587–595.

Cannavan A., Haggan A.S. and Kennedy D.G. (1998). Simultaneous determination of thiabendazole and its major metabolite, 5-hydroxythiabendazole, in bovine tissues

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

using gradient liquid chromatography with thermospray and atmospheric pressure chemical ionisation mass spectrometry. *Journal of Chromatography B,* 718, 103–113

Carra I., Santos-Juanes L., Acién Fernández FG., Malato S., Sánchez Pérez J.A. (2014). New approach to solar photo-Fenton operation. Raceway ponds as tertiary treatment technology. *J hazard Mater.*, 30, 322-329.

Castillio M d P, Terstensson L and Stenstrom J, (2008). Biobeds for Environmental Protection from Pesticide Uses: A Review, *J. Agric. Food Chem., 56,* 6206–6219

Castillo L.E., Martinez E., Ruepert C., Savage C., Gilek M., Pinnock M.E. (2006) Solis,Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica, *Sci. TotalEnviron*. 367 418–432.

Castillo M. d. P. and Torstensson L, (2007). Effect of biobed composition, moisture and temperature on the degradation of pesticides. *J.Agric. Food Chem., 55,* 5725–5733

Castillo M.d.P., Ander P., Stenstrom J. and Torstensson L., (2000). Degradation of the herbicide bentazon as related to enzyme production by *Phanerochaete chrysosporium* in two solid substrate fermentation systems. *World Journal of Microbiology and Biotechnology*, **16**, 289-295.

Ccanccapa A., Masia A., Andreu V., Pico Y., (2016) Spatio-temporal patterns of pesticide residues in the Turia and Júcar Rivers (Spain). *Science Total Environment* 540:200-210.

Chu B.L., Guo B.Y., Peng Z., Wang Z., Guo G., Lin J.M., (2007). Studies on degradation of imazalil enantiomers in soil using capillary electrophoresis. *Journal of Separation Science* 30(6) 923-929

Cnubben N, Elliott G.R., Hakkert B.C., Meuling W.J. and van de Sandt J.J. (2002). Comparative *in Vitro–in Vivo* Percutaneous Penetration of the Fungicide *ortho*-Phenylphenol. *Regulatory Toxicology and Pharmacology,* **35,** 198–208

Cooper J., Dobson, H. (2007) The benefits of pesticides to mankind and the environment. Crop Prot. *26,* 1337-1348.

Coppola L., Castillo M. d. P, Monaci E and Vischetti C (2007). Adaptation of the biobed composition for chlorpyrifos degradation to southern Europe conditions. *J. Agric. Food Chem., 55,* 396–401.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Cunningham S. D., Anderson T. A., Schwab P. A. and Hsu F. C., (1996). Phytoremediation of Soils Contaminated with Organic Pollutants, *Advances in Agronomy*, 56, 55-114

Dadrasnia A, Shahsavari N and Emenike CU, (2013). Remediation of Contaminated Sites, Chapter from the book Hydrocarbon.

Damalas C. A. and Eleftherohorinos I. G., 2011. "Pesticide exposure, safety issues, and risk assessment indicators". *Int. J. Environ. Res. Public Health*, 8: 1402-1419.

Damalas, C.A. (2009). Understanding benefits and risks of pesticide use. Sci. Res. Essays, *4*, 945-949

Danaher M., De Ruyckb H., Crooks S.R.H., Dowling G. and O'Keeffe M. (2007). Review of methodology for the determination of benzimidazole residues in biological matrices. *Journal of Chromatography B*, 845, 1–37.

De Koning AJ and Mol TH, (1989). Lipid determination in fish meal: an investigation of three standard methods applied to stabilised and non-stabilised anchovy meals at increasing stages of maturity. *J Sci Food Agric* 46:259±266.

De Koning AJ, A new method for measuring efficacies of antioxidants in fish meal. Int J Food Properties 1:255±261 (1998).

De Wilde T, Spanoghe P, Debaer C, Ryckeboer J, Springael D and Jaeken P. (2007). Overview of on-farm bioremediation systems to reduce the occurrence of point source contamination. *Pest Manage. Sci., 63*, 111–128.

Dietary guidelines for Americans, (2005). U.S. Department of Health and Human Services U.S. Department of Agriculture.

Drewhurst, "Ethoxyquin. JMPR Evaluations," (1998). http:// www.inchem.org/documents/jmpr/jmpmono/v098pr09.htm

Drzyzga O. and Blotevogel K.H. (1997). Microbial degradation of Diphenylamine under anoxic conditions. *Current microbiology*, 35, 343–347.

Drzyzga O., 2003. "Diphenylamine and derivatives in the environment: A Review". *Chemosphere*, 53: 809-818.

Drzyzga O., Jannsen S. and Blotevogel K.H. (1995). Toxicity of Diphenylamine and some of its nitrated and aminated derivatives to the luminescent bacterium *Vibrio fischeri*. *Ecotoxicology and environmental safety*, 31, 149-152.

Duus Børgensen C., Fomsgaard I.S., Plauborg F., Kirsten Schelde and Spliid N.H., (2015). Fate of pesticides in agricultural soils, *DCA - Danish Centre for Food and Agriculture*, scientific report 062.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Echols R.K, Brumbaugh W.G., Orazio C.E., May T.W., Poulton B.C. and Peterman P.H. (2008). Distribution of Pesticides, PAHs, PCBs, and Bioavailable Metals in Depositional Sediments of the Lower Missouri River, USA. *Archie Environmental Contaminante Toxicology,* 55, 161–172.

EFSA (European Food Safety Authority), 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance ethoxyquin. EFSA Journal 2010;8(9):1710, 38 pp. doi:10.2903/j.efsa.2010.1710.

Environmental Protection Agency (EPA) of USA (2009). Registering Pesticides, Available online: http://www.epa.gov/pesticides.htm

European Commission (EC), (2000). Opinion of the scientific committee on plants regarding the evaluation of thiabendazole in the context of council directive 91/414/EEC concerning the placing of plant protection products on the market - The Scientific Committee on Plants. CP/THIABEN/002—Final, p. 7.

European Commission (EC), (2001). Review report for the active substance thiabendazole, 7603/VI/97-final.

European Commission (EC), (2006). Commission Directive 2006/19/EC amending Council Directive 91/414/EEC to include 1-methylcyclopropene as active substance. *OJ*, L 44: 15-17.

European Commission (EC), (2007). Review report for the active substance imazlil, 688/VI/97-Final.

European Commission (EC), (2007a). Draft Assessment Report - Initial risk assessment provided by the rapporteur member state Ireland for the existing active substance diphenylamine, 1, pp 1-60.

European Commission (EC), (2008). Draft Assessment Report - Initial risk assessment provided by the rapporteur member-state Spain for the existing active substance 2-phenylphenol. Volume 1, April 2008

European Commission (EC), (2009). Draft Assessment Report, Initial risk assessment provided by the rapporteur member state the Netherlands for the existing substance imazalil. Volume 1, pp. 1-151.

European Commission (EC), (2009b). Final Review report for the active substance 2-phenylphenol. Available at http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_2_phenylphenol_en.pdf

European Commission (EC), (2010a). Commission Directive 2010/57/EU of 26 August 2010 amending Annex I to Council Directive 91/414/EEC to renew the

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

inclusion of imazalil as active substance. Official Journal of the European Union L225/5. Available at http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010: 225:0005:0009:EN:PDF

European Commission (EC), (2010b). Residue analytical methods. For post-registration control. SANCO/825/00-rev.8-1.

European Commission (EC), (2010c). Final- Review report for the active substance 2-phenylphenol. SANCO/10698/09 - rev. 2.

European Commission (EC), (2013). Instituto Nacional de Investigation y Tecnologia Agraria y Alimentaria (INIA), Draft renewal assessment report prepared according to the Commission Regulation (EU) N.1141/2010, Second program for the renewal of the inclusion of the following active substance under Regulation (EC) 1107/2009, Thiabendazole, Vol. 1, Report and proposed decision.

European Community (EC) REGULATION No 1107/2009 of the Europian parliament of the council (2009) concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC

European Food Safety Authority (EFSA), (2008). Conclusion regarding the peer review of the pesticide risk assessment of the active substance diphenylamine. *EFSA Scientific Report*, 188: 1-60

European Food Safety Authority (EFSA), (2012). Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine. *EFSA J*., 10 (1): 1426.

European Parliament and Council (EPA), 2002. Decision 1600/2002/EC laying down the Sixth Community Environment Action Programme. OJ L 242, pp. 1-15.

Eurostat Statistic Explained 2016. Pesticide sale statistics. Available at http://ec.europa.eu/eurostat/statistics-explained/index.php/Pesticide_sales_statistics#Data_sources_and_availability

Eurostat Statistical Books (2015). Agriculture, forestry and fishery. Available at http://ec.europa.eu/eurostat/statistics-explained/index.php/Agriculture,_forestry_and_fisheries_statistics_introduced

FAO, 1989. International Code of Conduct on the Distribution and Use of Pesticides, Rome, Italy.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Fogg P, Boxall A, Walker A and Andrew Jukes A, (2004). Degradation and leaching potential of pesticides in biobed systems, *Pest Manag Sci* 60:645–654

Garcia-Portillo, M., Avino, E.S., Vicente, J.O., De Andres, R.L., Jimenez, M.d.l.A.S., Blanco J.P.L., (2004). Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States Patent, US 6,709,585 B1, pp. 9

Gavrilescu M, (2005). Fate of Pesticides in the Environment and its Bioremediation. Engineering, *Life Sciences*, 5, 497-526

Giacomazzi S. and Cochet N., (2004). Environmental Impact of Diuron Transformation: a Review. *Chemosphere,* 56, 1021-1032

Gidarakos E and Aivalioti M (2007). Large scale and long term application of bioslurping: the case of a Greek petroleum refinery site. *J Hazard Mater* 149:574–581.

Guyot C and Chenivesse D (2006). A simple and affordable system to prevent water contamination. ICM EDITION, Bayer CropScience,September 2006, 31-33.

Hatzinger PB, Whittier MC, Arkins MD, Bryan CW, and Guarini WJ (2002) In situ and ex-situ bioremediation options for treating perchlorate in groundwater. *Remediat J* 12 69–86

Hu Y., Yang X., Wang C., Zhao J., Li W. and Wang Z. (2008). A sensitive determination method for carbendazim and thiabendazole in apples by solid-phase microextraction high performance liquid chromatography with fluorescence detection. *Food Additives and Contaminants*, 25, 314–319

Hu Y., Yang X., Wang C., Zhao J., Li W. and Wang Z. (2009). A sensitive determination method for carbendazim and thiabendazole in apples by solid-phase microextraction high performance liquid chromatography with fluorescence detection. *Food Additives and Contaminants*, 25, 314–319.

Imbeah M., (1998). Composting piggery waste: a review. *Bioresource Technology*, 63., 197-203.

Iranzo M, Sainz- Pardo I, Boluda R, Sánchez J, and Mormeneo S, (2001). The Use of Microorganisms in Environmental Remediation. *Annals of Microbiology*, 51, 135-143.

Jimenez, M., Maldonado, M.I., Rodriguez, E.M., Hernandez-Ramirez, A., Saggioro, E., Carrae, I., Sanchez, J.A., (2015). Supported TiO2 solar photocatalysis at semi-pilot scale: degradation of pesticides found in citrus processing industry

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

wastewater, reactivity and influence of photogenerated species. *J. Chem. Technol. Biotechnol*. 90, 149–157.

Johnson RL, Johnson PC, McWhorter DB and Hinchee RE GI, (2007). An overview of in situ air sparging, *Ground Water Monit Remed*,13 127-135.

Jung S-K and Watkins CB (2008). Superficial scald control after delayed treatment of apple fruit with diphenylamine (DPA) and 1-methylcyclopropene (1-MCP). *Post Harv Biol Technol* 50:45–52

Karanasios E, Tsiropoulos N and Karpouzas D.G., (2012) On-farm biopurification systems for the depuration of pesticide-wastewaters: recent advances and future perspectives. *Biodegradation* 23, 787-802

Karanasios, E., Tsiropoulos, N., Karpouzas, D.G., Menkissoglu-Spiroudi, U., (2010a). Novel biomixtures based on local Mediterranean ligninocellulosic materials: evaluation for use in biobeds. *Chemosphere* 80, 914–921

Karas P., Perucchon C., Exarhou C., Ehaliotis C., Karpouzas DG., (2011) Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. *Biodegradation* 22: 215-228.

Khan FI, Husain T and Hejazi R (2004). An overview and analysis of site remediation technologies, *Journal of Environmental Management*, 71(2), 95-122

Khodja, A.A., Sehili, T., Pilichowski, J.-F., Boule, P., 2000. Photocatalytic degradation of 2-phenylphenol on TiO2 and ZnO in aqueous suspensions. *J. Photochem. Photobiol. A Chem. 1*41, 231–239.

Kodama S., Yamamoto A., Ohura T., Matsunaga A. and Kanbe T. (2003). Enantioseparation of Imazalil residue in orange by Capillary Electrophoresis with 2-hydroxypropyl-*â*-cyclodextrin as a chiral selector. *Journal of Agricultural Food and Chemistry*, 51, 6128-6131

Kohler HPE, Kohler-Staub D and Focht DD, (1988). Degradation of 2-hydroxybiphenyl and 2,2′-dihydroxybiphenyl by Pseudomonas sp. strain HBP1. *Appl EnvironMicrobiol* 54(11):2683–2688

Korner, W., Bolz, U., Submuth, W., Hiller, G., Schuller, W., Hanf, V., Hagenmaier, H., (2000). Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 40, 1141–1142.

MacRae IC, and Alexander M, (1965). Microbial degradation of selected herbicides in soil. *J. Agric. Food Chem*, 13:72-76.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Magkos, F, Arvaniti, F and Zampelas, A. (2006) Organic Food: Buying more safety or just peace of mind? A critical review of the literature. *Crit. Rev. Food Sci. Nutr.*, *46*, 23-55

Mari M., Bertolini P. and Pratella G.C. (2003). Non-conventional methods for the control of post-harvest pear diseases: a review. *Journal of Applied Microbiology*, 94, 761–766.

Maruyama T., Komatsu C., Michizoe J., Sakai S. and Goto M. (2007). Laccase-mediated degradation and reduction of toxicity of the postharvest fungicide imazalil. *Process Biochemistry*, 42, 459–461

Masiá, A., Campo, J., Vázquez-Roig, P., Blasco, C., Picó, Y., (2013). Screening of currently used pesticides in water, sediments and biota of the Guadalquivir River Basin (Spain). *J. Hazard. Mater*. 263 (Part 1), 95–104.

McIntyre T, (2003). Phytoremediation of heavy metals from soils. *Advance Biochemical Engineering Biotechnology*, 78:97–123.

Mokrini A., Oussi D. and Esplugas S., (1981). "Oxidaiton of Aro- matic Compounds with UV Radiation/Ozone/Hydrogen Peroxide," *Water Science and Technology*, Vol. 35, (4), 95-102 65

Monaci E, Coppola L, Casucci C, Perucci P and Vischetti C, (2009). Retention capacity of an organic bio-mixture against different mixtures of fungicides used in vineyards. *J Environ Sci Health B* 44:724–729.

Muto N. Hirai H., Tanaka T., Itoh N. and Tanaka K. (1997). Induction and inhibition of cytochrome P450 isoforms by imazalil, a food contaminant, in mouse small intestine and liver. *Xenobiotica*, 27, 1215-1223.

Narayanasamy, P. *Postharvest Pathogens and Disease Management*; John Wiley & Sons: New York, NY, USA, 2006.

Niklasch H and Joergensen RG (2001) Decomposition of peat, biogenic municipal waste compost, and shrub/grass compost added in different rates to a silt loam. *J Plant Nutr Soil Sci* 164:365–369

Nunes C., Usall J., Teixido N. and Vinas I. (2001). Biological control of postharvest pear diseases using a bacterium, *Pantoea agglomerans* CPA-2. *International Journal of Food Microbiology*, 70, 53–61.

OECD guideline for testing of chemicals: Adsorption – Desorption Using a Batch Equilibrium Method. No. 106, adopted 21 January, 2000).

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Oerke, E.C.; Dehne, H.W. (2004). Safeguarding production-losses in major crops and the role of crop protection. Crop Prot, *23*, 275-285.

Omirou, M., Dalias, P., Costa, C., Papastefanou, C., Dados, A., Ehaliotis, C., and Karpouzas D.G., 2012. Exploring the potential of biobeds for the depuration of pesticide-contaminated wastewaters from the citrus production chain: Laboratory, column and field studies. Environ. Pollut. 166, 31-39.

Ortelli D., Edder P. and Corvi C. (2005). Pesticide residues survey in citrus fruits. *Food Additives and Contaminants*, 22, 423–428.

Ortiz-Hernandez M, Sanchez-Salinas E, Dantan-Gonzalez E. and castrejon-Godines ML. (2013). Pesticide biodegradation: Mechanisms, Genetics and Strategies to Enhance the Process. Chapter of the book "Biodegradation – Life of science" edited by Chamy R. and Rosenkranz F.

Palma P, Köck-Schulmeyer M, Alvarenga P, Ledo L, Barbosa IR, López de Alda M and Barceló D (2014). Risk assessment of pesticides detected in surface water of the Alqueva reservoir (Guadiana basin, southern of Portugal). *Science of Total Environment*, (488-499), 208-219.

Papadopoulou E. S., Perruchon C., Omirou M., Stamatopoulou N., Karpouzas D. G., (2015). Bioaugmentation as a depuration treatment for soils polluted with pesticides from the fruit-packaging industry wastewater. 2nd Environmental Conference of Thessaly, Skiathos Island, Greece, 26-28 September 2015.

Papadopoulou E.S., Tsachidou P., Sulowic S., Menkissoglu-Spiroudi U., Karpouzas D.G., (2016). Land spreading of wastewaters from the fruit packaging industry and potential effects on soil microbes: Effects of the antioxidant ethoxyquin and its metabolites on ammonia oxidizers. *Applied and Environmental Microbiology* 82: 747-755

Pastor S., Creus A., Parrón T., Cebulska-Wasilewska A., Siffel C., Piperakis S. and Marcos R. (2003).Biomonitoring of four European populations occupationally exposed to pesticides: Use of micronuclei as biomarkers. *Mutagenesis*, *18*, 249-258.

Perruchon C., Batianis C., Zouborlis, S., Papadopoulou E.S., Ntougias S., Vasileiadis S., Karpouzas D.G., (2015). Isolation of a diphenylamine-degrading bacterium and characterization of its degradation capacities, bioremediation and bioaugmentation potential. *Environmental Science and Pollution Research* 22: 19485-19496

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Perruchon C., Chatzinotas A., Omirou M., Vasileiadis S., Menkissoglu-Spiroudi U., Karpouzas D.G., (2016b) Isolation of a bacterial consortium able to degrade the fungicide thiabendazole and determination of its metabolic pathway: the key role of a *Sphingomonas* phylotype. *Applied Microbiology and Biotechnology (submitted)*

Perruchon C., Patsioura V., Vasileiadis S., Karpouzas D.G, (2016a). Isolation and characterization of a *Sphingomonas* strain able to degrade the fungicide *ortho*-phenylphenol. *Pest Management Science* 72(1): 113-124

Philp JC, Atlas RM (2005) Bioremediation of contaminated soils and aquifers. In: Atlas RM, Philp JC (eds) Bioremediation: applied microbial solutions for real-world environmental cleanup. American Society for Microbiology (ASM) Press, Washington, pp 139–236

Power, A.G. (2010). Ecosystem services and agriculture: Tradeoffs and synergies. *Phil. Trans. R. Soc. B, 365*, 2959-2971.

Racke K. D. and Coats J. R., (1990). "Enhanced Biodegradation of Pesticides in the Environment," *American Chemical Society*, No. 426, Washington DC, 28

Ramwell, C.T., Johnson, P.D., Boxall, A.B.A., Rimmer, D.A., (2004). Pesticide residues on the external surfaces of field-crop sprayers: environmental impact. *Pest Manage. Sci.* 60, 795–802.

Rayu S, Karpouzas DG. and Singh BK. (2012). Emerging technologies in bioremediation: constraints and opportunities. Biodegradation, 23:917–926

Reichenberger S., Bach M., Skitschak A. and Frede H.-G. 2007. Mitigation strategies to reduce pesticide inputs into ground- and surface water and their effectiveness; A review. *Sci. Total Environ*., 384, 1-35.

Ritenour M.A., Zhang J., Wardowski F.W. and Brown G.E. (2003). Postharvest Decay Control Recommendations for Florida Citrus Fruit. *University of Florida IFAS extension*, circular 359A

Ross G. (2005). Risks and benefi ts of DDT. *The Lancet* 366 (9499): 1771.

Rushing J. W., Cook W. P., Spell L., (1995). "Accumulation of pesticides in tomato packinghouse wastewater and the influence of integrated pest management on reducing residues". *HortTechnology*, 5 (3): 243-247.

Sanchez Perez, J.A., Carra, A., Sirtori, C., Aguera, A., Esteban, B., 2014. Fate of thiabendazole through the treatment of a simulated agro-food industrial effluent by combined MBR/Fenton processes at mg/L scale. *Water Res*. 51, 55–63.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Santiago, D.E., Melian, E.P., Rodriguez, C.F., Mendez, J.A.O., Perez-Baez, S.O., Dona- Rodriguez, J.M., (2011). Degradation and detoxification of banana postharvest treatment water using advanced oxidation techniques. *Green Sustain. Chem.* 1, 39–46

Schulz M. and Matthies M., (2007). Runoff of Pesticides: Achievements and Limitations of Modelling Agrochemical Dislocation from Non-Point Sources at Various Landscape Related Scales, *Living Rev. Landscape Res.*, 1, 1-28

Sharma V.K., Canditelli M., Fortuna F., Cornacchia G., (1997). Processing of urban and agro-industrial residues by aerobic composting: review. *Energy Convers. Mgmt*, 38, 453-478.

Shin KA and Spain JC (2009). Pathway and evolutionary implications of diphenylamine biodegradation by *Burkholderia sp. strain JS667*. *Appl Environ Microbiol* 75:2694–2704

Shukla KP, Singh NK, Sharma S (2010). Bioremediation: developments, current practices and perspectives. Genet. Eng. Biotechnol. J., 3: 1-20.

Simon, L., Spiteller, M., Haisch, A., Wallnofer, P.R., (1992). Influence of soil properties on the degradation of the nematicide fenamiphos. *Soil Biol. Biochem*. 24, 769– 773.

Singh BK (2009). Organophosphorus-degrading bacteria: ecologyand industrial applications. Nat Rev Microbiol 7:156–16.

Singh D. and Chen S. (2008). The white-rot fungus Phanerochaete chrysosporium: conditions for the production of lignin-degrading enzymes. *Applied Microbiology and Biotechnology*, 81, 399–417

Singh, B.K., Walker, A., Morgan, J.A.W. and Wright, D.J. (2003) Effect of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. *Appl. Environ. Microbiol.* 69, 5198–5206.

Sirtori C, Agüera A, Carra I, Sanchéz Pérez JA. (2014). Identification and monitoring of thiabendazole transformation products in water during Fenton degradation by LC-QTOF-MS. Anal Bioanal Chem 406**:** 5323-5337

Sniegowski K., Springael D., (2014). Establishment of multiple pesticide biodegradation capacities from pesticide-primed materials in on-farm biopurification system microcosms treating complex pesticide-contaminated wastewater, *Pest Manag. Sci.* 71 986–995.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Stoate, C., Boatman, N.D., Borralho, R.J., Rio Carvalho, C., de Snoo, G.R., and Eden, P. (2001). Ecological impacts of arable intensification in Europe. *J. Environ. Manag.*, *63*, 337-365.

Storck, V., Karpouzas D.G., Martin-Laurent F (2016) Towards a new pesticide registration policy. Science of the Total Environment http://dx.doi.org/10.1016/j.scitotenv.2016.09.167

Tani S., Yonezawa Y., Morisawa S. and Nishioka H. (2007). Development of a new *E. coli* strain to detect oxidative mutation and its application to the fungicide *o*-phenylphenol and its metabolites. *Mutation Research*, 628, 123–128

Topp E, Vallayes T, and Soulas G, 1997. Pesticides: Microbial degradation and effects on microorganisms. In: Modern soil microbiology, Pp, 547–575.

Torstensson (2000). L. Experiences of biobeds in practical use in Sweden *Pestic. Outlook, 11*, 206–212.

Torstensson, L (1996). Herbicides in the Environment. *Proceedings of the Second International Weed Control Congress*, Copenhagen, Denmark, 25-28 June 1996; Vol. 1-4, 267-274.

Torstensson, L. and Castillo, M. d. P. (1997). Use of biobeds in Sweden to minimize environmental spillages from agricultural spraying equipment. *Pestic. Outlook, 8*, 24–27.

Tortella R.G. and Diez M.C. (2005). Fungal Diversity and Use in Decomposition of Environmental Pollutants. *Critical Reviews in Microbiology*, 31, 197–212

Tuomela M., Vikman M., Hatakka A., Itavaara M., (2000). Biodegradation of lignin in a compost environment: a review. *Bioresource Technology*, 72, 169-183.

U.S. Environmental Protection Agency (EPA), (1995). Protocol for Equipment Leak Emission Estimates, EPA-453/R-95-017

U.S. Environmental Protection Agency (EPA), (1998). Reregistration Eligibility Decision (RED) DEET, EPA738-R-98-010

U.S. Environmental Protection Agency (EPA), (2002). Reregistration Eligibility Decision (RED) Thiabendazole, EPA738-R-02-xxx

U.S. Environmental Protection Agency (EPA), (2003). Reregistration Eligibility Decision for imazalil, chemical list B, case number 2325

U.S. Environmental Protection Agency (EPA), (2009). Exposure factors handbook: 2009. External review Draft, EPA/600/R-09/052A.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

U.S. Environmental Protection Agency, EPA (2006). Reregistration Eligibility Decision for 2-phenylphenol and Salts (Orthophenylphenol or OPP). 739-R-06-004

USDA (2015). Citrus world markets and trade, July 2015. http://usda.mannlib.cornell.edu/usda/fas/citruswm/2010s/2015/citruswm-07-23-2015.pdf

Vidali M., (2001). "Bioremediation. An overview". *Pure Appl. Chem.*, 73 (7): 1163–1172.

Vinggaard A.M., Hnida C., Breinholt V. and Larsen J. C. (2000). Screening of selected pesticides for inhibition of CYP19 aromatase activity *in* V*itro*. *Toxicology in Vitro*, 14, 227-234

Vischetti C, Capri E, Trevisan M, Casucci C and Perucci P (2004). Biomassbed: A biological system to reduce pesticide point contamination at farm level. *Chemosphere, 55*, 823–828

Walker A (1978). The degradation of methazole in soil. I. Effects of soil type, soil temperature and soil moisture content. *Pest Management Science* 9:326-332

Walker A., Jurado- Exposito M, Bending GD, and Smith V J R, (2001). Spatial variability in the degradation rate of isoproturon in soil. *Environmental Pollution* 111, 407-415.

Watanabe-Akanuma M., Ohta T., Sasaki Y., (2005). "A novel genotoxic aspect of thiabendazole as a photomutagen in bacteria and cultured human cells". *Toxicol. Lett.*, 158: 213-219.

Whelan MJ, Coulon F, Hince G, Rayner J, McWatters R, Spedding T and Snape I (2015). Fate and transport of petroleum hydrocarbons in engineered biopiles in polar regions. *Chemosphere* 131, 232–240.

Zamora T., Hidalgo C., Lopez F.J. and Hernandez F. (2004). Determination of fungicide residues in fruits by coupled-column liquid chromatography. *J. Sep. Sci.*, **27**, 645-652

Zheng C., Focks A., Ellebrake K., Eggers T., Fries E., (2011). Sorption of ortho-phenylphenol to soils. *Clean- Soil, Air, Water*, 39: 116-120.

Zmora-Nahum S, Hadar Y, Chen Y (2007) Physico-chemical properties of commercial composts varying in their source materials and country of origin. *Soil Biol Biochem* 39:1263–12

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Ζιώγας Β. και Μαρκόγλου Α. (2007). Γεωργική φαρμακολογία. Βιοχημεία,φυσιολογία, μηχανισμοί δράσης και χρήσεις των φυτοπροστατευτικών προϊόντων, Αθήνα.

Institutional Repository - Library & Information Centre - University of Thessaly
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Chapter 2

Dissipation, metabolism and sorption of pesticides used in fruit-packaging plants in sewage sludge and biobeds packing materials - Lab studies

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

Karas P.A., Metsoviti A., Zisis V., Ehaliotis C., Omirou M., Papadopoulou ES., Menksissoglu-Spiroudi U., Manta S., Komioti D., Karpouzas D.G., (2015) Dissipation, metabolism and sorption of pesticides used in fruit-packaging plants: Towards an optimized depuration of their pesticide-contaminated agro-industrial effluents. *Science of the Total Environment* 530-531: 129-139

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2.1. INTRODUCTION

Upon their harvest fruits are transported to fruit-packaging plants where they are treated with fungicides (thiabendazole (TBZ), imazalil (IMZ), *ortho*-phenylphenol (OPP) or antioxidants (diphenylamine (DPA), ethoxyquin (EQ)) to minimize losses due to fungal infestations or physiological disorders during storage (Smilanick et al. 2008; Jung and Watkins 2008). Postharvest treatments of fruits result in the production of large wastewater volumes which are characterized by low BOD/COD values but high concentrations of pesticides which should be detoxified prior to environmental release (Santiago et al. 2011). This need has been laid down on the registration documents of all relevant pesticides. For example authorization was granted to IMZ only under the clause that *appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place* (EC 2010). The only full-scale treatment system currently in place is based on pesticide sorption onto granular activated carbon (Garcia Portillo et al., 2004). Although this system is particularly efficient in the removal of TBZ from wastewaters (EC, 2000) its cost is prohibitive for small to medium enterprises which constitute the majority of fruit-packaging plants in the Mediterranean region. Recent semi-pilot studies showed that combination of membrane bioreactor and advanced oxidation processes could effectively remove TBZ from wastewaters (Sachez-Perez et al. 2014). Similarly, TiO_2 solar photocatalysis showed high depuration efficiency for the removal of IMZ, TBZ (Jimenez et al. 2015) and OPP (Khodja et al. 2001) from wastewaters. However those methods produce several oxidized metabolites which are of unknown toxicity compared to the parent compound plus their full scale implementation is still pending.

In the absence of appropriate and established treatment methods, fruit-packaging plants tend to discharge their wastewater into municipal wastewater treatment plants, abandoned fields or evaporation ponds. Previous studies have provided indirect evidence for the limited removal capacity of municipal wastewater treatment plants for IMZ, TBZ (Campo et al. 2013) and OPP (Jonkers et al. 2010). This combined with the inappropriate disposal methods currently in place for these wastewaters have resulted in the frequent detection of these pesticides in receiving water bodies (Castillo et al. 2000, 2006). Thus an efficient, cost-effective and

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sustainable treatment system for the depuration of those effluents is needed. Omirou et al. (2012) provided first evidence for the potential use of modified biobed systems for the depuration of wastewaters from the fruit-packaging industry. Such modified systems should be packed with organic materials which ensure effective dissipation of the particularly persistent (TBZ (EC 2013) and IMZ (Kreuzig et al. 2010)) and toxic pesticides (OPP (EFSA 2008), EQ (EFSA 2010b) and DPA (EFSA 2012)) contained in those agro-industrial effluents. The optimum composition of biobed packing material includes a lignocellulosic material like straw, soil and a humified substrate like peat or compost (Castillo et al. 2008). Spent mushroom substrate (SMS) of the fungus *Pleurotus ostreatus* has been found to accelerate the biodegradation potential of on-farm biobed systems (Karanasios et al. 2010a). SMS is produced in large quantities in several areas of the Mediterranean basin and the mushroom production sector is seeking sustainable and environmental-friendly uses for this material (Herrero-Hernandez et al. 2011).

To date little is known regarding the basic processes controlling the dissipation of pesticides contained in the wastewaters from the fruit packaging plants. Only a few studies have investigated the dissipation of IMZ, TBZ and OPP in soil (Kreuzig et al. 2010; Kesavan et al. 1976), municipal wastewater treatment plants (Campo et al. 2013; Korner et al. 2000) and organic substrates (Omirou et al. 2012), while even less are known for DPA. In sewage sludge Gardner et al. (1992) reported the metabolism of DPA to aniline, 4-hydroxy-DPA and indole. More recently Shin and Spain (2009) isolated a soil bacterium that metabolized to Krebs cycle intermediates via formation of aniline. No information are available regarding the dissipation and metabolism of EQ in the environment. Metabolic studies for EQ are only available on fish feed, fish meals and fruits which identified the formation of several metabolites like a dimer of EQ and quinone imine (QI) (He and Ackman 2000).

The main aims of this work were (a) to assess the capacity of sewage sludge, anaerobically digested or liquid aerobic, to degrade the pesticides contained in the wastewaters from the fruit-packaging industry; providing a measure of the removal capacity of municipal wastewater treatment plants (b) to identify the most potent organic biomixture, with SMS as a key component, regarding its dissipation capacity for the pesticides contained in the effluents from fruit packaging plants. Apart from

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pesticide dissipation, the sorption of these pesticides on the organic substrates was also assessed to evaluate the contribution of the different processes in the removal of pesticides from those wastewaters. In addition, the metabolism of EQ in all materials was determined considering that most of the metabolic products of this antioxidant compound are equally active and toxic as the parent compound (Blaszczyk et al. 2013). These data will provide (a) information on the depuration potential of the currently followed strategies for treatment of those effluents and (b) a first lab-scale assessment of the optimum organic biomixture which could be used as packing material in full-scale biobed systems used for the biodepuration of wastewaters from the fruit packaging industry (tested in Chapter 4).

2.2. MATERIALS AND METHODS

2.2.1. Pesticides

Analytical standards of IMZ (99.8% Pestanal®), TBZ (99% Pestanal®), OPP (99.9% Pestanal®), DPA (99.9% Pestanal®) and EQ (99% Pestanal®) were purchased from Fluka, Sigma-Aldrich. For residue analysis, pesticides stock solutions in methanol were initially prepared (1000 mg L^{-1}) and used for obtaining a series of dilutions at the range of 0.1–50 mg L^{-1} which were used for the construction of calibration curves for quantification of pesticides concentrations by HPLC. Particularly for EQ, preliminary studies indicated a rapid oxidation of EQ (*m/z* 218 $[M+H]^+$, 202 $[M^+-CH_3]$, 174 $[202-C_2H_4]$ and retention time (Rt) 9.9 min) in the substrates tested to two metabolites which were tentatively identified via LC-MS/MS analysis as (1) *2,6-dihydro-2,2,4-trimethyl-6-quinone imine* (QI) (*m/z* 188 $[M+1]^+$, 172 $[M^+- CH_3]$, 159 $[M^+- CO]$, 144 $[159-CH_3]$, Rt 9.2) and (2) *2,4-dimethyl-6-ethoxyquinoline* (EQNL) (*m/z* 202 $[M+1]^+$, 173 $[M^+- C_2H_4]$, 144 [173-CHO], Rt 10.1 min) (Fig. 2.1).Therefore, their concentration along with this of the parent compound were determined in all studies as is described below and will be referred as ‘total residues of EQ’. In the absence of commercial analytical standards, the two EQ metabolites were synthesized in the Laboratory of Organic Chemistry, University of Thessaly, Department of Biochemistry and Biotechnology according to the procedure described by Thorisson et al (1992). Their structure was verified by NMR analysis: (a) ^1H NMR spectrum of EQNL: 1.38 (3H, t, *J* 7.5 Hz, CH_2CH_3), 2.39 (3H, s, CH_3), 2.60 (3H,

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s, CH_3), 4.00 (2H, q, *J* 7.5 Hz, -CH_2-), 6.97 (1H, s, C(3) H), 7.02 (1H, d, *J* 3 Hz, C(5) H), 7.35 (1H, dd, *J* 3 and 11 Hz, C(7)H) and 8.01 ppm (1 H, d, *J* 11 Hz, C(8)H) and (b) 1H NMR spectrum of QI:1.40(6H, s, (CH3)2), 1.98 (3H, d, J 2Hz, CH3), 6.32(1H, m, C(3)H), 6.45 (1H , m, C(5) H), 6.62 (1H, dd, J 2.5 and 12.5 Hz, C(7) H) and 7.17 ppm (1H, d , J 12.5 Hz, C(8)H).

Commercial formulation of the pesticides were used in all fortification experiments described below including TECTO® 50 SC (TBZ), FUNGAZIL® 50 EC (IMZ), FRUITGARD® 20 SL (OPP), NO SCALD® 31.8 EC (DPA) and XEDAQUINE® 50 EC (EQ).

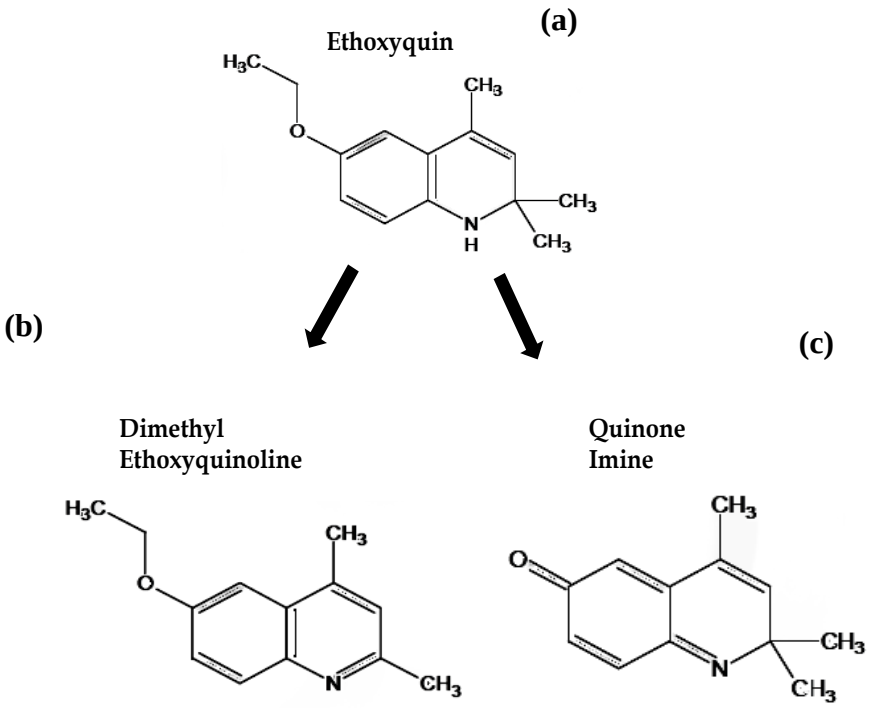

Figure 2.1 The chemical structures of the parent compound ethoxyquin (EQ) (a) and its metabolites dimethyl ethoxyquinoline (EQNL) (b) and quinine imine (QI) (c).

2.2.2. Organic substrates

Anaerobically digested sewage sludge (10 kg) was obtained from the municipal wastewater treatment facility of the city of Larissa, Greece. It is produced after

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anaerobic digestion at mesophilic temperatures (35°C) in anaerobic digesters of continuous flow, allowing complete mixing, and operated at high load rates. The sludge which was digested was collected from the primary settling and mixed with small amounts of sludge from the secondary settling. The sludge produced had a water content of 70% and its properties are shown in Table 2.1. Upon its production it was stored at aerobic conditions in the municipal wastewater treatment plant prior to its collection. Anaerobically digested sewage sludge was partially dried (to 50% of its water holding capacity) and it was sieved to pass through a 3 mm mesh. Liquid aerobic sewage sludge was collected from the secondary settlers of the municipal wastewater treatment facility of the city of Larissa, Greece. The liquid aerobic sewage sludge was used immediately after its collection to avoid prolonged storage which might suppress the elevated metabolic activities of the microbial biomass.

Table 2.1. Physicochemical properties of the substrates used to assess the dissipation and sorption of the pesticides studied.

Substrates	pH	Organic Carbon (%)	Total N (%)	C/N
Soil[a]	7.55	1.05	0.13	8.1
Straw	7.15	79.2	0.80	97.8
SMS	6.83	71.0	1.20	59.2
SMS/Soil (50:50)	7.20	16.9	0.33	51.2
SMS/Straw/Soil (50:25:25)	7.10	29.3	0.30	97.7
Straw/Soil (50:50)	7.40	6.6	0.13	50.8
Straw/SMS/Soil (50:25:25)	7.20	23.5	0.20	117.5
Anaerobically Digested Sewage Sludge	6.95	10.2	2.1	4.8

[a] Soil texture: sand 37%, Clay 31%, Silt 32% (clay loam)

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SMS, soil and straw were mixed in different volumetric ratios to prepare the various organic materials. A soil collected from a farm of the National Agricultural Research Foundation of Greece in Larissa, Greece was used for the preparation of the different organic biomixtures. It was sieved to homogenize (2 mm) and stored at 4^{o}C prior to use. Straw was chopped into small pieces (1-3 cm) and passed through a 4.75 mm sieve. SMS was obtained from a *P. ostreatus* edible mushroom production unit (Mpoulogeorgos-Meteora, Trikala, Greece). It was chopped into small pieces and stored at 4°C until further use. The physicochemical properties of the raw materials (soil, straw and SMS), and of their biomixtures produced, are given in Table 2.1. Total organic C and N content were determined by the wet digestion (Walkley and Black 1934) and the Kjeldahl digestion method (McKenzie 1994) respectively. pH was determined in a mixture of 1:2.5-5 air dried solid substrate:water (w:v). Soil texture was determined with the Bouyoucos hydrometer method (Sheldrick and Wang 1993).

2.2.3. Dissipation of pesticides in anaerobically digested sewage sludge

Anaerobically digested sewage sludge was divided into 5 bulk samples (600 g). These were treated with appropriate amounts of aqueous solutions of the pesticides DPA, OPP, IMZ, TBZ and EQ (2000 mg L^{-1}), prepared by their commercial formulations, aiming to a final concentration of 35 mg kg^{-1} for DPA, IMZ, TBZ, EQ and 45 mg kg^{-1} for OPP. The application of OPP generates much higher wastewater volumes and their disposal is expected to result in higher concentrations in the receiving matrices. Upon treatment with pesticides, the moisture content of the anaerobically digested sewage sludge was adjusted to 60% of its water holding capacity with addition of ddH_2O. Subsequently the bulk samples were mixed by hand to ensure uniform distribution of pesticides and were divided into 27 subsamples of 20 g which were placed in airtight plastic bags. All subsamples were incubated in the dark at 25°C. Immediately after pesticide application, and at regular intervals thereafter, triplicate sub-samples from each treatment were removed from the incubator and stored at -20°C until analyzed by HPLC-UV.

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2.2.4. Dissipation of pesticides in liquid aerobic sewage sludge

Thirty 200-ml samples of liquid aerobic sewage sludge were transferred in 500 ml stoppered glass bottles. Half of the samples were autoclaved at 121°C for 30 min. Triplicate sterilized and non sterilized liquid aerobic sewage sludge samples were treated with TBZ, IMZ, EQ, OPP and DPA to give concentrations of 15 mg L^{-1}. Pesticides were added in the form of aqueous pesticide solutions (2000 mg L^{-1}) prepared by their commercial formulations. Upon pesticide treatments, all sewage sludge samples were briefly agitated to ensure uniform dissolution of the pesticides and were placed in an orbital shaking platform incubator at 100 rpm and 25ºC. Immediately after pesticide application and at regular intervals thereafter subsamples (2 ml) were removed aseptically, extracted with an organic solvent as described below, and analyzed in HPLC-UV.

2.2.5. Dissipation of pesticides in organic substrates

The dissipation of pesticides in different organic biomixtures prepared by mixing of soil, straw and SMS in variable ratios was assessed. For each of the five materials; Soil, Soil+SMS (50:50), SMS+Straw+Soil (50:25:25), Straw+Soil (50:50) and Straw+SMS+Soil (50:25:25) (all volumetric ratios) one bulk sample (1000 g d.w.) was prepared and separated into 27 sub-samples (30 g). These were individually treated with aliquots of aqueous solutions of the pesticides TBZ, IMZ, DPA, EQ and OPP aiming to a final concentration of 35 mg a.i. kg^{-1}d.w for the first four compounds and 45 mg a.i. kg^{-1}d.w for OPP. Those doses were calculated to represent a realistic loading scenario for a biobed system of 30 m^3 which receives in total 22 m^3 of wastewaters containing 10-15 mg L^{-1} of the pesticides studied. Such concentrations have been reported in recycled wastewaters from citrus fruit-packaging plants in Spain (Santiago et al. 2011). Pesticides were evenly mixed into the organic substrates and the moisture content was adjusted to 50% of their water holding capacity. All treatments were incubated in the dark at 25^{o}C for a period of 70 days. Moisture content was maintained by regular additions of deionized water. Immediately after pesticide application and at fixed intervals thereafter sub-samples from each treatment were removed and stored at -20^{o}C until analyzed for pesticide residues.

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2.2.6. Pesticides sorption in organic biomixtures

The sorption of TBZ, IMZ, DPA and OPP in the different organic substrates selected as organic biomixtures was determined using the standard batch equilibrium method according to the OECD guideline 106 (OECD, 2000). Preliminary kinetic studies were employed to determine the most appropriate substrate:solution ratios and equilibration times for all pesticides. Thus, the most appropriate solid substrate:solution ratios to achieve 20-80% sorption of TBZ, IMZ, OPP and DPA were 1:50, 1:100, 1:25 and 1:25 respectively. Equilibrium was achieved at 24 h for TBZ, IMZ and at 8 h for OPP and DPA. The shorter equilibration time for OPP and DPA was selected to avoid losses of those two non-persistent pesticides during the equilibration period. All materials tested were prepared, air-dried and stored at room temperature. Individual stock solutions of each pesticide in acetone (10000 μg ml^{-1}) were prepared. Appropriate amounts of the stock pesticide solutions were dissolved in 0.01M $CaCl_2$ solution leading to the preparation of four pesticide solutions at concentrations of 10, 20, 40 and 80 μg ml^{-1}. The only exception was OPP for which the concentration levels of the four solutions were 20, 40, 80 and 100 μg ml^{-1} considering the higher exposure expected in biobed systems for this molecule. Triplicate samples (1 to 2 g) were mixed with 50 or 100 ml of each of the above solutions in screw-cupped vials and shaken overnight on an orbital shaker (200 rpm) at room temperature. When equilibrium was reached, samples were centrifuged at 4500 rpm for 10 min and the supernatant was collected, extracted and analyzed by HPLC-UV as is described below.

2.2.7. Pesticides residue analysis

2.2.7.1. Pesticides extraction from liquid substrates

Extraction of TBZ, IMZ, EQ and its metabolites QI and EQNL was performed by mixing 2 ml from liquid aerobic sewage sludge with 8 ml of methanol in 20-ml screw glass vials. The mixture was shaken vigorously by vortex for a minute and the extract was passed through a 0.45μm syringe filter (PTFE Syringe Filter). The filtrate was collected in a glass tube and stored at -20°C until analyzed. Regarding extraction of OPP and DPA the same extraction procedure as above was followed with the only exception that acetonitrile instead of methanol was used.

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2.2.7.2. Pesticides extraction from solid substrates

Regarding TBZ extraction, 10 ml of methanol were mixed with 5 g of solid substrate in a conical flask. The mixture was shaken for an hour in an orbital shaker (200 rpm), centrifuged for 5 min at 11000 rpm and the clear supernatant was recovered. The remaining soil was re-extracted with further 10 ml of methanol and the clear supernatant from the two extraction cycles were combined. The extract was passed through a 0.45μm syringe filter (PTFE Syringe Filter) and kept at -20°C until analyzed. For the extraction of IMZ from the organic substrates, 5 g of substrate were mixed with 1 ml of NaOH 1N and 10 ml of methanol. Samples were shaken for 30 min and centrifuged at 11000 rpm for 5 min, the supernatant was collected in a glass bottle, and the soil was re-extracted with another 10 ml of methanol. After 30 min shaking and centrifugation, the clear supernatant from the two extraction cycles were combined and stored at -20°C. For the extraction of OPP and DPA, 10 g of soil were mixed with 25 ml of acetonitrile. The mixture was agitated for 1.5 hours in an orbital shaker at 200 rpm and then centrifuged at 11000 rpm for 5 minutes. The supernatant was collected and stored at -20°C.

The extraction of EQ and its metabolites was performed according to the original buffered QuEChERS method, slightly modified (Anastassiades et al., 2003). Due to the general instability of EQ, special care was taken during the extraction to minimize transformation of EQ to its oxidation derivatives. Thus, all extractions were conducted into a dark cold-room at 4°C. Briefly, 5 g of solid substrate were mixed in a teflon tube with 5 ml of cold ddH_2O and were agitated manually for a minute. Subsequently, 10 ml of acetonitrile were added and the mixture was vortexed for 1 min. The samples were subsequently amended with a mixture of salts (4g $MgSO_4$, 1g NaCl and 1.5g $C_6H_5Na_3O_7 \cdot 2\ H_2O$), vortexed for 1 min and centrifuged for 5 min at 7500 rpm. Then 1 ml of the clear supernatant was mixed with 150 mg $MgSO_4$ and 25 mg PSA and the mixture was vortexed for 30sec and centrifuged for 1 min at 3000 rpm. The final extracts for all pesticides were filtered through a syringe filter 0.45μm (PTFE Syringe Filter) and analyzed by HPLC.

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2.2.7.3 HPLC analysis

Pesticide residues were analyzed in a Marathon III (LabAlliance, USA) HPLC - UV system equipped with a Grace Smart RP C18 (150 mm x 4.6 mm). TBZ and OPP residues were detected at 254 nm using a mobile phase of acetonitrile/water/25% NH_3 solution (by volume) with different elution strength (39/60.5/0.5 and 55:44.5:0.5 respectively). Under these conditions, the retention time (Rt) of TBZ and OPP were 3.3 and 3.4 min respectively. IMZ residues were detected at 204 nm using a mobile phase of 80:20 methanol: NH_3 solution 0.25% (by volume). Under those conditions the Rt of IMZ was 5 min. DPA residues were determined at 210 nm using a mobile phase of 60:30:10 acetonitrile:water:methanol (by volume) with a Rt of 3.5 min. Residues of EQ and its metabolites were determine at 254 nm using a mobile phase of 69:30:1 acetonitrile:water:NH_3 (by volume). Under these chromatographic conditions EQ, QI and EQNL were eluted at Rt of 5.7, 4.1 and 5.4 min respectively. In all cases a flow rate of 1 ml min^{-1} was used.

2.2.7.4. Analytical methods validation

Analysis of fortified samples was conducted to verify the extraction efficiency of the methods described above. Recovery tests were performed in soil and organic substrates (1, 5 and 50 mg Kg^{-1} dw), anaerobically digested sewage sludge (0.5. 5 and 20 mg kg^{-1} dw) and liquid substrates (0.1, 1 and 10 mg L^{-1}) at three concentration levels. Triplicates for each concentration level were processed. The mean percentage recovery of TBZ, IMZ and OPP in soil and organic substrates were 80.8%, 94.0% and 82.7%, respectively, (CV $\leq$ 5.0%), while the recoveries of DPA, EQ, QI, and EQNL were 83.2%, 84.7%, 101.5 and 92.8%, respectively (CV $\leq$ 6.3%). Similarly average recoveries of TBZ, IMZ, OPP, DPA, EQ, QI and EQNL in anaerobically digested sewage sludge and liquid aerobic sewage sludge ranged from 83.6% to 102.0% and 81.9% to 94.2% respectively. The limit of detection ranged from 0.001 μg mL^{-1} for IMZ to 0.007 for EQNL, while the limit of quantification was $\geq$ 0.05 mg Kg^{-1} substrate dw for all pesticides in the solid media tested and $\geq$ 0.05 mg L^{-1} for all pesticides in the liquid media.

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2.2.8. Pesticides dissipation kinetics

The four kinetic models proposed by the FOCUS workgroup on pesticide degradation kinetics (FOCUS 2006) were used for fitting the dissipation data. The single first order (SFO) kinetic model and three biphasic models: hockey-stick (HS), first order multi-compartment model (FOMC) and the double first order in parallel (DFOP) model were used. SFO is based on the assumption that the change in a given pesticide concentration with time (dC/dt) is directly proportional to the actual concentration of the pesticide at this time. HS involves two sequential first-order degradation phases with different rates (k_1 and k_2) and having a break point between them (t_b). DFOP describes degradation by a sum of two normal first-order degradation phases each in a different part of the soil compartment, while FOMC is based on the division of soil into a number of subcompartments each with a different first-order degradation rate. The mathematical equations describing the kinetics models used are shown in Table 2.2. The χ^2 test as well as visual inspection and the distribution of the residuals were used as criteria to assess the agreement between calculated and observed data for a given fit. The χ^2 was calculated with the following equation:

$$x^2 = \sum \frac{(C - O)^2}{(\frac{err}{100} \cdot \bar{O})^2}$$

where C is the calculated value, O is the observed value, $\tilde{O}$ the mean of observed values and err the measurement error. Parameters of the kinetic models and their standard errors were obtained by least square non-linear regression analysis using the statistical program R.

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Table 2.2. The mathematical equations of the kinetic models used to describe the dissipation of the pesticides in the different treatments

Kinetic Model	**Mathematic equation**	**DT_{50}**	**DT_{90}**
Single first order (SFO)	$C = C_0 e^{-kt}$	$DT_{50} = ln2/K$	$DT_{90} = ln10/K$
Hockey Stick (HS)	$C = C_0 e^{-K1t}$ *for* $t \leq t_b$	$DT_{50} = ln2/K_1$	$DT_{90} = ln10/K_1$
	$C = C_0 e^{-k1t} e^{-k2(t-tb)}$ *for* $t > t_b$	$DT_{50} = t_b + (ln2 - k_1 t_b)/k_2$	$DT_{90} = t_b + (ln10 - k_1 t_b)/k_2$
First order multi-compartment (FOMC)	$C = C_0/(t/\beta + 1)^{\alpha}$	$DT_{50} = \beta(2^{1/\alpha} - 1)$	$DT_{90} = \beta(10^{1/\alpha} - 1)$
Double first order in parallel (DFOP)	$C = C_0(ge^{-k_1 t} + (1-g)e^{-k_2 t})$	*Iterative method*	*Iterative method*

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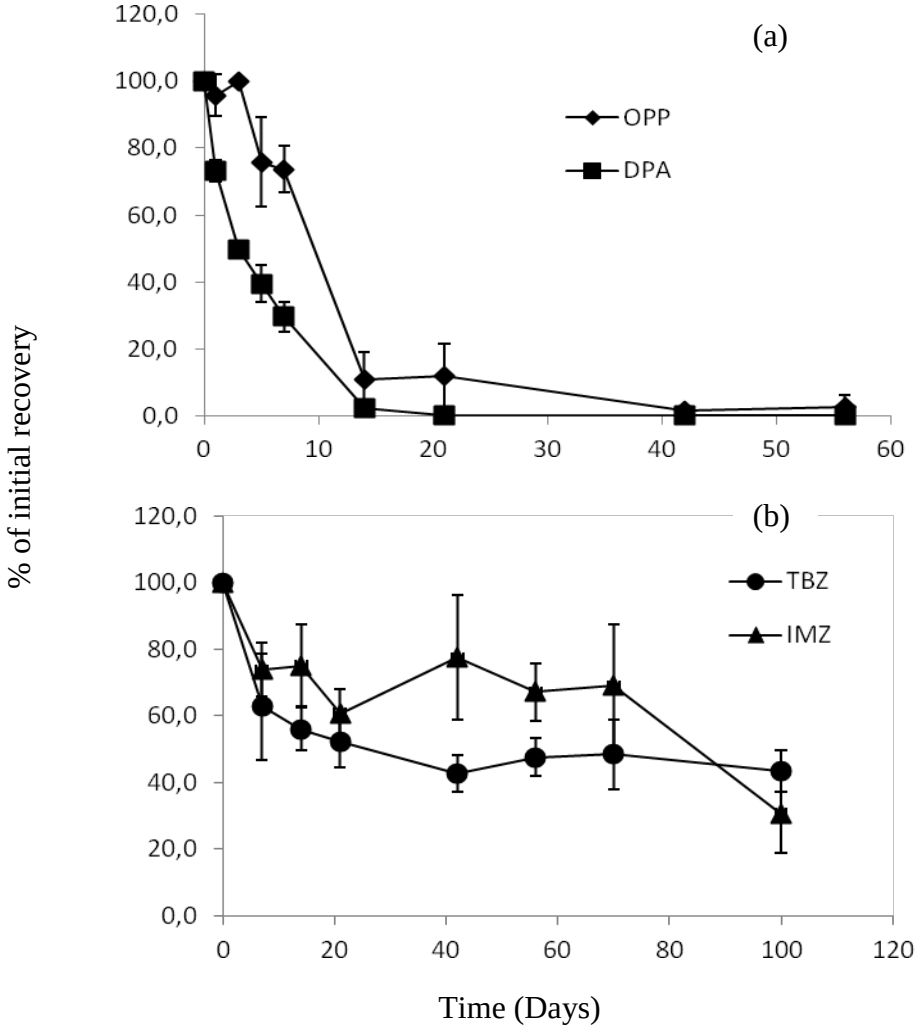
2.3. RESULTS

2.3.1. The dissipation of pesticides in anaerobically digested sewage sludge

The dissipation patterns of OPP, DPA, TBZ, IMZ and of the total residues of EQ are shown in Fig 2.2. A rapid dissipation of OPP and DPA was evident in anaerobically digested sewage sludge with DT_{50s} of 9.3 and 3.6 days respectively (Fig 2a, Table 2.3). Similarly, a rapid dissipation of the total residues of EQ was observed with DT_{50} of < 1 day. From the two EQ metabolites, EQNL was formed in low amounts but constituted the only detectable residue from 7 days onwards, while only trace amounts of QI were detected (Fig. 2.2c). In contrast, TBZ and IMZ showed moderately to high persistence with DT_{50s} of 32.3 and 108.3 days respectively (Fig. 2.2b, Table 2.3).



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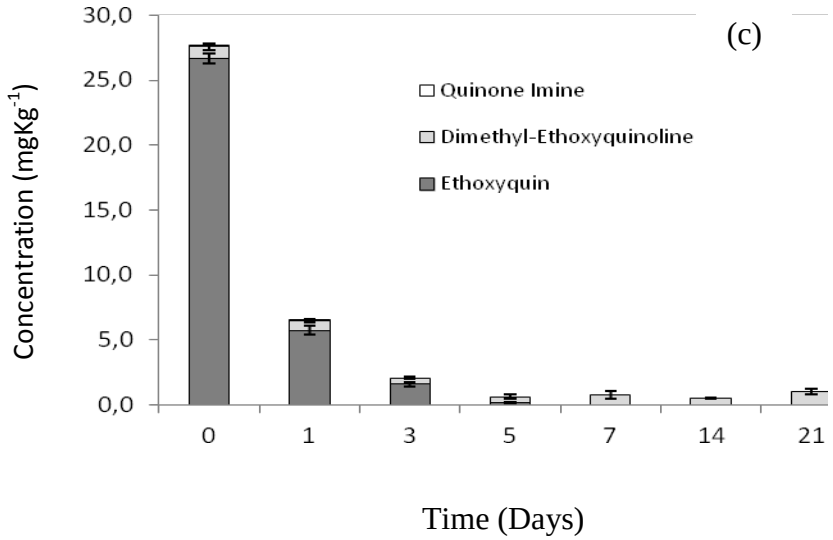

Figure 2.2. Dissipation of *ortho*-phenylphenol (OPP) and diphenylamine (DPA) (a), thiabendazole (TBZ) and imazalil (IMZ) (b) and ethoxyquin (EQ) (c) in anaerobically digested sewage sludge. Each value is the mean of three replicates with error bars representing the standard deviation of the mean

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Table 2.3. The half-life values (DT_{50s}) of *ortho*-phenylphenol (OPP), diphenylamine (DPA), imazalil (IMZ), thiabendazole (TBZ) and total residues of ethoxyquin (EQ) in anaerobically digested sewage sludge and in liquid aerobic sewage sludge.

Pesticides	Anaerobically digested sewage sludge			Liquid aerobic sewage sludge					
				Non Sterilized			Sterilized		
	DT_{50} (d)	χ^2 (%)	Model[a]	DT_{50} (d)	χ^2 (%)	Model	DT_{50} (d)	χ^2 (%)	Model
OPP	9.3	16.0	HS	1.3	38.8	SFO	36.0	3.3	SFO
DPA	3.6	9.5	SFO	1.5	5.13	SFO	28.7	5.3	SFO
IMZ	108.3	13.4	SFO	257.6	2.81	SFO	942.0	3.3	SFO
TBZ	32.3	3.6	FOMC	76.9	10.8	SFO	208.7	5.8	SFO
EQ+QI+EQNL[b]	0.46	8.0	SFO	0.18	0.001	SFO	4.7	5.6	SFO

[a] SFO: Single First Order; HS: Hockey-Stick; FOMC: First Order Multi-Compartment

[b] Calculations were made with the sum of residues of ethoxyquin (EQ), quinone imine (QI) and dimethyl ethoxyquinoline (EQNL)

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2.3.2. The dissipation of pesticides in liquid aerobic sewage sludge

The dissipation patterns of the five pesticides in sterilized and non-sterilized liquid aerobic sewage sludge are shown in Figure 3. A rapid dissipation of OPP, DPA and total residues of EQ was evident with DT_{50s} of 1.3, 1.5 and <1 d respectively (Table 2.3, Fig. 2.3a and 2.3c). Regarding the metabolism of EQ, no residues of the parent compound were detected from 3 days onwards with QI being the major component of the total residues at day 1, whereas EQNL became the major component from day 3 onwards (Fig. 2.3c). In contrast, a slow dissipation of TBZ and IMZ was observed with DT_{50s} of 76.9 and 257.6 d (extrapolated with the single first order kinetic model) respectively (Fig. 2.3b). Sterilization of liquid aerobic sewage sludge significantly inhibited the dissipation of all pesticides. This is clearly illustrated with EQ which remained the main component of the total residues of EQ for the first 7 days of the incubation. The two metabolites, QI and EQNL, became the major components of the total residues from day 14 onwards (Fig. 2.3d).

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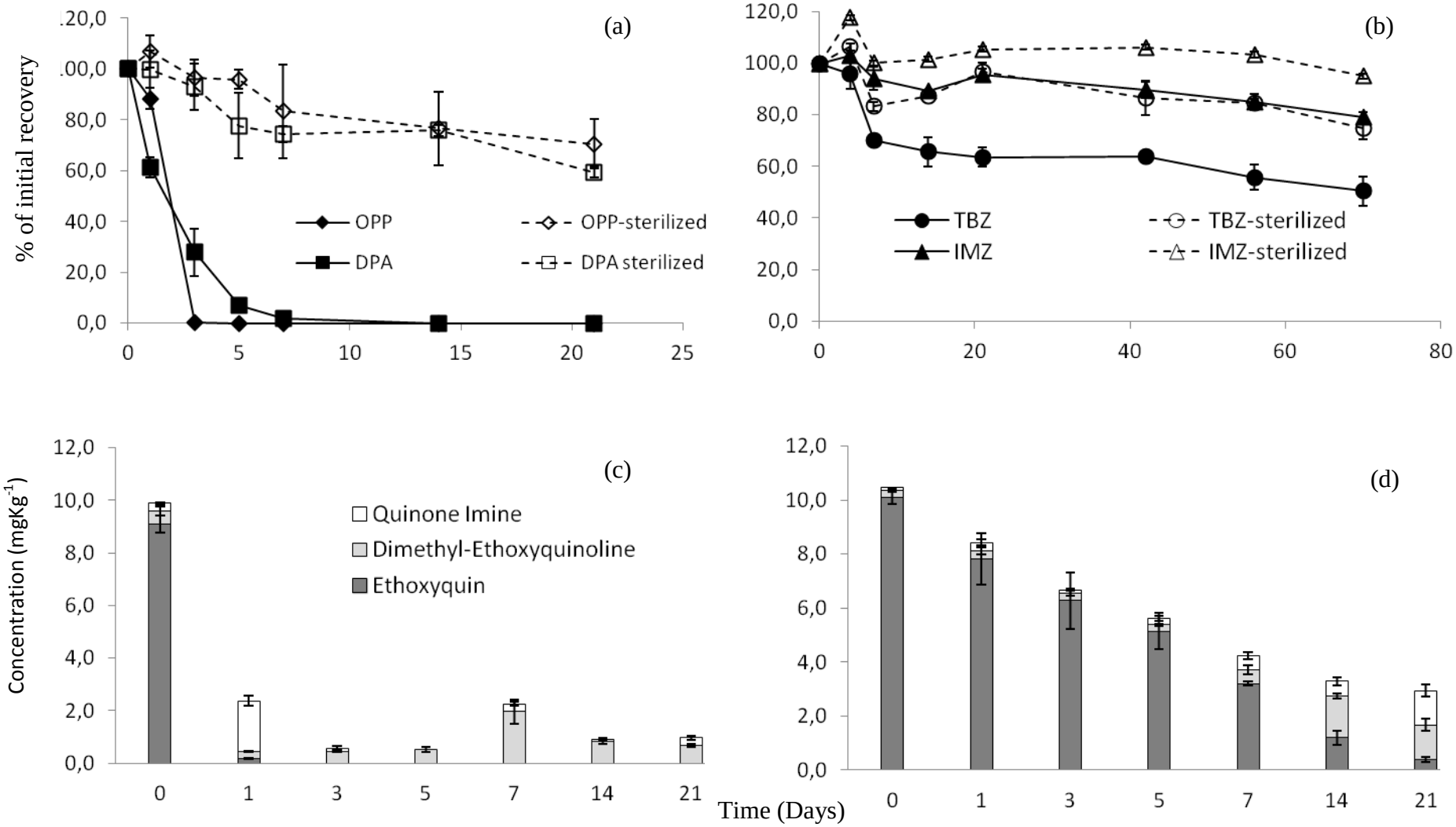

Figure 2.3. Dissipation of *ortho*-phenylphenol (OPP) and diphenylamine (DPA) (a), thiabendazole (TBZ) and imazalil (IMZ) (b) and dissipation and metabolism of ethoxyquin (EQ) and its metabolites QI and EQNL by non-sterilized (c) or sterilized liquid aerobic sewage sludge (d). Each value is the mean of three replicates with error bars representing the standard deviation of the mean

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2.3.3. The dissipation of pesticides in biobeds packing materials

The dissipation of OPP and DPA in the different substrates was very rapid within the first 7 days precluding the calculation of realistic DT_{50} values (Fig. 2.4a and 2.4c). Thus their dissipation was re-determined in an identical follow up experiment with focus on the first 72 h after application. The results indicated differences in the dissipation rates of the two compounds in the different substrates tested (Fig. 2.4b and 2.4d). For both compounds the higher dissipation efficiency was evident in SMS/Straw/Soil (50:25:25) with DT_{50s} of 0.34 and 1 d for OPP and DPA respectively. On the other hand, the slowest dissipation rates for those two compounds were observed in soil with DT_{50s} > 4 d (Table 2.4).

In accordance with the dissipation studies in sewage sludge, TBZ and IMZ were again the most persistent chemicals (Fig. 2.4e and 2.4f). For both pesticides the most rapid dissipation was evident in the substrates where SMS was the major component (SMS/Soil and SMS/Straw/Soil) (50% by volume) with DT_{50s} of 20-29 d for IMZ and 22.4 - 28.3 d for TBZ (Table 2.4). In contrast, the slowest dissipation for those compounds was observed in soil and Straw/Soil (50:50) for IMZ (DT_{50} = 79.3 d and 58.3 d) and in Straw/Soil (50:50) for TBZ (DT_{50} = 236 days).

The dissipation of EQ and its metabolites was very rapid within the first 7 days after its application with QI being the major component of the total residues of EQ even at 0 days (Fig. 2.5). This could be attributed to the very rapid transformation of EQ to its metabolites during the 4-h interval between pesticide application and collection and storage of T0 samples. To get a more focused view of the dissipation and metabolism kinetics of EQ, a follow up study was undertaken to measure the dissipation of EQ and its metabolites during the first 24 h after application. The slowest dissipation of EQ and its metabolites was evident in soil (DT_{50} = 2.7 d), where the parent compound was immediately transformed to QI which constituted the major component of the total residues at 24 h (Fig. 2.5a). In contrast in the other substrates tested, EQ was more gradually transformed to QI (Fig. 2.5b to 2.5e) with DT_{50s} of less than 0.6 days.

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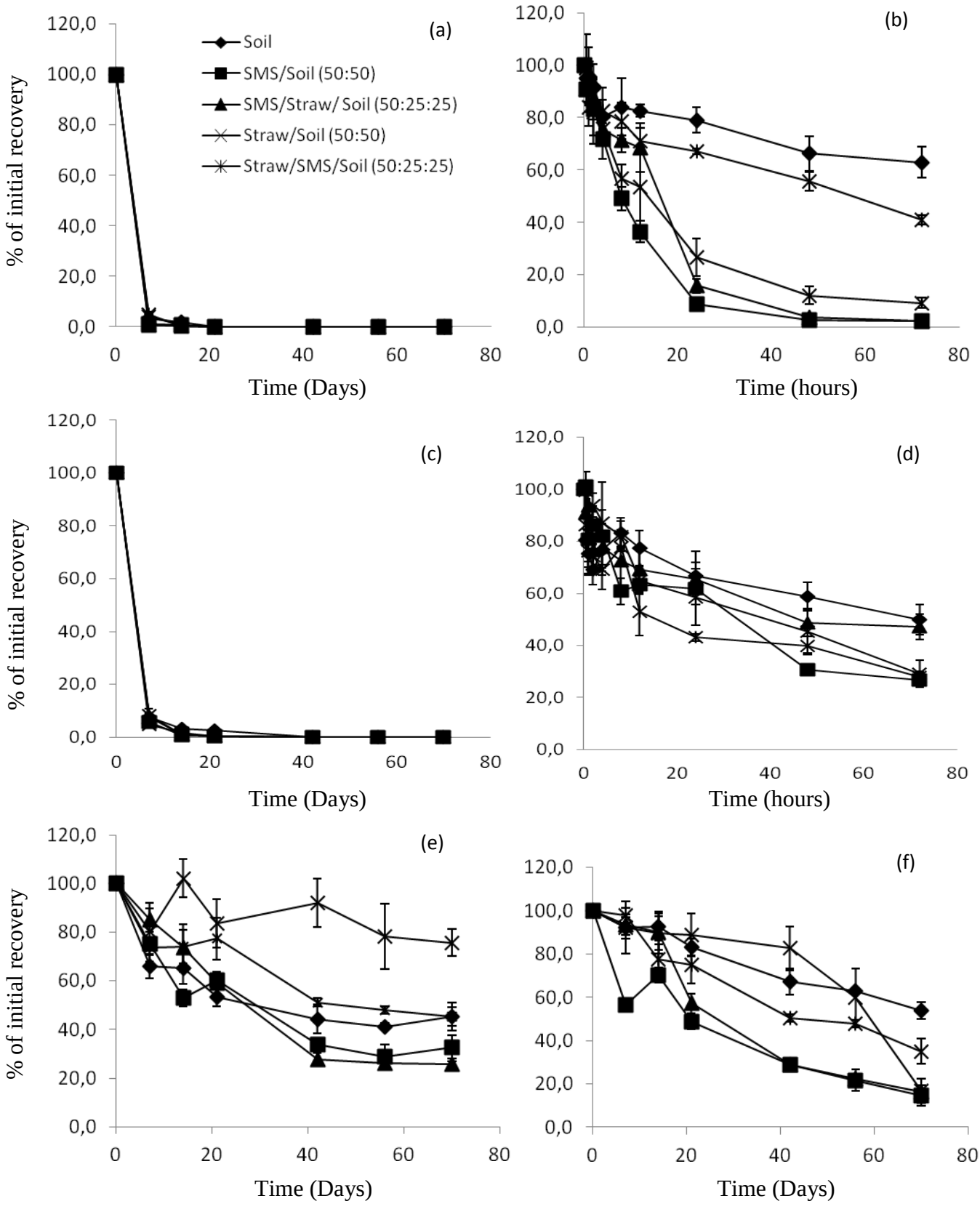

Figure 2.4. Dissipation patterns of *ortho*-phenylphenol (OPP) (a & b) diphenylamine (DPA) (c & d) within 70 days or 72 hours after their application in different organic substrates. The dissipation patterns of thiabendazole (TBZ) (e) and imazalil (IMZ) (f) in the same organic substrates are also shown. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

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Table 2.4. The half-life values (DT_{50s}) of *ortho*-phenylphenol (OPP), diphenylamine (DPA), imazalil (IMZ), thiabendazole (TBZ) and total residues of ethoxyquin (EQ) in soil and other organic substrates as they were estimated by fitting the best - fitted kinetic model.

Pesticides	Soil			SMS/Soil (50:50)			SMS/Straw/Soil (50:25:25)			Straw/Soil (50:50)			Straw/SMS/Soil (50:25:25)		
	DT50 (d)	χ^2 (%)	Model[a]	DT50 (d)	χ^2 (%)	Model	DT50 (d)	χ^2 (%)	Model	DT50 (d)	χ^2 (%)	Model	DT50 (d)	χ^2 (%)	Model
OPP	4.65	4.98	HS	0.57	9.48	SFO	0.34	3.16	SFO	2.5	3.46	FOMC	0.56	5.17	FOMC
DPA	4.08	8.11	SFO	3.16	4.52	FOMC	1.01	8.43	FOMC	1.04	8.92	FOMC	1.46	5.57	FOMC
IMZ	79.3	2.02	SFO	19.9	12.26	HS	28.6	8.89	HS	58.3	1.97	HS	46.0	4.32	SFO
TBZ	31.7	4.49	FOMC	22.4	7.81	FOMC	28.3	6.14	SFO	236.5	7.61	FOMC	54.8	6.04	HS
EQ+QI+EQNL[b]	2.7	7.4	SFO	0.6	10.7	SFO	0.2	4.4	DFOP	0.1	6.9	HS	0.1	12.6	HS

[a] SFO: Single First Order; HS: Hockey-Stick; FOMC: First Order Multi-Compartment

[b] Calculations were made with the sum of residues of ethoxyquin, quinone imine (QI) and dimethyl ethoxyquinoline (EQNL)

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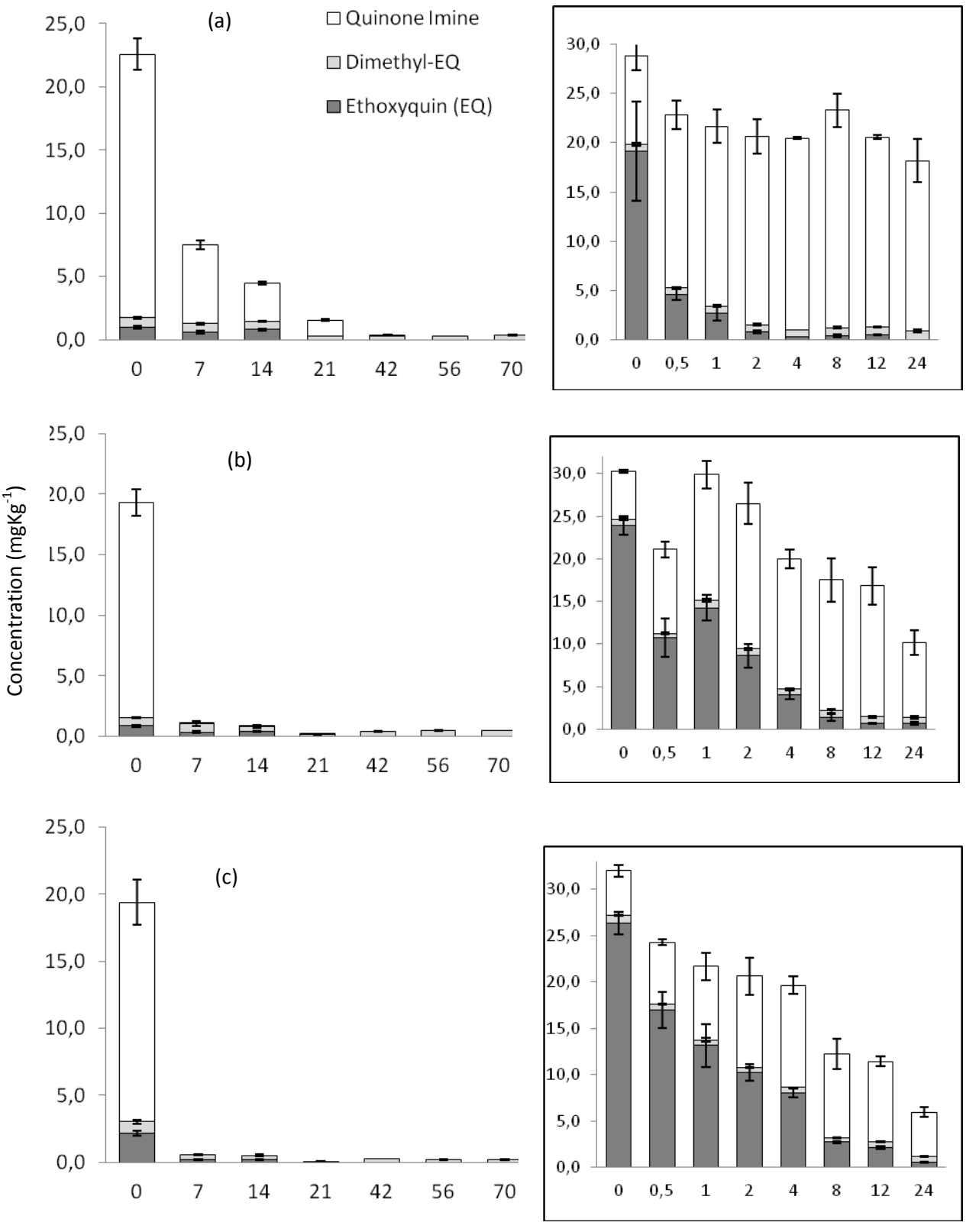

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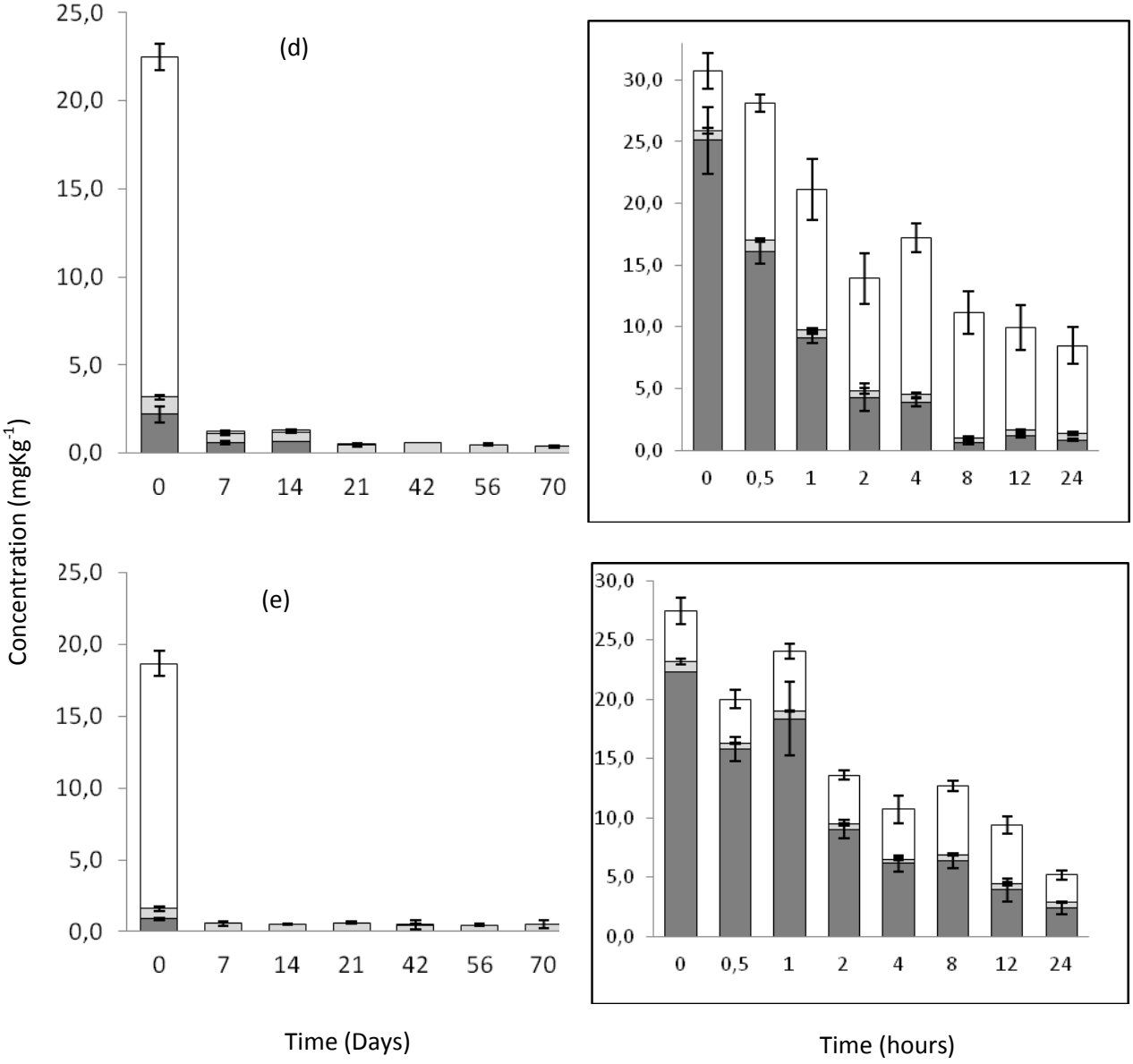

Figure 2.5. Dissipation and metabolism of ethoxyquin (EQ) at different time frames, 70 days (left) and 24 hours (right), after their laboratory application into soil (a) and into various organic substrates like SMS/Soil (50:50) (b), SMS/Straw/Soil (50:25:25) (c), Straw/Soil (50:50) (d) and Straw/SMS/Soil (50:25:25) (e) (all ratios are by volume). Each value is the mean of three replicates with error bars representing the standard deviation of the mean

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2.3.4. Pesticides sorption onto biobeds packing materials

The pesticide sorption isotherms are shown in Fig 2.6. Pesticides sorption in all cases was well described by the Freundlich equation which was used for calculation of the sorption parameters (K_f, N) (Table 2.4). The Freundlich equation is given by the following formula:

$$Cs = K_f C_w^{\ N}$$

where K_f is the Freundlich sorption coefficient and N is the Freundlich exponent.

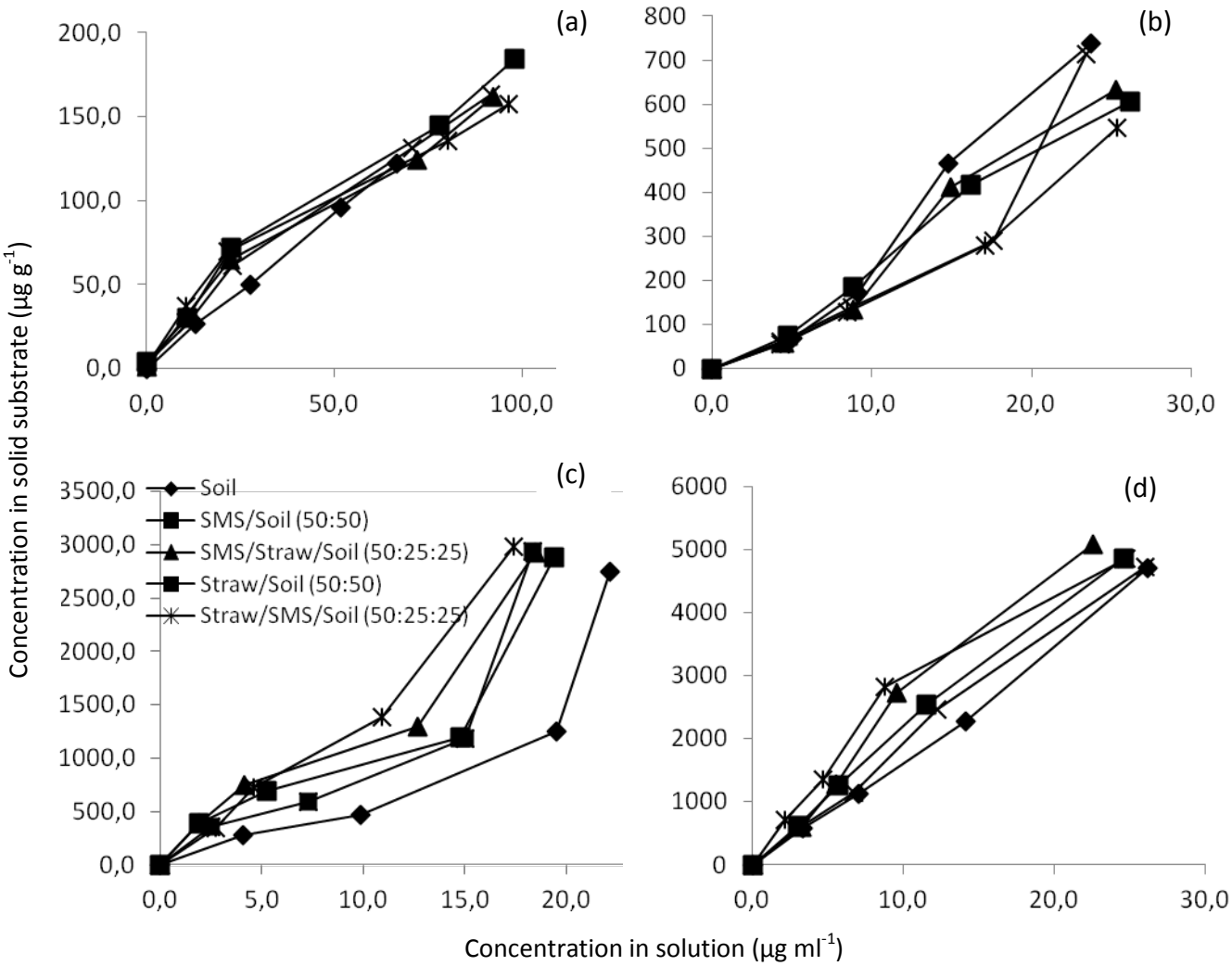

Figure 2.6. Sorption isotherms of *ortho*-phenylphenol (OPP) (a), diphenylamine (DPA) (b), thiabendazole (TBZ) (c) and imazalil (IMZ) (d) in soil and in various organic substrates. Each value is the mean of three replicates.

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OPP and DPA showed weak sorption with the lowest K_f values observed in soil (2.47 and 5.57 g ml^{-1}) and the highest in Straw/SMS/Soil (50:25:25) (30.3 and 12.02 g ml^{-1}) respectively. IMZ and TBZ showed higher sorption affinity in the organic substrates which were characterized by higher organic matter content compared to soil where, again the lowest K_f values for both pesticides were measured (47.2 and 83.4 g ml^{-1} respectively). In agreement with OPP and DPA, the highest sorption of IMZ was seen in Straw/SMS/Soil (50:25:25) (Table 2.5). When K_f was normalized for the organic carbon content of each substrate K_{foc} values increased in the order OPP<DPA<TBZ<IMZ (Table 2.5.)

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Table 2.5. Sorption parameters K_f (g ml^{-1}), K_{foc} (g ml^{-1}) and N for the pesticides *ortho*-phenylphenol (OPP), diphenylamine (DPA), imazalil (IMZ) and thiabendazole (TBZ) in soil and organic substrates used in the study

Pesticides	Soil			SMS/Soil (50:50)			SMS/Straw/Soil (50:25:25)			Straw/Soil (50:50)			Straw/SMS/Soil (50:25:25)		
	K_f	K_{foc}[a]	N	K_f	K_{foc}	N	K_f	K_{foc}	N	K_f	K_{foc}	N	K_f	K_{foc}	N
OPP	2.47	235.2	1.065	5.01	29.6	1.195	11.67	39.8	1.374	8.01	120.0	1.273	30.3	128.9	1.573
DPA	5.57	530.5	1.574	11.57	68.5	1.245	6.37	21.7	1.456	7.82	117.9	1.353	12.02	51.1	1.245
TBZ	47.2	4965.7	1.141	217.2	1285.2	0.755	226.8	774.1	0.800	128.4	1936.7	0.926	120.3	511.9	1.091
IMZ	83.4	7942.9	1.013	222.3	1315.4	0.976	186.2	635.5	1.100	183.6	2769.2	1.005	412.4	1754.9	0.798

[a] K_{foc} was calculated by the following equation $K_{foc} = K_f / f_{oc}$, where K_f is the Freundlich sorption coefficient and f_{oc} is the fraction of organic carbon

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2.4. DISCUSSION

2.4.1. Pesticides dissipation in sewage sludge

We initially investigated the dissipation of the pesticides contained in the wastewaters from the fruit-packaging industry (a) in anaerobically digested sewage sludge, a by-product of municipal wastewater treatment systems which is increasingly used as soil amendment in agriculture (del la Herras et al. 2005) and (b) in liquid aerobic sewage sludge, which constitutes the metabolically active biomass found in the biological treatment systems of municipal wastewater treatment plants. Both substrates were efficient in rapidly dissipating OPP, DPA, EQ and its metabolites (QI and EQNL) with consistently faster dissipation observed in the liquid aerobic sewage sludge. It should be noted that the liquid aerobic sewage sludge used in the current study was not acclimated with the tested substances in contrast to several previous studies which found that an acclimation period was essential to achieve effective removal of other pesticides (Gonzalez et al. 2006). Our findings are in agreement with previous studies which reported a DT_{50} of 1.4 days for DPA in a bioreactor (Christodoulatos et al. 1997) and a rapid metabolism of DPA in sewage sludge with formation of aniline, imine and 4-hydroxy-DPA (Gardner et al 1992). Similarly previous studies in a municipal wastewater treatment plant in Germany reported the complete removal of OPP, although the metabolic pathway of OPP was not shown (Korner et al. 2000). Regarding EQ the only other study that have explored its behavior in biological wastewater treatment systems showed that it is largely recalcitrant at both anaerobic and aerobic conditions and can induce inhibitory effects on the methanotrophic microbial community at 300 mg L^{-1}(Shah et al. 2005), which are well above the levels tested in our study.

EQ showed a slightly different transformation patterns in the anaerobically digested sewage sludge compared to the liquid aerobic sewage sludge. This might reflect the different microbial communities in the two substrates: Liquid aerobic sewage sludge is expected to be dominated by microorganisms accustomed to high metabolic activities and oxidative degradation of organic matter like proteobateria and firmicutes (Yang et al. 2011) compared to anaerobically digested sewage sludge where anaerobic digestion has drastically altered the microbial community (Pascual et al. 2008). Considering that the main use of EQ is as preservative of fish meal and fruits (indoor uses) the vast majority of studies have looked into its metabolism in animal and plant tissues and a range of metabolites detected including QI, dimeric

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EQ, methyl-EQ, EQNL, dihydro-EQ and demethyl-EQ (Gupta and Buddis 2005; JMPR 2005). On the other hand there is lack of knowledge regarding its metabolism in municipal wastewater treatment plants and environmental compartments. Although QI and EQNL were detected in our study, no residues of the other metabolites reported above were observed. Our study provides first evidence for the metabolism of EQ and its oxidation products in liquid aerobic sewage sludge.

Both types of sewage sludge showed a limited capacity to dissipate TBZ and IMZ. This is in agreement with previous findings which showed that municipal wastewater treatment plants acted as point sources for the contamination of surface water bodies (Campo et al. 2013; Masia et al. 2013). However, the possibility that higher dissipation efficiency for TBZ and IMZ could be achieved through acclimation of the liquid aerobic sewage sludge cannot be ruled out and should be tested in future studies. During our study no metabolites of TBZ and IMZ were measured. The metabolism of these two fungicides in sewage sludge is largely unknown. Recent studies showed that TBZ could be transformed via oxidation (Fenton/ PhotoFenton process) to OH-TBZ, thiazole-4-carboxamidine and other derivatives produced upon fusion of the benzyl ring (Sanchez Perez et al. 2014; Sirtori et al. 2014).

Sterilization of liquid aerobic sewage sludge resulted in drastic inhibition of pesticides dissipation stressing the microbial nature of the decay observed. It should be noted that EQ oxidation to QI and EQNL was also hampered in the sterilized liquid aerobic sewage sludge suggesting that these transformation steps are mostly biologically-driven, an information which was largely unknown. Overall our data suggest that the direct discharge of wastewaters from fruit-packaging industry onto municipal wastewater treatment plants are expected to effectively remove DPA, OPP, EQ and its derivatives but not the persistent fungicides TBZ and IMZ which entail a risk for the ecological integrity of receiving ecosystems.

2.4.2. Pesticides dissipation in biobeds packing materials

The recalcitrance of TBZ and IMZ in sewage sludge suggests that other treatment methods should be applied to effectively eliminate those fungicides from those agro-industrial effluents. Previous studies by Omirou et al. (2012) showed that biobeds could be an efficient method for the depuration of wastewaters from the citrus fruit-packaging plants. To further exploit this, we investigated not only the dissipation of

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TBZ, IMZ, OPP, previously studied by Omirou et al. (2012), but also of preservatives like DPA and EQ which are used in packaging plants of pears and apples. In addition we introduced SMS as a potentially effective organic substrate to ameliorate the depuration efficiency of biobed systems receiving wastewaters from the fruit packaging industry.

Overall, the persistence of pesticides in soil and in the different organic biomixtures increased in the following order EQ<DPA<OPP<TBZ=IMZ in accordance with the sewage sludge dissipation patterns. The long persistence of IMZ (US EPA 2003; Kreuzig et al. 2010) and TBZ (Kesavan et al 1976; EC 2013) in soil is well documented. Regarding organic biomixtures, our results are in agreement with Omirou et al (2012) who identified OPP and IMZ as the least and most persistent chemicals respectively, in different organic biomixtures derived from by-products of the winery and olive oil agro-industries compared to our SMS-rich biomixtures. No possible metabolites of TBZ, IMZ, OPP and DPA were found in the substrates tested although a more sensitive and high-resolution analytical approach is needed to verify this. Little is known regarding the metabolism of those fungicides in soil and they are mostly coming from regulatory documents. TBZ dissipation in soil was followed by formation of negligible amounts of benzimidazole and 5-OH-TBZ (EC 2013), while for IMZ its main metabolite was IMZ-ethanol which was detected at low amounts (EFSA 2010a).

Organic biomixtures showed a higher dissipation capacity compared to soil for all pesticides tested. Substrates with the highest % of SMS such as SMS/Straw/Soil (50:25:25) and SMS/Soil (50:50) showed the highest dissipation potential for all pesticides tested. In particular, the DT_{50s} obtained for TBZ and IMZ in those organic biomixtures were amongst the lowest ever reported verifying the enhanced dissipation efficiency of those organic materials (Kesavan et al. 1976; Kreuzig et al. 2010; Omirou et al. 2012). Our findings are in accordance with the positive correlation between % of SMS in biobed substrates and pesticide biodegradation observed by Karanasios et al (2010a). This substrate is generally rich in complex and partly degraded organic C macromolecules (cellulose, hemicellulose, lignin) and N substrates which could support the growth of a particularly active microbial community able to degrade pesticides (Marin-Benito et al. 2009). The contribution of *P. ostreatus* by the SMS on the higher dissipation capacity of the SMS-augmented materials is not clear. Previous studies have shown that the role of this fungus on the

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degradation of pesticides in similar organic biomixtures is negligible (Karanasios et al. 2010b) and its mycelium is progressively surpassed by other fast-growing microorganisms when mixed with soil (Tuomela et al. 2002). The significant role of white rot fungi like *P. ostreatus* on the degradation of pesticides is mostly documented in peat-based organic biomixtures where their survival and activity is favored by the acidic pH of those materials (Castillo et al. 2008). In contrast the neutral to alkaline pH of the organic biomixture used in our study are not expected to favor their survival. The indirect role of *P. ostreatus* in partly degrading and modifying the properties of the raw mushroom substrate leading to an optimized co-substrate for pesticide degradation may be critical for the observed SMS performance. Overall, the beneficial effect of SMS on the dissipation efficiency of biobeds packing material provides an option to the mushroom units in the Mediterranean basin for the sustainable, environmental-friendly and effective exploitation of this waste.

The inclusion of high proportions of straw (50%) in the organic substrates substantially increased the persistence of most pesticides with TBZ showing the most prominent increase. Despite the well documented beneficial effect of straw on pesticides dissipation in biobed systems (Castillo et al. 2008), its high organic C content could enhance the sorption affinity of lipophilic substances like TBZ and IMZ resulting in higher persistence due to reduced bioavailability. The higher K_f values reported for straw-rich packaging materials in our sorption isotherm results are in line with this.

EQ showed similar metabolic patterns in soil and organic substrates with rapid conversion to QI, which constituted the major component of the total residues of EQ 24 h after application. On the other hand EQNL was a minor metabolite, which was formed at low amounts but persisted until 70 days post application. This metabolic pattern deviates from the metabolic pattern observed in anaerobically digested sewage sludge. This could be attributed to the different composition of those materials which are expected to support microbial communities with different metabolic capacities: anaerobically digested sewage sludge is mostly composed of hydrocarbons, amino acids and lipids compared to biobed materials which are mostly composed of cellulose, hemicellulose and lignin (Rodriguez - Cruz et al. 2012) and may favor aerobic oxidation processes. This is the first study providing data for the fate and the transformation of EQ in soil and biobed packing material.

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2.4.3. Pesticides sorption onto biobeds packing materials

Sorption of pesticides in soil and organic biomixtures provided explanations for the dissipation patterns observed. In particular, OPP and DPA showed a weak sorption affinity in agreement with previous studies which also showed moderate sorption for those pesticides with soil K_{foc} values of 894-1793 ml g^{-1} (Zheng et al., 2011) and 1212-6593 ml g^{-1} (US EPA 1998) respectively. On the other hand, TBZ and IMZ were strongly sorbed onto soil and organic biomixtures which is in accordance with previous soil sorption studies with K_{foc} values of 4059 (Kreuzig et al. 2010) to 4357 ml $g^{-1}$for IMZ (EFSA 2010a) and of 1104 to 22467 ml g^{-1} for TBZ (EC 2001). The strong sorption of IMZ and TBZ combined with their limited biodegradability explain the general recalcitrance of those chemicals. Omirou et al. (2012) also showed in column and full-scale biobeds that OPP was mobile but dissipated rapidly compared to TBZ and IMZ which remained in the top layers of the biobed (an indication of high sorption affinity) and dissipated at low rates.

Regarding the impact of substrate on the sorption behavior of pesticides, soil showed a substantially lower sorption affinity compared to the organic substrates tested. This is in agreement with previous studies which have attributed this to the higher organic C content of the latter providing more sorption sites for non-polar pesticides (De Wilde et al. 2009). Amongst the organic biomixtures tested, Straw/SMS/Soil (50:25:25) showed the highest sorption capacity for OPP, DPA and IMZ and SMS/Straw/Soil (50:25:25) for TBZ. This is in accordance with the higher organic C content of those two substrates compared to the rest of the substrates tested (Table 2.1). Pesticides desorption was not measured in the current study. Previous studies have suggested a limited reversibility of pesticides sorption in soil amended with SMS (Marin-Benito et al. 2009) and various biobeds packing materials (Karanasios et al. 2010b) compared to soil. On the one hand, this might favor the efficient removal of pesticides from the agro-industrial effluents but on the other hand it might result in limited bioavailability and retardation of the degradation of the retained pesticide residues.

2.5. CONCLUSIONS

Wastewaters from the fruit packaging industry constitute a serious point source contamination of natural water resources with pesticides. Our findings suggest that

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municipal wastewater treatment plants are expected to effectively remove OPP, DPA, EQ and its oxidation products but not TBZ and IMZ stressing the need for the implementation of more efficient but still simple and low-cost depuration methods like biobeds. SMS-rich organic biomixtures accelerated the dissipation of all pesticides particularly of the recalcitrant TBZ and IMZ suggesting that biobeds packed with such organic biomixtures could effectively depurate the wastewaters from the fruit-packaging industry. Further tests will focus on the assessment of the depuration of the most efficient organic substrates in semi-field (leaching column studies) and full scale conditions described in Chapters 3 and 4 respectively.

2.6. REFERENCES

Anastassiades, M., Lehotay, S.J., Stajnbaher, D., Schenck, F.J. 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J.AOAC Intern. 86, 412-431

Baszczyk, A.B., Augustyniak, A.,Skolimowski, J., 2013.Ethoxyquin: An antioxidant used in animal feed. Intern. J. Food Sci.Article ID 585931,http://dx.doi.org/10.1155/2013/585931

Campo, J., Masia, A., Blasco, C., Pico, Y., 2013. Occurrence and removal efficiency of pesticides in sewage treatment plants of four Mediterranean River Basins. J. Haz. Mater. 263P, 146-157

Castillo, L.E., Martinez, E., Ruepert, C., Savage, C., Gilek, M., Pinnock, M., Solis E. 2006. Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. Sci. Total Environ. 367, 418-432

Castillo, L.E., Ruepert, C., Solis, F. 2000. Pesticide residues in the aquatic environment of banana plantation areas in the North Atlantic Zone of Costa Rica. Environ.Toxicol.Chem. 19, 1942 – 1950

Castillo, M.d.P., Torstensson L., Stenstrom J., 2008. Biobeds for environmental protection from pesticides use – a review. J. Agric. Food Chem. 56, 6206-6219

Christodoulatos, C., Koutsospyros, A.B., Brodman, B.W., Korfiatis, G.P. 1997. Biodegradation of diphenylamine by selected microbial cultures. J. Environ. Sci. Health Part A32, 15-30.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

De Wilde, T., Spanoghe, P., Ryckeboer, J., Jaeken, P., Springael, D., 2009. Sorption characteristics of pesticides on matrix substrates used in biopurification systems. Chemosphere 75, 100-108.

del la Herras, J., Manas, P., Labrador, J., 2005. Effects of several applications of digested sewage sludge on soil and plants. J. Environ.Sci. Health, A40, 437–451.

Environmental Protection Agency (EPA) USA, 1998. R.E.D. Facts-Diphenylamine. EPA-738-F-97-010.

Environmental Protection Agency (EPA) USA, 2003. Reregistration eligibility decision for imazalil Case No. 2325. p. 74.

European Commission (EC), 2000. Opinion of the scientific committee on plants regarding the evaluation of thiabendazole in the context of council directive 91/414/EEC concerning the placing of plant protection products on the market – The Scientific Committee on Plants, SCP/THIABEN/002-Final, p 7

European Commission (EC), 2001. Review report for the active substance thiabendazole finalised in the Standing Committee on Plant Health at its meeting on 12 December 2000 in view of the inclusion of thiabendazole in Annex I of Directive 91/414/EEC. 7603/VI/97-final

European Commission (EC), 2010. Draft Assessment Report, Initial risk assessment provided by the rapporteur member state the Netherlands for the existing substance imazalil. Volume 1, pp. 1-151

European Commission (EC), 2013. Draft renewal assessment report prepared according to the Commission Regulation (EU) N.1141/2010, Second programme for the renewal of the inclusion of the following active substance under Regulation (EC) 1107/2009, Thiabendazole, Vol. 1, Report and proposed decision

European Food Safe Authority (EFSA). 2008. Conclusion on pesticide peer review. Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217, 1-67.

European Food Safety Authority (EFSA). 2010a. Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil. EFSA J., 8(3), 1526

European Food Safety Authority (EFSA). 2010b. Conclusion on the peer review of the pesticide risk assessment of the active substance ethoxyquin. EFSA J., 8(9), 1710

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

European Food Safety Authority (EFSA). 2012. Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine. EFSA J., 10(1), 2486-2545

FOCUS, 2006. Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration. Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference Sanco/10058/2005 version, 2.0, 2006, 434 pp.

Garcia-Portillo, M., Avino, E.S., Vicente, J.O., De Andres, R.L., Jimenez, MdlAS, Blanco JPL., 2004. Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States Patent, US 6,709,585 B1, p. 9.

Gardner, A.M., Alvarez, G.H., Ku, Y., 1982. Microbial degradation of ^{14}C-diphenylamine in a laboratory model sewage sludge system. Bull. Environ. Contamin. Toxicol. 28, 91–96.

Gonzalez, S., Muller, J., Petrovic, M., Barcelo, D., Knepper, T.D. 2006. Biodegradation studies of selected priority acidic pesticides and diclofenac in different bioreactors. Sci. Total Environ. 144, 926-932

Gupta, P.K., Boobis, A. 2005. Ethoxyquin (Addendum), http://www.inchem.org/documents /jmpr/jmpmono/v2005pr-10.pdf

He, P., Ackman, R.G. 2000. HPLC determination of ethoxyquin and its major oxidation products in fresh and stored fish meals and fish feeds. J. Sci. Food Agric. 80, 10-16

Herrero-Hernandez, E., Andrades, M.S., Marın-Benito, M.S., Sanchez-Martın, M.J., and Rodrıguez-Cruz,M.S. 2011. Field-scale dissipation of tebuconazole in a vineyard soil amended with spent mushroom substrate and its potential environmental impact. Ecotox. Environ. Safety 74, 1480-1488

Jimenez, M., Maldonado, M.I., Rodriguez, E.M., Hernandez-Ramirez, A., Saggioro, E., Carrae,I., Sanchez, J.A. 2015. Supported TiO2 solar photocatalysis at semi-pilot scale: degradation of pesticides found in citrus processing industry wastewater, reactivity and influence of photogenerated species. J. Chem. Technol. Biotech. 90, 149-157

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

JMPR, 2005. “Pesticide residues in food—2005. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues,” JMPR Report, Ethoxyquin 035, http://www.fao.org/ag/AGP/AGPP/Pesticid/JMPR/Download/99eva/14Ethoxyquin.pdf

Jonkers, N., Sousa, A., Galante-Oliveira, S., Barroso, C.M., Kohler, H.P.E., and Giger, W. 2010. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro, Portugal. Environ. Sci. Pollut. Res. 17, 834–843

Jung, S-K., Watkins, C.B., 2008. Superficial scald control after delayed treatment of apple fruit with diphenylamine (DPA) and 1-methylcyclopropene (1-MCP). Posth. Biol. Technol. 50, 45-52.

Karanasios, E., Tsiropoulos, N., Karpouzas, D.G., Ehaliotis C., 2010b. Degradation and adsorption of pesticides in compost-based biomixtures as potential substrates for biobeds in south Europe. J. Agric. Food Chem.58, 9147-9156

Karanasios, E., Tsiropoulos, N., Karpouzas, D.G., Menkissoglu-Spiroudi, U., 2010a. Novel biomixtures based on local Mediterranean ligninocellulosic materials: evaluation for use in biobeds. Chemosphere 80, 914-921

Kesavan, R., Van Wambeke, E., Van Assche, C., 1976. The effects of certain factors on the persistence of thiabendazole, 2-(4-thiazolyl) benzimidazole in the soil environment. Meded. Fak. Landbouwwet. R. U. Gent 41(2), II.

Khodja, A.A., Sehili, T., Pilichowski, J.-F., Boule, P., 2001. Photocatalytic degradation of 2-phenylphenol on TiO_2 and ZnO in aqueous suspensions. J. Photochem.Photobiol. A: Chem. 141, 231-239

Korner, W., Bolz, U., Submuth, W., Hiller, G., Schuller, W., Hanf, V., Hagenmaier, H., 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 40, 1141-1142

Kreuzig, R., Hartmann, C., Teigeler, J., Höltge, S., Cvetkovic, B., Schlag P., 2010. Development of a novel concept for fate monitoring of biocides in liquid manure and manured soil taking ^{14}C-imazalil as an example. Chemosphere 79, 1089-1094

Marin-Benito, J.M., Sanchez-Martin, M.J., Andrades, M.S., Periz-Clavijo, M., Rodriguez-Cruz.2009. Effect of spent mushroom substrate amendment of vineyard soils on the behavior of fungicides:1. adsorption-desorption of penconazole and metalaxyl by soils and subsoils. J. Agric. Food Chem. 57, 9634-9642

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Masia, A., Campo, J., Vazquez-Roig, P., Blasco, C., Pico, Y., 2013. Screening of currently used pesticides in water, sediments and biota of the Guadalquivir River Basin (Spain). J. Haz. Mater. 263P, 95-104

McKenzie H.A., (1994). The Kjeldahl determination of nitrogen: retrospect and prospect. *Tren. Anal. Chem*. 13: 138

OECD 106, (2000). OECD guideline for the testing of chemicals: adsorption-desorption using a batch equilibrium method.

Omirou, M., Dalias, P., Costa, C., Papastefanou, C., Dados, A., Ehaliotis, C., and Karpouzas D.G., 2012. Exploring the potential of biobeds for the depuration of pesticide-contaminated wastewaters from the citrus production chain: Laboratory, column and field studies. Environ. Pollut. 166, 31-39.

Pascual, I., Aviles, M., Aguirreola, J., Sanchez-Diaz, M. 2008. Effect of sanitized and non-sanitized sewage sludge on soil microbial community and the physiology of pepper plants. Plant Soil 310, 41-53.

Rodriguez-Cruz, M.S., Herrero-Hernandez, E., Ordaz, J.M., Marin-Benito, J.M., Draoui, K., Sanchez-Martin, M.J. 2012. Adsorption of pesticides by sewage sludge, grape marc, spent mushroom substrate and by amended soils. Int. J.Environ.Anal. Chem. 92, 933–948

Sanchez Perez, J.A., Carra, A., Sirtori, C., Aguera, A., Esteban, B., 2014. Fate of thiabendazole through the treatment of a simulated agro-food industrial effluent by combined MBR/Fenton processes at mg/L scale. Water Res. 51, 55-63

Santiago, D.E., Melian, E.P., Rodriguez, C.F., Mendez, J.A.O., Perez-Baez, S.O., Dona-Rodriguez, J.M. 2011. Degradation and detoxification of banana postharvest treatment water using advanced oxidation techniques. Green Sustain. Chem. 1, 39-46

Shah, A.G., Pierson, J.A., Pavlostathis, S.G. 2005. Fate and effect of the antioxidant ethoxyquin on a mixed methanogenic culture. Water Res. 39, 4251-4263

Sheldrick B.H. and Wang C.C, (1993). Particle Size Distribution: Soil Sampling and Methods of Analysis, ed. by Carter MR. Canadian Society of Soil Science, Lewis Publishers, Ann Arbor, MI, pp. 477–512.

Shin, K.A., Spain, J.C. 2009. Pathway and evolutionary implications of diphenylamine biodegradation by *Burkholderia* sp. strain JS667. Appl. Environ. Microbiol. 75: 2694-2704

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Sirtori, C., Aguera, A., Carra, I., Sanchez Perez, J.A. 2014. Identification and monitoring of thiabendazole transformation products in water during Fenton degradation by LC-QTOF-MS. Anal. Bioanal. Chem. 406: 5323–5337

Smilanick, J.L., Mansour, M.F., Gabler, F.M., Sorenson, D., 2008. Control of citrus postharvest green mold and sour rot by potassium sorbate combined with heat and fungicides. Posth. Biol. Technol. 47, 226-238.

Thorisson, S., Gunstone, F.D., Hardy, R., 1992. Some oxidation products of ethoxyquin including those found in autoxidising systems. Chem. Phys. Lipids 60, 263-271

Tuomela, M., Oivanen, P., Hatakka, A., 2002. Degradation of synthetic ^{14}C-lignin by various white-rot fungi in soil. Soil Biol. Biochem. 34, 1613–1620

Walkley A.J. and Black I.A., (1934). Estimation of soil organic carbon by chromic acid titration method. *Soil Sci.*, 37 29–38

Yang, C., Zhang, W., Liu, R., Li, Q., Li, B., Wang, S., Song, C., Qiao, C., Mulchandani, A., 2011. Phylogenetic diversity and metabolic potential of activated sludge microbial communities in full-scale wastewater treatment plants. Environ. Sci. Technol. Lett. 4, 7408-7415

Zheng, C., Focks, A., Ellebrake, K., Eggers, T., Fries, E., 2011. Sorption of ortho-phenylphenol to soils. Clean- Soil, Air, Water, 39, 116-120

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Chapter 3

The potential of organic substrates based on mushroom substrate and straw to dissipate fungicides contained in effluents from the fruit-packaging industry – Leaching column studies

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

Karas P.A., Makri S., Papadopoulou E.S, Ehaliotis C., Menkissoglu-Spiroudi U., Karpouzas D.G., (2016). The potential of organic substrates based on mushroom substrate and straw to dissipate fungicides contained in effluents from the fruit-packaging industry – Is there a role for *Pleurotus ostreatus*?. *Ecotoxicology and Environmental Safety* 124: 447-454

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3.1. INTRODUCTION

Fruit packaging plants constitute a serious point-source contamination of natural water resources with pesticides like imazalil (IMZ) and *ortho*-phenylphenol (OPP). These are used mostly in citrus fruit packaging plants for the control of fungal infestations during storage (Kinay et al. 2007). IMZ is toxic to aquatics, persistent in soil with DT_{50s} of 44 - 137 days (US EPA 2003; EC 2009) and of limited mobility in soil (Kreuzig et al. 2010). On the other hand, OPP is non persistent with $DT_{50soil} < 1$ d (EFSA 2008), relatively mobile in soil (Zheng et al. 2011), non-toxic to mammals and birds but highly toxic to aquatics (EFSA 2008). Monitoring studies in water bodies adjacent to areas where fruit packaging plants operate reported the presence of high concentrations of IMZ and OPP (Castillo et al. 2006; Jonkers et al. 2010). Considering the environmental risk imposed by the mishandling of pesticides used in the fruit packaging plants, the European Commission (EC) granted authorization for use until 2021 and 2019 for OPP and IMZ respectively, under the clause that *member states should pay particular attention to ensure that appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place* (EC 2009; EC 2010). Although several studies have addressed this issue using physicochemical approaches like photocatalysis (Khodja et al. 2010) or sorption (Garcia Portillo et al. 2004), their full implementation was hampered by their high cost, high engineering needs for operation and maintenance and the risk for production of toxic intermediates which require further treatment.

Biological treatment of those effluents could be a possible solution either in the form of bioreactors inoculated with tailored-made pesticide-degrading inocula (Perruchon et al. 2015) or through biobeds. These are simple on-farm systems packed with organic materials like soil/straw/peat (Castillo et al. 2008) or compost (Omirou et al. 2012) or spent mushroom substrate (SMS) (Karanasios et al. 2010). SMS has been proposed as a key component of biobed packing material (biomixtures) that could promote their biodepuration capacity (Gao et al. 2015) and at the same time facilitate the sustainable recycling of this waste produced by mushroom units (Herrero-Hernandez et al. 2011; Phan and Sabaratnam 2012). Results presented in Chapter 2 showed that SMS-rich substrates were the most efficient in dissipating persistent fungicides like thiabendazole and IMZ used in fruit packaging plants. The exact mechanism through which SMS accelerates the dissipation of pesticides in

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biomixtures is not yet known and the contribution of the white rot fungi (i.e. *Pleurotus ostreatus* or *Agaricus bisporus*) present in the SMS in the degradation of pesticides is not clear. For example, Garcia-Delgado et al. (2015) showed that soil incorporation of sterilized SMS of *A. bisporus* accelerated the degradation of 3-ring PAHs via stimulation of heterotrophic bacteria, while incorporation of non sterilized SMS enhanced the removal of 5, 6-ring PAHs stressing the involvement *A. bisporus* in the removal high molecular weight PAHs. Knowledge of the key microbial component of the SMS and of their role in the dissipation of pesticides would allow the directed optimization of SMS application in biobed systems.

Full scale biobeds packed with a compost-based biomixture and modified to cope with the high wastewater volumes produced by the citrus fruit packaging plants were successfully tested by Omirou et al. (2012). However little is known regarding the processes controlling the dissipation of the pesticides contained in these effluents and their interactions with the microbial community of biomixtures. Knowledge of the processes which dominate the depuration of those effluents is essential. Biological systems where degradation predominates over sorption are preferable (Karanasios et al. 2012) since the opposite might result in the accumulation of high pesticide loads in the biomixture which when replaced will require detoxification, increasing the overall implementation cost of biobeds (De Wilde et al. 2010). In turn information on the interactions of pesticides with the microbiota colonizing biobeds will facilitate the optimized operation of those systems through prevention of toxicity effects and maximization of the microbial catabolic activity. Previous studies have reported a clear correlation between phenoloxidase activity and pesticide degradation in low pH biomixtures which favor fungal activity (Castillo and Torstensson 2007), whereas others did not observe any correlation between microbial indicators and pesticide degradation (Karanasios et al. 2010).

The main aim of this study was to further evaluate the performance of SMS, identified by studies in Chapter 2 as a main component of the best performing biobed packing material, to depurate pesticides contained in effluents from the fruit packaging industry. The study focused on IMZ and OPP as (a) they constitute the most representative pesticides used in citrus fruit-packaging plants in Europe and (b) they represent chemicals with contrasting physicochemical properties and environmental behaviour (IMZ persistent vs OPP non persistent). Within this frame a leaching column study was employed (a) to evaluate the capacity of different

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biomixtures composed of SMS, straw and soil mixed at various combinations to depurate effluents containing IMZ and OPP; (b) to explore the contribution of *P. ostreatus* from SMS on pesticides dissipation by comparison with the depuration capacity of fresh mushroom substrate (FMS) of *P. ostreatus* and (c) to investigate the interaction of those pesticides with the microbial community in biobed systems.

3.2. MATERIALS AND METHODS

3.2.1. Pesticides

Analytical standards of IMZ (99.8%, Pestanal®) and OPP (99.9%, Pestanal®) were purchased from Fluka. Pesticides stock solutions in methanol were prepared (1000 mg L^{-1}) from analytical standards and used for analytical purposes. Commercial pesticides formulation like FUNGAZIL® 50EC (IMZ) and FOAMER®20EC (OPP) were used for the preparation of the aqueous pesticides solutions discharged on the leaching columns. The main physicochemical properties of the two pesticides studied are shown in Table 1.4.

3.2.2. Organic substrates

SMS, soil and straw were mixed in different volumetric ratios to prepare two of the four substrates tested in the leaching column study: SMS/Straw/Soil (50/25/25 by volume) and Straw/Soil (75/25 by volume). The former was amongst the best performing substrates regarding the dissipation of the pesticides contained in the effluents of fruit packaging plants (see Chapter 2). The soil used was collected from a farm of the National Agricultural Research Foundation of Greece in Larissa, Greece. It was sieved (2 mm) and stored at 4^{o}C prior to use. Wheat straw was chopped into small pieces (1-3 cm). SMS was obtained from a *P. ostreatus* edible mushroom unit (Mpoulogeorgos-Meteora, Trikala, Thessaly) after two harvest cycles, while fresh mushroom substrate of *P. ostreatus* (FMS) was obtained from the company DIRFYS, Euvoia, Greece. Mushroom substrates were chopped into small pieces (1-2 cm long) with a blender and stored at 4°C for a maximum of 10 days until further use. The physicochemical properties of all materials were determined as described in Chapter 2 and they are given in Table 3.1. FMS was more acidic than SMS. The latter was characterized by lower C/N ratio which is attributed to the gradual decomposition of

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the more easily degradable fractions of its organic matter (mostly hemicellulose and cellulose) (Koutrotsios et al. 2014).

Table 3.1. Physicochemical properties of the substrates used to assess the dissipation of the pesticides studied.

Substrates	pH	Organic Carbon (%)	Total N (%)	C/N
Soil[a]	7.55	1.05	0.13	8.1
Straw	7.15	38.9	0.80	48.6
SMS	6.83	35.5	1.20	29.6
FMS	5.50	42.0	0.72	58.3
SMS/Straw/Soil (50:25:25)	7.10	8.82	0.54	16.3
Straw/Soil (75:25)	7.35	3.26	0.26	12.5

[a] Soil texture: Sand 37%, Clay 31%, Silt 32% (clay loam)

3.2.3. Leaching column study

In total 12 PVC columns of 12.5 cm i.d. and 90 cm long were used (Photographs 3.1.). Triplicate columns for each substrate were prepared: SMS, Straw/Soil, SMS/Straw/Soil and FMS. A metal sieve was installed at the bottom of all columns to prevent passage of the packing material in the drainage of the columns. The columns were packed with the following materials from the bottom to the top: a) a 7-cm layer of thoroughly washed gravel (2-3 cm i.d); b) an 80-cm layer of biomixture and c) a 3-cm layer of well washed gravel (2-3 cm i.d.) to ensure uniform wetting and distribution of the pesticide solution into the biomixture. Pesticide solutions applied to the columns were loaded in 2 L separatory funnels with their outlet linked to a plastic tube through which pesticides solutions were discharged at the top of the columns. A flow controller was installed on the plastic tube to adjust solution flow rate and the flow on each column was individually calibrated (flow rates are given below) to ensure uniform delivery of pesticides solution on all columns. A plastic funnel was placed at the bottom of each column to collect the leachates in amber 2.5-L bottles.

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Right before pesticides application the columns were saturated with water and were left to drain for 4 days.

Photograph 3.1: The installation and the experimental set up of the leaching columns.

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Columns were treated in a sequential mode with aqueous solutions of OPP (first) and IMZ (secondly). The sequential treatment scheme employed simulated a realistic wastewater production scenario from a citrus fruit packaging plant (treating annually approximately 15000 tones of citrus fruits) treating oranges with the fungicide OPP for a period of 3 months (January to March) followed by the application of IMZ to tangerines (April). During the application of OPP two types of wastewater are produced: (i) a dense OPP aqueous solution (5 g L^{-1}) produced three times per season (approximate total volume 14 m^3) which is expected to be discharged on the biobeds and (ii) a diluted wastewater containing 5 mg L^{-1} of OPP (fruits rinsates) which is produced daily at volumes of 25 m^3 and is currently land-filled in nearby field sites. Regarding IMZ, its application later in the season results in the production of approximately 10 m^3 of dense effluent (1.2 g L^{-1}). Based on the above industrial scenario, the dense OPP- and IMZ-containing wastewaters are discharged in a 45 m^2 biobed system of 1 m depth.

In the experiment employed, columns were initially treated for a period of 60 days (day 1 to day 60) with aqueous solutions of OPP (2.6 g L^{-1}). These values are much higher than the maximum recommended dose of OPP (0.6 g L^{-1}) but they were used based upon consultation of citrus fruit packaging plants which have probably concentrated OPP in the final effluent upon accumulation of several treatment cycles. During this period, OPP solutions were delivered continuously (24 h d^{-1}) onto the columns at a flow rate of 12 ml h^{-1} resulting in a total wastewater volume and OPP amount discharged in each column of 17.25 L and 44.85 g respectively (4062.5 g of OPP per m^3 of substrate). At the end of the 60-day OPP treatment period, the columns were left to drain for 5 days (days 61 to 65) followed by application of aqueous solutions of IMZ (0.275 g L^{-1}) for a further period of 46 days (days 66 to 112). IMZ aqueous solutions were delivered to the columns every other day at a flow rate of 17 ml/h (24 h/d) resulting in a total wastewater volume and IMZ amount discharged in each column of 9.4 L and 2.6 g respectively (235.5 g of IMZ per m^3 of substrate). Regular analysis of the OPP and IMZ aqueous solutions loaded on the separatory funnel showed that both pesticides were stable with less than 10% losses observed during the storage period. Leachates were collected from the bottom of the columns on 3-day intervals. At each sampling day, the volume of the leachate collected was measured and a 100-ml sub-sample was transferred into plastic bottles which were stored at -20ºC until analyzed. A 10-ml fraction from each leachate sample was

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removed before storage and used for the measurement of enzymatic activities as described below.

Upon completion of the treatment period, the amounts of OPP and IMZ retained in the packing materials of the columns were determined to perform a mass balance analysis. Leaching columns were dismantled and their content was divided into three layers (0-20, 20-50 and 50-80 cm) (Photograph 3.2). The amounts of pesticides retained in the different layers of the leaching columns were extracted by sequential extractions with water and acetonitrile as described below. The total amount of pesticide recovered by the substrate at the end of the study, plus the amount of pesticide leached were deducted from the total pesticide amount applied on the columns and this amount was considered as 'dissipated'. This was a lump process including degradation and non extractable residues formation (bound residues).

Photograph 3.2: The packing material of the leaching columns after completion of the treatment period.

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3.2.4. Pesticides residue analysis

Extraction of IMZ and OPP from water samples was performed by mixing 2 ml of leachate with 8 ml of methanol or acetonitrile respectively. The mixture was vortexed for 1-2 minute and the extract was passed through a 0.45μm syringe filter (PTFE Syringe Filter, Whatman) prior to analysis. In all cases tests at three fortification levels (0.1, 1 and 10 mg L^{-1}) showed recoveries > 80%.

Extraction of pesticides from the packing material of the columns was achieved by sequential extractions performed initially with water and subsequently with organic solvents. The water-extracted pesticide residues represented the fraction which is readily available, whereas the fraction extracted with the organic solvent constitutes the less available fraction. Based on this, 4 g of biomixture were mixed with 40 ml of ddH_2O and extracted *via* agitation in an orbital shaker at 200 rpm for 30 min. The extract was centrifuged for 5 min at 7000 rpm and the clear supernatant was collected. Aqueous extraction was repeated two more times and the supernatants from each extraction step were combined (120 ml water extract) and subsequently extracted as described above for aqueous samples. Upon the third aqueous extraction cycle the solid substrate remaining in the flasks were extracted with 10 ml of acetonitrile *via* agitation for 90 min in an orbital shaker as described above. The extract was subsequently centrifuged as above and the clear supernatant was collected, filtered through a syringe filter (0.45 μm PTFE, Whatman) and stored at -20°C for subsequent HPLC analysis. Tests at three fortification levels (0.2, 2 and 20 mg kg^{-1}) for the different substrates tested showed recoveries >80% in all cases.

Pesticide residues were analyzed in an HPLC-UV Marathon III system equipped with a Grace Smart RP C18 (150 mm x 4.6 mm) column. OPP residues were detected at 254 nm using a mobile phase of 55:44.5:0.5 of acetonitrile:water:25%NH_3 solution (by volume), while IMZ was detected at 204 nm using a mobile phase of 80:20 methanol: 0.25% NH_3 solution (by volume). The flow rate was always 1ml min^{-1} and the retention times of OPP and IMZ were 3.4 and 5 min respectively. The limit of quantification (LOQ) for the two pesticides in solid substrates and water samples were 0.08 mg kg^{-1} and 0.05 mg L^{-1} respectively.

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3.2.5. Microbial measurements

3.2.5.1. Enzymatic activity measurements in the leachates

The activity of laccase and manganese peroxidase, commonly produced by *P. ostreatus*, were determined in the leachates of all columns throughout the experimental period. Laccase activity was determined spectrophotometrically at 425 nm by oxidation of 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (Bourbonnais and Paice 1990). The activity of manganese peroxidase was determined spectrophotometrically at 590 nm by oxidative coupling of 3-methyl-2-benzothiazoline hydrazone and 3-dimethylaminobenzoic acid (Ngo and Lenhoff 1980).

3.2.5.2. Phospholipid Fatty Acid Analysis (PLFAs)

Samples from all the substrates used in the column study were collected prior to the initiation of the study and upon completion of the leaching study and they were analyzed for their content in microbial Fatty Acids Methyl Esters (FAME) as described by Papadopoulou et al. (2011). For analysis of the data obtained by the PLFA analysis, FAMEs 15:0, a15:0, i15:0, i16:0, 17:0, i17:0 were used as indicators of Gram positive (GP) bacteria; 18:1ω9*cis/trans*, 16:1ω7, cy17:0, cy19:0 were used as indicators of Gram negative (GN) bacteria; 16:0 was considered as a general microbial indicator; 18:2ω6,9*cis/trans* were considered as indicators of fungi and 10Me16:0, 10Me17:0, and 10Me18:0 were considered as indicators of actinobacteria (Frostegård and Bååth 1996, Findlay 2004).

3.2.6. Statistical analysis

The data obtained from mass balance analysis, pesticides distribution in the column layers and total PLFA yields were subjected to two-way-ANOVA followed by Tukey's posthoc test to identify significant differences between the substrates studied. Relative abundance data of FAME indicators of GP and GN bacteria, actinobacteria and fungi were subjected to one way ANOVA to identify significant differences in the abundance of those microbial groups in the different column layers for each substrate tested.

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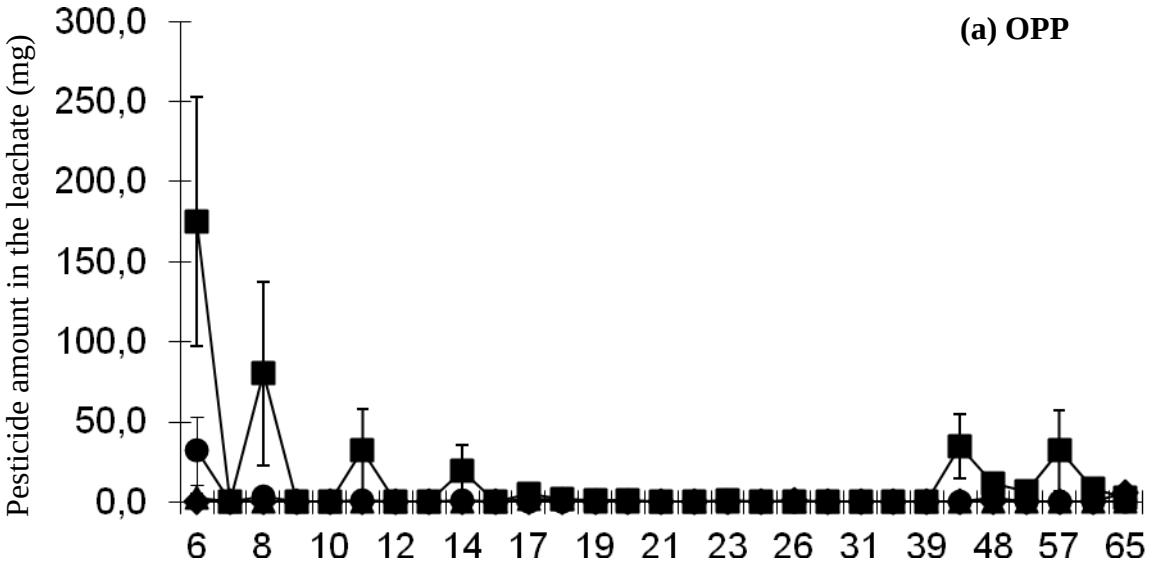
3.3. RESULTS

3.3.1. Leaching of OPP and IMZ

In all columns the first leaching event of OPP occurred on day 6 with a peak amount observed in the leachate of SMS and SMS/Straw/Soil columns (Fig. 3.1a). Thereafter significant amounts of OPP (80, 32, 19 35 and 32 mg) were found mostly in the leachate of SMS-columns at days 8, 11, 14, 43 and 57 days respectively. In general, the total amount of OPP found in the leachates of FMS, Straw/Soil and SMS/Straw/Soil columns did not significantly differ ($p>0.05$) (0.014, 0.017 and 0.120% of the total OPP amount applied respectively) compared to the SMS-columns where significantly higher % leaching ($p<0.05$) was observed (1.1%) (Table 3.2).

Regarding the temporal pattern of IMZ leaching, an early peak of IMZ (1.8 and 1 mg) was observed in the leachate of the SMS- and the SMS/Straw/Soil-columns on day 70 (Fig. 3.1b). Thereafter, IMZ was detected at considerable amounts (1.5-2.5 mg) in the leachates of the SMS columns at 79, 83, 96 and 106 days. Leaching from all the other packing materials peaked between days 87-90 (2.1 mg in Straw/Soil on day 87 and 2.3 mg in FMS on day 90). With the exception of the SMS-columns, the residues of IMZ dropped to levels below the LOQ in the leachates from day 104 onwards. Overall, a tendency for higher % leaching of IMZ was observed in the SMS columns (0.420% of total IMZ amount discharged on each column) followed by FMS (0.322%), Straw/Soil (0.220%) and SMS/Straw/Soil (0.120%) although the differences observed were not statistically significant (p>0.05) (Table 3.2).



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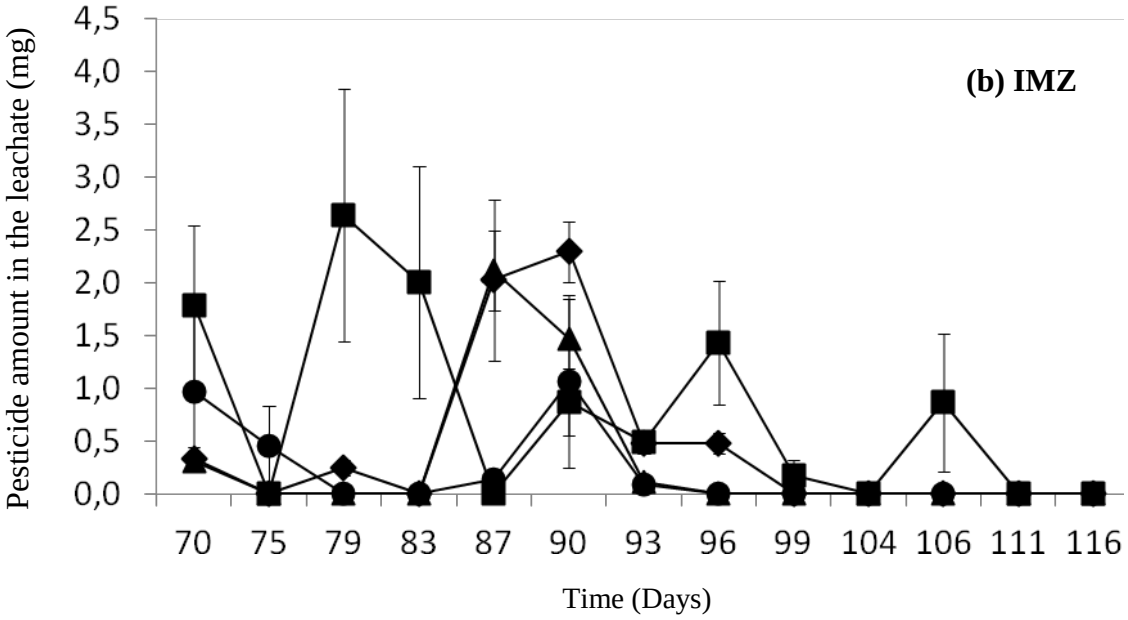

Figure 3.1. The temporal patterns of OPP (a) and IMZ (b) amounts (mg) detected in the leachates of the SMS (■), SMS/Straw/Soil (50/25/25 by volume) (●), Straw/Soil (75/25 by volume) (▲) and FMS (◆) columns. Each value is the mean of three replicate columns ± the standard deviation.

3.3.2. Mass Balance Analysis

3.3.2.1. Ortho-phenylphenol

More than 65% of the total amount of OPP applied to the columns was recovered by the packing materials of the columns at the end of the study (Table 3.2). The only exception was the FMS-columns where *ca.* 57% of OPP was recovered although this difference was not statistically significant ($p>0.05$). Furthermore, over 74% of the recovered amount was extracted with water. When the distribution of OPP residues in the different horizons of the columns was examined no significant differences between biomixtures ($p<0.05$) were observed with 99-100% of OPP recovered at the top 0-20 cm (Fig. 3.2a). No significant differences ($p>0.05$) between substrates in the amounts of OPP dissipated were observed with the highest values, 43%, observed in the SMS/Straw/Soil-columns and the lowest, 30% in the SMS-columns (Table 3.2).

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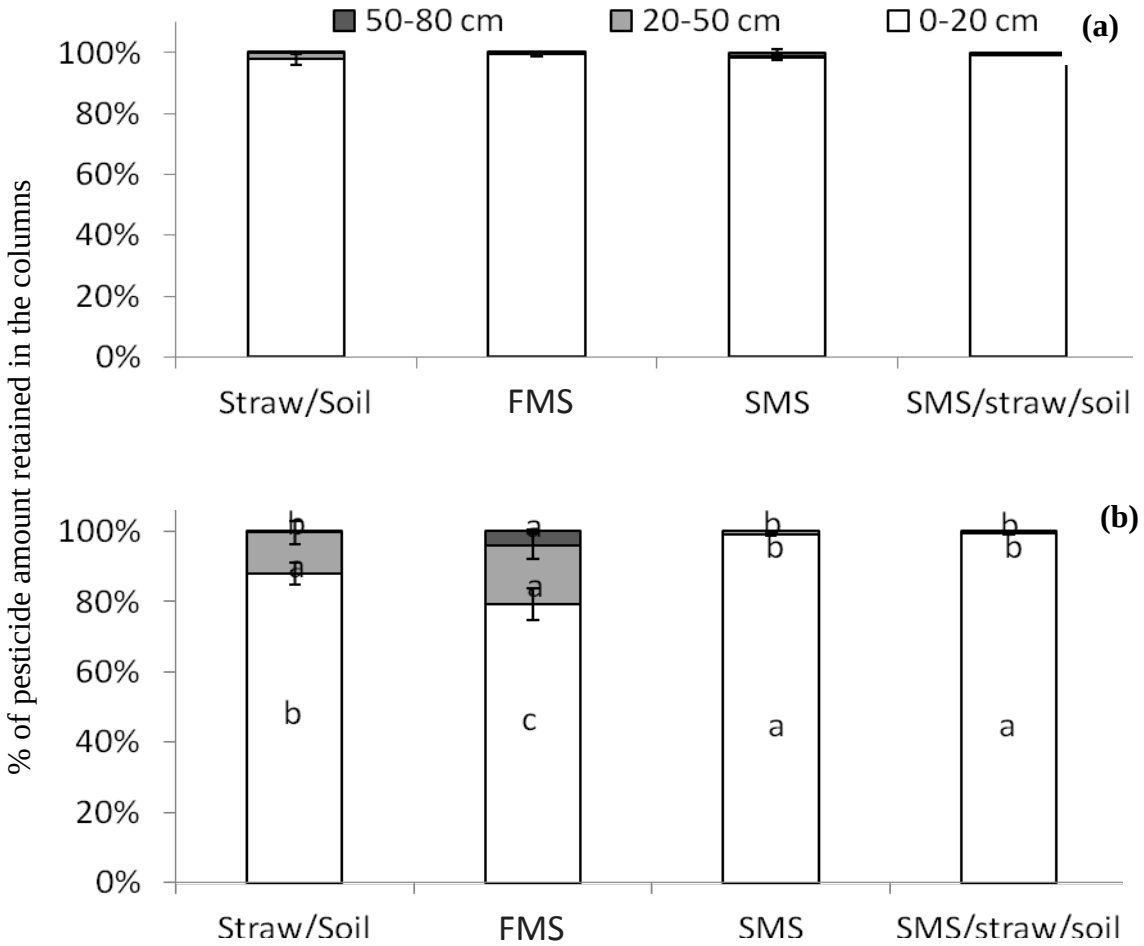

Figure 3.2. The distribution of the residues of OPP (a) and IMZ (b) in the three layers (0-20, 20-50 and 50-80 cm) of the Straw/Soil (75/25 by volume), FMS, SMS and SMS/Straw/Soil (50/25/25 by volume) columns. Data are presented as % of the amount of pesticide retained in the columns and extracted by water and acetonitrile (sum is presented). Each value is the mean of three replicate columns ± standard deviation. Different letters indicate significant differences ($p<0.05$) in the amount of pesticide leached, dissipated or retained in the different biomixtures. The absence of letters in column layers indicates that no significant difference were found.

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Table 3.2. The mass balance analysis for *ortho*-phenylphenol (OPP) and imazalil (IMZ) in the columns packed with the different substrates. Within each row, different letters indicate significant differences ($p<0.05$) in the amount of pesticide leached, dissipated or retained in the different biomixtures. Absence of letters in pesticide fractions indicate that no significant differences ($p>0.05$) were found.

Pesticides	Fraction (% of initially applied)	Substrates			
		Straw/Soil	FMS	SMS	SMS/Straw/Soil
Ortho-phenylphenol	Leached	0.014**b**	0.017**b**	1.100**a**	0.120**b**
	Retained-extracted with water	50.4	48.2	51.8	47.8
	Retained-extracted with acetonitrile	17.4	8.8	17.0	17.0
	Dissipated	32.2	42.9	30.1	35.1
Imazalil	Leached	0.220	0.322	0.420	0.120
	Retained-extracted with water	8.1	10.9	19.7	12.5
	Retained-extracted with acetonitrile	45.9	46.4	31.0	29.8
	Dissipated	45.8	42.5	48.9	57.6

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3.3.2.2. Imazalil

No significant differences in the amount of IMZ recovered by the different biomixtures were observed ($p>0.05$). Approximately 57, 54 and 51% of the applied IMZ were recovered from the FMS-, Straw/Soil- and SMS-columns respectively compared to 42% recovered from the SMS/Straw/Soil-columns respectively (Table 3.2). In contrast to OPP, 72% (SMS/Straw/Soil) to 85% (Straw/Soil) of IMZ recovered by the columns at the end of the study was extractable with acetonitrile. No significant differences ($p>0.05$) between substrates in the amounts of IMZ considered as dissipated were observed with the highest values 57.6%, observed in the SMS/Straw/Soil-columns and the lowest, 48.9% in the SMS-columns (Table 3.2).

Significant differences ($p<0.05$) in the distribution of IMZ residues in the column profiles of the different substrates were observed (Fig. 3.2b). Thus, in the columns packed with SMS/Straw/Soil and SMS nearly all IMZ was recovered from the top 0-20 cm, whereas significantly lower ($p<0.05$) amounts (88 and 79%) were detected in the top layer of the Straw/Soil- and FMS-columns. In turn, significantly higher ($p<0.05$) amounts of IMZ (10 and 25% respectively) were recovered from the 20-50 cm of the Straw/Soil and FMS-columns, while in the latter significant amounts of IMZ (3.6%) were even found at the 50-80 cm layer (Fig. 3.2b).

3.3.3. Microbial activity and dynamics in the leaching columns

3.3.3.1. Peroxidases activity

No manganese peroxidase activity was detected in the leachates of the columns throughout the study. Laccase activity was detected only in the leachates of the columns packed with FMS and SMS, with significantly higher values ($p<0.05$) observed in the former (Fig. 3.3). Laccase activity showed a similar temporal pattern in the leachates of both substrates with a peak observed at the first leaching event of OPP (day 6). Thereafter laccase activity decreased to negligible levels in the leachate of both substrates from day 10 onwards.

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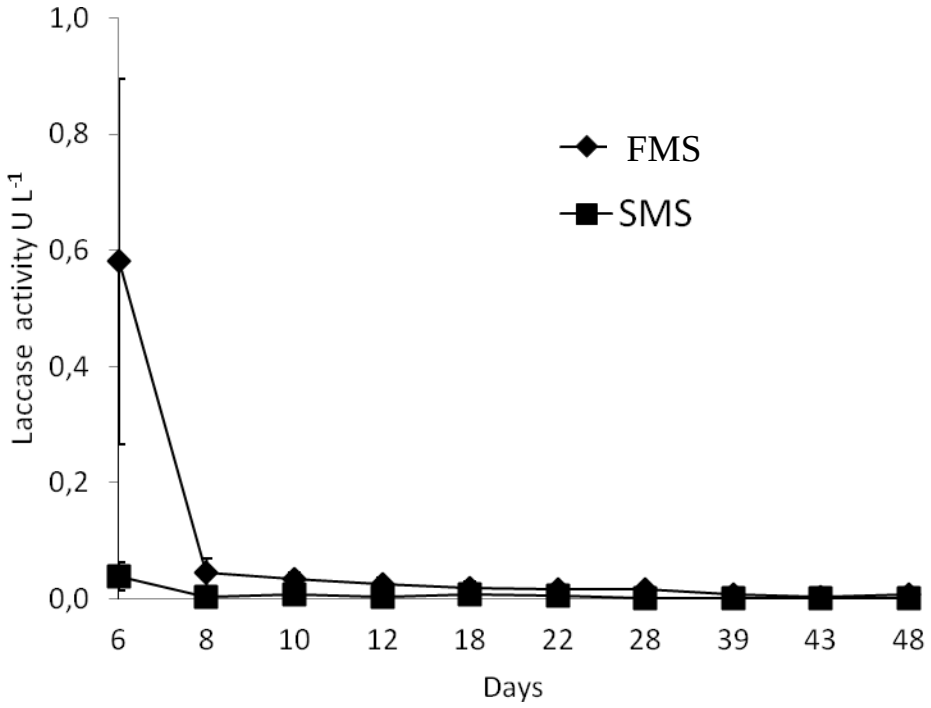

Figure 3.3. Laccase activity detected in the leachates of the columns packed with FMS and SMS. Results are presented until day 48 (OPP-treatment period) since no activity of laccase was detected from this day onwards. Each value is the mean of three replicates ± the standard deviation. No laccase activity was detected in the leachates of the columns packed with Straw/Soil and SMS/Straw/Soil.

3.3.3.2. PLFAs

No significant main effects ($p>0.05$) of column layer and organic substrate on total PLFA yields were observed, whereas significant interactions between these two factors were observed ($p<0.05$). Post-hoc tests showed that the total PLFA yields did not significantly differ in the different layers for each of the substrates tested (Fig. 3.4a). However, significantly higher PLFA yields ($p<0.05$) were observed at the 50-80 cm layer of FMS compared to the PLFAs yields measured at the same layer in SMS/Straw/Soil and SMS (Fig. 3.4a). Regarding the microbial community structure, GN bacteria were dominant in all substrates, especially in the top layer, whereas fungi showed significantly lower values ($p<0.05$) in the same layer (Fig. 3.4b). Apart from the universal reduction in the abundance of fungi in the surface layer of all substrates, GP bacteria and actinobacteria also showed a significantly lower relative abundance ($p<0.05$) in the surface layer of FMS and SMS/Straw/Soil respectively (Fig. 3.4b).

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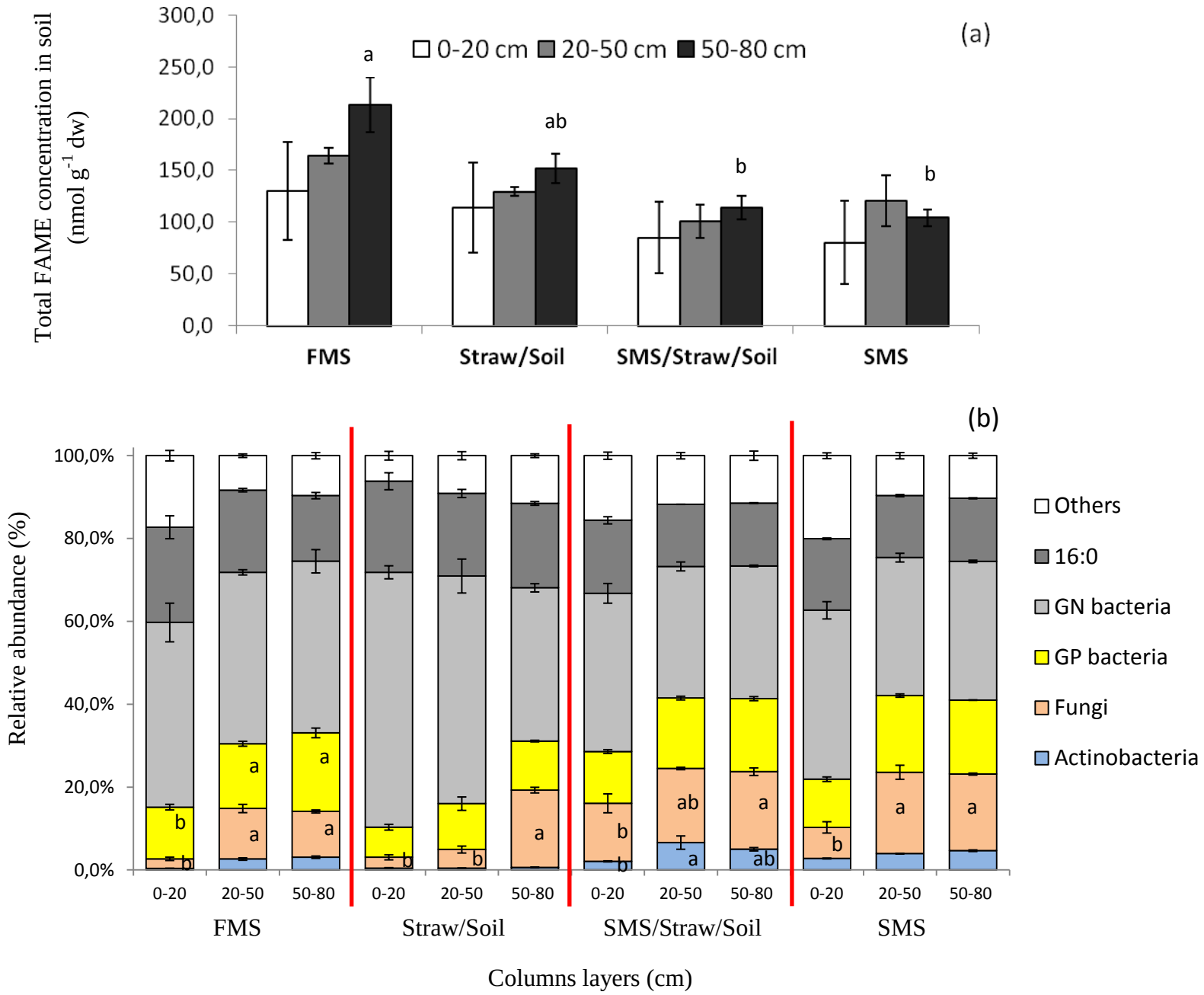

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Figure 3.4. (a) The total concentration of fatty acid methyl esters (FAME) and (b) in the relative abundance of the actinobacteria, fungi, GP bacteria and GN bacteria, and 16:0 (general microbial indicator) in the different layers of the biomixtures tested at the end of the study. Each value is the mean of three replicates ± the standard deviation. Bars designated by the same letters in graph (a) indicate non-significant differences ($p>0.05$) in the total PLFA yield measured in the same column layer in the different substrates, whereas stacked bars designated by the same letters in graph (b) indicate non significant differences ($p>0.05$) within each substrate in the relative abundance of the different microbial in the different column layers. Absence of letters indicates non significant differences.

3.4. DISCUSSION

3.4.1 Pesticides mass balance analysis

Pesticide leaching was generally low from all columns with OPP showing equal or lower leaching compared to IMZ. This is in contrast to the generally lower sorption affinity of OPP (K_f = 5.01 -30.3 g ml^{-1}) compared to IMZ (K_f = 183.6 - 412.4 g ml^{-1}) in similar organic substrates as shown in Chapter 2. In a column study Omirou et al., (2012) observed a higher mobility of OPP over IMZ, however different organic substrates, lower hydraulic and pesticide loadings and a different overall application scheme were employed in their study. The higher % leaching of IMZ compared to OPP observed in our study could be attributed to the disposal scenario employed in our study which took into account the temporal pattern of wastewater production and their pesticide content in a running citrus fruit packaging plant. Thus the preceding application of large volumes of OPP-contaminated wastewater might have saturated the substrates, limiting their capacity to retain the following application of IMZ despite its higher lipophilicity and thus higher sorption affinity.

Regarding the processes that contribute to the capacity of the different substrates to retain OPP, more than half of the amount of the fungicide applied on the columns was recovered from the substrates at the end of the study suggesting a predominance of sorption over dissipation processes. This is in contrast to the limited persistence of OPP in similar organic biomixtures and soil (Chapter 2). This

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discrepancy could be attributed to the particularly high amounts of OPP disposed of in the columns in our study compared to our data in Chapter 2 and previous studies (Omirou et al. 2012). This combined with the high retention capacity of the tested substrates resulted in the accumulation of high OPP concentrations in the biomixtures and sub-optimal conditions for its degradation. Although more than 70% of the amount of OPP recovered by the columns was extractable with water, in agreement with its generally weak sorption (Zheng et al. 2011) and high water solubility (EFSA 2008), 99% of it remained in the top layer (0-20 cm) of the columns. These results suggest that the mobility of OPP in biobeds packed with those substrates is expected to be limited.

Similarly to OPP, more than 51% of the total IMZ applied was recovered by the different substrates at the end of the study. The only exception was SMS/Straw/Soil in which less than 50% of the totally applied IMZ was recovered suggesting higher contribution of dissipation processes. This is particularly important considering the general recalcitrance of IMZ (US EPA 2003; Kreuzig et al. 2010). The more important role of dissipation over sorption in SMS/Straw/Soil is in line with our data in Chapter 2, which showed a lower persistence of IMZ in SMS/Straw/Soil (DT_{50} = 26 d) compared to other biomixtures used in this study like Straw/Soil (50/50) (DT_{50} = 58 d). In contrast, IMZ residues retained in the columns were mostly extractable with acetonitrile, in line with its low water solubility and stronger sorption affinity (Kreuzig et al. 2010; EFSA 2008). Another point which should be noted is the variable distribution of IMZ residues in the different columns at the end of the study. More than 99% of its residues in the SMS/Straw/Soil and SMS columns were retained in the 0-20 cm layer, suggesting a limited potential for further leaching, in contrast to Straw/Soil or FMS where significant levels of IMZ were found below 20 cm. Overall, these results suggest that SMS/Straw/Soil appears as the most desirable substrate for the removal of IMZ from the effluents of citrus fruit packaging plants.

It should be noted that our experimental set-up does not allow a distinction between the different dissipation processes contributing to OPP and IMZ loss. However, the overall high degradation rate (Chapter 2), high water solubility (Table 1) and low sorption affinity of OPP (Zheng et al. 2011) indicate that degradation could be the dominant dissipation process in the substrates studied. Regarding IMZ, its longer persistence (Kreuzing et al. 2010; Chapter 2), lower water solubility (Table

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1) and higher sorption affinity (EFSA, 2010) imply that the formation of non-extractable residues might have contributed to the pool of pesticide amount considered as dissipated. However, the short period of IMZ application (46 days) which is not known to simultaneously form non extractable residues (EC 2009) and its reported accelerated degradation in substrates like SMS/Straw/Soil (DT50 = 26 d), as shown in Chapter 2, are in support of a significant contribution of degradation in the dissipation of IMZ in the substrates tested.

3.4.2. The role of *P. ostreatus*

Contrasting results for the two pesticides regarding the removal efficiency of FMS vs SMS were observed. The increasing removal efficiency of FMS over SMS for OPP, was opposed to the generally equal removal efficiency of the two substrates for IMZ. This result might indicate that *P. ostreatus* actively growing on a fresh substrate (FMS) is more efficient in the degradation of phenolic molecules (OPP) compared to the fungal mycelium still present in the SMS which is though depleted of nutrients and energy sources. The higher acidity of FMS over SMS and other substrates tested might have also contributed to the higher enzymatic activity of *P. ostreatus* in the former at the initial phase of the experiment (Castillo et al. 2008). In line with this is the significantly higher activity of laccase in the leachates of the FMS columns compared to SMS in the first 8 days of the experiment and during the application period of OPP (1-60 days). Karas et al. (2011) showed that *P. ostreatus* actively degraded OPP via its lignolytic enzymatic system, whereas it only partially degraded IMZ. Apart from the limited capacity of the fungus to degrade IMZ, the preceding application of OPP might have resulted in the gradual elimination of *P. ostreatus* efficiency which was not enzymatically active in the substrate when the application of IMZ-containing effluents commenced. In line with this is the negligible activity of laccases in the leachates of FMS- and SMS-columns during the IMZ application period. Previous studies have verified the limited capacity of white rot fungi such as *P. ostreatus* to survive competition under wastewater treatment conditions (Libra et al. 2003; Gao et al., 2008). In addition, Cordova Juarez et al. (2011) showed that storage of the mushroom substrate leads to a drastic reduction of the enzymatic activity of *Pleurotus pulmonarious* with consequences on its degrading activity

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against chlorothalonil. However based on the mode of action of IMZ and OPP on fungal cells and the high application rates tested in our study, inhibitory effects on non-target fungi like *P. ostreatus* present in the substrates tested cannot be ruled out, thus limiting its role in the depuration efficiency of biobed systems.

3.4.3. Interactions of pesticides with the microbial community

Despite that the performance of biobeds relies mostly on their high biodegradation capacity, little is known regarding the interactions of pesticides with the microbial community in those systems. Using PLFA analysis, we observed a rather uniform distribution of the total microbial biomass in the different layers of the substrates tested at the end of the study. In contrast the relative abundance of the different microbial groups in the column layers of the different substrates tested varied and the differences observed were in agreement with the distribution of pesticides residues in the columns. The significantly lower abundance of fungi at the surface layer of all substrates, and of actinobacteria and GP bacteria at the surface layer of SMS/Straw/Soil- and FMS-columns, is in accordance with the accumulation of the studied fungicides in the surface layer of those substrates. So far no studies have investigated the impact of those fungicides on the microbial community and especially on fungi. IMZ acts by inhibiting the biosynthesis of ergosterol, the main sterol of the cellular membranes not only of Ascomycetes, which constitute the main target of IMZ (Guan et al. 1992), but also of Basidiomycetes and Zygomycetes (Weete et al. 2010). Similarly OPP acts by generating active oxygen radicals which destroy components of the fungal membranes in a non-selective mode (Dekker 1999). Based on their mode of action and their high application rates in the current study, adverse effects on off-target fungi should be expected. Previous studies by Marinozzi et al. (2013) in similar organic substrates showed different responses of fungi upon exposure to fungicides with penconazole inducing higher reductions in the abundance of total fungi compared to cyprodynil and axosystrobin. The differences observed were attributed to differences in the inherent toxicity of the three pesticides to microorganisms.

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3.5. CONCLUSIONS

The data presented in Chapter 3 reinforce our initial observations from laboratory studies (Chapter 2) that SMS-rich substrates may enhance the depuration capacity of biobeds receiving effluents from citrus fruit packaging plants containing OPP and IMZ. Despite its known capacity to degrade organic pollutants, *P. ostreatus* present in the SMS did not seem to actively contribute to the degradation of those fungicides. Overall the high depuration capacity of the SMS-rich substrates coupled with the associated benefit of recycling an organic waste of agricultural origin, further stress their potential for application in full-scale systems. Further studies, described in Chapter 4, will explore the capacity of pilot-scale biobed systems packed with SMS-based biomixtures to dissipate the pesticides contained in the effluents of the fruit packaging plants.

3.6. REFERENCES

Bourbonnais, R., Paice, M.G. 1990. Oxidation of non-phenolic substrates:an expanded role for laccase in lignin biodegradation. FEBS Lett. 267, 99–102

Castillo, L.E., Martinez, E., Ruepert, C., Savage, C., Gilek, M., Pinnock, M., Solis E. 2006. Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. Sci. Total Environ. 367, 418-432

Castillo, M.d.P. Torstensson, L. 2007. Effect of biobed composition, moisture and temperature on the degradation of pesticides. J. Agric. Food Chem. *55*, 5725-5733

Castillo, M.d.P., Torstensson L., Stenstrom J., 2008. Biobeds for environmental protection from pesticides use – a review. J. Agric. Food Chem. 56, 6206-6219

Cordova Juarez RA., Gordillo Dorry LL., Bello-Mendoza R., Sanchez JE., 2011. Use of spent substrate after *Pleurotus pulmonarius* cultivation for the treatment of chlorothalonil containing wastewater. J. Environ. Manag. 92, 948-952

De Wilde, T., Debaer, C., Ryckeboer, J., Springael, D., Spanoghe, P. 2010. The influence of small-and large-scale composting on the dissipation of pesticide residues in a biopurification matrix. J. Sci. Food Agric. 90, 1113–1120.

Dekker M., 1999. Handbook of pest management. Ruberson J. R. ed., New York

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

European Food Safe Authority (EFSA). 2008. Conclusion on pesticide peer review. Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217, 1-67.

European Food Safe Authority EFSA (2008). Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217: 1-67

European Food Safe Authority (EFSA), (2010). Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil. EFSA J. 8(3):1526

Environmental Protection Agency (EPA) USA. 2003. Reregistration eligibility decision for imazalil Case No. 2325. p. 74.

European Commission (EC) 2009. Final Review report for the active substance 2-phenylphenol. Available at http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_2_phenylphenol_en.pdf

European Commission (EC) 2010. Commission Directive 2010/57/EU of 26 August 2010 amending Annex I to Council Directive 91/414/EEC to renew the inclusion of imazalil as active substance. Official Journal of the European Union L225/5. Available at http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:225:0005:0009:EN:PDF

Findlay RH., 2004. Determination of microbial community structure using phospholipids fatty acid profiles. Molecular Microbial Ecology Manual, Second Edition 4.08, Kluwer Academic Publishers, Netherlands, pp 983-1004.

Frostegård, Å., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol. Fert. Soils 22, 59-65

Garcia-Delgado C., D'Annibale A., Pesciaroli L., Yunta F., Crognale S., Petruccioli M., Eymar E., 2015. Implications of polluted soil biostimulation and bioaugmentation with spent mushroom substrate (*Agaricus bisporus*) on the microbial community and polycyclic aromatic hydrocarbons biodegradation. Sci. Total Environ. 508: 20-28

Gao D., Zheng Y., Wen X., Qian Y. 2008. Competition strategies for the incubation of white rot fungi under non-sterile conditions. Proc. Biochem. 43,937–944

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Gao W., Liang J., Pizzul L., Feng X.M., Zhang K., Castillo MdP. 2015. Evaluation of spent mushroom substrate as substitute of peat in Chinese biobeds. Int. Biodeter. Biodeg. 98, 107-112

Garcia-Portillo, M., Avino, E.S., Vicente, J.O., De Andres, R.L., Jimenez, MdlAS, Blanco JPL., 2004. Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States Patent, US 6,709,585 B1, p. 9.

Guan J., Van Leemput L., Posthumus MA., De Waard MA., 1992. Metabolism of imazalil by wild-type and DMI-resistant isolates of *Penicillium italicum*. Neth. J. Pl. Path. 98, 161-168

Herrero-Hernandez, E., Andrades, M.S., Marın-Benito, M.S., Sanchez-Martın, M.J., and Rodrıguez-Cruz, M.S. 2011. Field-scale dissipation of tebuconazole in a vineyard soil amended with spent mushroom substrate and its potential environmental impact. Ecotox. Environ. Safety 74, 1480-1488

Jonkers, N., Sousa, A., Galante-Oliveira, S., Barroso, C.M., Kohler, H.P.E., Giger, W. 2010. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro, Portugal. Environ. Sci. Pollut. Res. 17, 834–843

Karanasios, E., Tsiropoulos, N**.,** Karpouzas, D.G., Ehaliotis C., 2010. Degradation and adsorption of pesticides in compost-based biomixtures as potential substrates for biobeds in south Europe. J. Agric. Food Chem. 58, 9147-9156.

Karanasios E., Papadi-Psyllou A., Karpouzas D.G., Tsiropoulos N. 2012. Optimization of water management and biomixture composition for maximizing the pesticide depuration of peat-free biobed systems. J. Environ. Qual. 41, 1787-1795

Karas P., Perucchon C., Exarhou C., Ehaliotis C., Karpouzas DG., 2011. Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. Biodegradation 22, 215-228

Khodja, AA.,Sehili, T., Pilichowski, J-F., Boule, P., 2001. Photocatalytic degradation of 2-phenylphenol on TiO_2 and ZnO in aqueous suspensions. J. Photochem. Photobiol. A: Chem. 141, 231-239

Kinay P, Mansour MF, Gabler MF, Margosan DA, Smilanick JL. 2007 Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Prot. 26, 647-656

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Koutrotsios G., Mountzouris K.C., Chatzipavlidis I., Zervakis G.I. 2014. Bioconversion of lignocellulosic residues by *Agrocybe cylindracea* and *Pleurotus ostreatus* mushroom fungi – Assessment of their effect on the final product and spent substrate properties. Food Chem. 161, 127-135

Kreuzig, R., Hartmann, C., Teigeler, J., Höltge, S., Cvetkovic, B., Schlag P., 2010. Development of a novel concept for fate monitoring of biocides in liquid manure and manured soil taking ^{14}C-imazalil as an example. Chemosphere 79, 1089-1094

Marinozzi M, Coppola L., Monaci E, Karpouzas D.G., Papadopoulou E, Menkissoglu-Spiroudi U., Vischetti C., 2013. The dissipation of three fungicides in a biobed organic substrate and their impact on the structure and activity of the microbial community. Environ. Sci. Pollut. Res. 20, 2546-2555

Ngo TT., Lenhoff HM., 1980. A sensitive and versatile assay for peroxidase and peroxidase-coupled reactions. Anal. Biochem. 105, 389–397

Omirou, M., Dalias, P., Costa, C., Papastefanou, C., Dados, A., Ehaliotis, C., Karpouzas D.G. 2012. Exploring the potential of biobeds for the depuration of pesticide-contaminated wastewaters from the citrus production chain: Laboratory, column and field studies. Environ. Pollut. 166, 31-39.

Papadopoulou E.S., Karpouzas D.G., Menkissoglu-Spiroudi U., 2011. Extraction parameters significantly influence and the quantity and the profile of PLFAs extracted from soil. Microb. Ecol. 62, 704-714

Perruchon C., Patsioura V., Vasiliadis, S. Karpouzas D.G., 2015. Isolation and characterisation of a *Sphingomonas strain* able to degrade the fungicide ortho-phenylphenol. Pest Manag. Sci. DOI: 10.1002/ps.3970 (early view)

Phan C-W., Sabaratnam V. 2012. Potential uses of spent mushroom substrate and its associated lignocellulosic enzymes. Appl. Microbiol. Biotechnol. 96, 863-873

Wette, J.D., Abril, M., Blackwell, M. 2010. Phylogenetic distribution of fungal sterols. Plos One 5, e10899.

Zheng, C., Focks, A., Ellebrake, K., Eggers, T., Fries, E., 2011. Sorption of ortho-phenylphenol to soils. Clean- Soil, Air, Water, 39, 116-120

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Chapter 4

Integrated biodepuration of pesticide-contaminated wastewaters from the fruit-packaging industry in pilot-scale biobed systems: Bioaugmentation, risk assessment and optimized management

The work presented in Chapter 4 is included in the following article:

Karas P.A. Perruchon C., Karanasios E., Papadopoulou E.S, Manthou E., Sitra., S., Ehaliotis C., Karpouzas D.G., (2016) Integrated biodepuration of pesticide-contaminated wastewaters from the fruit-packaging industry: Bioaugmentation, risk assessment and optimized management. *Journal of Hazardous Materials* 320: 635-644

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4.1. INTRODUCTION

Postharvest treatment of fruits with pesticides guarantees their protection from fungal infestations and physiological disorders during storage. However, it leads to the production of large volumes of pesticide-contaminated effluents whose discharge without prior treatment entails serious environmental risks (Castillo et al. 2006). This is exemplified by the high aquatic toxicity of the pesticides used in this industrial sector like thiabendazole (TBZ) (EC. 2001) imazalil (IMZ) (EC. 2009), *ortho*-phenylphenol (OPP) (EFSA, 2008) and diphenylamine (DPA) (EFSA, 2012).

The need for the treatment of those effluents is stressed in the relevant pesticide regulatory documents which state that member-states should ensure *that appropriate waste management practices to handle the waste solution remaining after application are put in place* (EC, 2012). Several methods have been tested for the treatment of those effluents but integrated full scale implementation has not been achieved yet. Garcia-Portilo et al. (2004) patented a treatment system based on activated carbon which showed high removal efficiency for TBZ. However its high construction cost have prevented its wide implementation in fruit-packaging plants. Recent studies by Sanchez Perez et al. (2014) proposed a combined membrane biological reactor/Fenton- Photo Fenton process for the dissipation of TBZ. However this study was performed at pesticide levels (0.1 mg L^{-1}) which are multifold lower than the pesticides concentrations found in the effluents. In addition, those treatments lead to the formation of oxidation products of unknown toxicity (Sirtory et al. 2014). In the absence of treatment systems industries dispose their effluents in municipal sewage treatment plants which are not effective in the removal of those pesticides transferring the contamination to receiving water systems (Campo et al. 2013).

Biological treatment systems like biobeds could be a possible solution for the treatment of those effluents. They are simple to construct, economic and efficient systems used up to now for the depuration of pesticide-contaminated effluents at on farm level (Castillio et. al. 2008). In their simplest form they are composed of a pit or a container filled with a mixture of bioorganic material (De Wilde et al. 2007). Omirou et al. (2012) first tested biobeds for the depuration of wastewater produced by the citrus fruit production chain (from on-farm and post-farm activities), thus pesticides like DPA used in pome fruit-packaging plants were not considered. Our

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previous lab and column studies presented in Chapters 2 and 3 respectively demonstrated the capacity of SMS-rich biobeds packing materials to degrade and retain pesticides. Though their performance at full-scale biobeds for the depuration of those effluents is still pending.

In the study of Omirou et al. (2012), TBZ and IMZ were retained by the biobed packing material leading to a potential build-up of high pesticide residues stressing the need for decontamination of the spent packing material. This is still a key regulatory issue withholding the wider adoption of biobeds (Karanasios et al. 2012). Despite that only a few studies have addressed this problem (Terstensson 2000: De Wilde et al. 2010). Little attention has been given also to the post-treatment handling of biobeds-treated effluents. Despite the high depuration performance of biobeds (De Wilde et al. 2007), pesticide residues are still present in their effluents and their environmental release should be allowed pending risk assessment. This is feasible for biobeds receiving wastewaters from the fruit-packaging industry where a limited number of pesticides is used, in contrast to on-farm systems which receive a much wider pesticide range and thus complex risk assessment approaches are required.

Biodegradation has been identified as the key process controlling the depuration efficiency of biobeds (Castillio et al. 2008). Despite that little is known about the composition of the microbial community in biobeds and the microbial dynamics driving the biodegradation process. Good knowledge of the microbiology of biobed systems will facilitate their optimization. Bioaugmentation has been explored as a strategy for optimization of biobeds performance. Karanasios et al. (2010) showed that the use of spent mushroom substrate (SMS) from the edible fungus *Pleurotus ostreatus* in biobeds accelerated pesticide dissipation. Sniegowski and Springael (2014) showed that the use of soil adapted to the rapid biodegradation of these pesticides as a component of the packing material could ameliorate the depuration capacity of biobeds. This strategy or bioaugmentation with tailored-made microbial inocula could be ideal in cases where biobeds receive effluents containing a limited number of known pesticides like in fruit packaging plants.

The main aim of this study was to evaluate the depuration performance of biobeds against pesticides used in fruit-packaging plants at pilot scale level. Pilot

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biobeds were constructed based on the data obtained from the lab and column studies presented in Chapters 2 and 3. Beyond this central aim, futher research and practical objectives were the a) assessment of bioaugmentation as an optimization strategy for biobeds depuration performance against recalcitrant chemicals, b) identification of the key microbial groups, phylogeneticaly and functionally relevant for biobed systems, c) estimation of the risk associated with the environmental disposal of the biobed-treated effluents and d) assessment of optimum methods for the decontamination of the spent biobed packing material.

4.2. MATERIALS AND METHODS

4.2.1. Pesticides

Analytical standards of IMZ (99.8%), TBZ (99%) OPP (99.9%) and DPA (99.9%) (Pestanal®, Sigma-Aldrich) were used for residue analysis. Commercial pesticides formulation like TECTO® 50%SC (TBZ), FUNGAZIL® 50% EC (IMZ) FRUITGARD® 20%SL (OPP) and NO SCALD® 31.8%EC (DPA) were utilized for the preparation of the aqueous solutions which were applied on biobeds.

4.2.2. Biobed packing material

Following the results of the laboratory and column studies presented in Chapters 2 and 3 a mixture of SMS, soil and straw (50:25:25 by volume) was used for the packing of the pilot biobeds. The soil used was collected from a field site in Larissa, Greece. It was sieved to homogenize (4 mm) prior to mixing with organic materials. Wheat straw was chopped into small pieces (1-3 cm) and passed through a 4.75 mm sieve. SMS was obtained from a *P. ostreatus* mushroom production unit (Mpoulogeorgos-Meteora, Trikala, Greece) and it was chopped into small pieces. Appropriate volumes of soil, straw and SMS were mixed thoroughly and they were left to mature for a month (Photograph 4.1). The properties of the raw materials and of the final mixture were determined as described in Chapter 2 (section 2.2.2) and are listed in Table 4.1

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Photograph 4.1: The homogenised biomixture of SMS, soil and straw (50:25:25 by volume) before being used for the packing of the pilot biobeds.

Table 4.1. Physicochemical properties of the individual organic substrates and of the biobed packing materials used in the pilot biobed systems.

Substrates	pH[b]	Organic Carbon (%)	Total N (%)	C/N
Soil[a]	7.55	1.05	0.13	8.1
Straw	7.15	38.9	0.80	48.6
SMS	6.83	35.5	1.20	29.6
SMS/Straw/Soil (50:25:25)	7.10	8.82	0.54	16.3

[a] Soil texture: Sand 37%, Clay 31%, Silt 32% (clay loam)

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4.2.3. Set up of pilot biobeds

Five pilot biobeds composed of plastic containers of 1.1 m^3 (3 biobeds) or 0.24 m^3 (2 biobeds) volume were set up. The bottom of the biobeds was covered with a metal wire mesh and on top of this a 5-cm layer of well-washed gravel (2-3 cm diameter) was placed (Photograph 4.2a and 4.2b). The remaining volume was filled with the packing material described above (Photograph 4.2c). A 10-cm diameter hole was made at the bottom of the biobeds to allow collection of the draining effluent. A plastic funnel was positioned under the outer side of the hole and it was connected to a plastic tube (15 mm i.d.) leading to a 2.5-L amber glass bottle where effluents were collected (Photograph 4.2d).

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Photograph 4.2: Photographs of the preparation and set up of the pilot biobed systems used in this study.

The pesticide solutions applied on the biobeds were prepared in three 100-L tanks each containing an aqueous solution of two pesticides: IMZ+DPA (Tank 1), OPP+IMZ (Tank 2) and TBZ+OPP (Tank 3) (Photograph 4.2e). The concentration of all pesticides in the aqueous solutions was 100 mg L^{-1} assuming a 10-fold dilution/reduction of their concentration in the water during the fruits treatment process and considering the pesticides recommended dose rates (0.6 g L^{-1} for OPP, 1.2 g L^{-1} for TBZ, 1 g L^{-1} for IMZ and 2 g L^{-1} for DPA). Pesticides combinations were established according to their use patterns: (a) IMZ + DPA are used in pome fruit packaging plants (b) OPP + IMZ or TBZ are used in citrus fruit-packaging plants. In total 1080 and 252 L of pesticide solutions were discharged into the large and the small pilot biobeds respectively within a total period of 160-d corresponding to the average operation period of a fruit-packaging plant (Valero and Serano 2010). Pesticide solutions were pumped (max capacity 10 L h^{-1}) into the biobeds daily (three 10-min application periods times per day) (pumps, timers and tubing for each biobed

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are shown in Photograph 4.2f). This resulted in a daily discharge of 7.5 and 2.0 L in the large and the small pilot biobeds respectively. Pesticide solutions were applied at the top of the pilot biobeds via a drip irrigation system ensuring their uniform application onto the surface of the biobeds (Photograph 4.2g). Prior to pesticides application, all biobeds were irrigated with clean water for three days and were left to drain for a week to allow for equilibration. Upon commencement of wastewater application biobeds leachates were collected on a regular basis. Each time, the volume of the leachate collected was recorded and a subsample (0.5 L) was stored at -20°C for analysis.

At the end of the 160-d period three cores were collected from each pilot biobed using a 90-cm long PVC plastic tube (8 cm i.d.). The packing material cores were sectioned into three layers: 0-20, 20-50 and 50-80 cm and stored at -20ºC until analyzed. Pesticides amounts retained in the different layers of the biobeds were determined by sequential extractions with water and acetonitrile as described previously in Chapter 3. The total amount of pesticides recovered by the substrate at the end of the study, plus the amount of pesticide leached were deducted from the total pesticide amount applied and it was considered as 'dissipated'. This was a lump process including degradation and non extractable residues formation.

4.2.4. Bioaugmentation of pilot biobeds

We evaluated bioaugmentation with OPP- (*Sphingomonas haloaromaticamans*) (Perruchon et al. 2016a), DPA- (*Pseudomonas putida*) (Perruchon et al. 2015) and TBZ-degrading bacteria (consortium comprised of proteobacteria where a *Sphingomonas* phylotype was the key degrader of TBZ) (Perruchon et al. 2016b) as a strategy for ameliorating the depuration performance of biobeds. The pesticide-degrading bacteria were grown in mineral salts media where the pesticide constituted the sole C (OPP, TBZ) or the sole C and N source (DPA) (Perruchon et al. 2015). Bacterial inocula were harvested at the mid-logarithmic phase, cells were washed three times with sterile ddH_2O and they were re-suspended to ddH_2O which was applied to the packing material of the two small pilot biobeds (biobed 2bioaug and biobed 3bioaug) aiming to a final inoculum density of 10^6 cells g^{-1} of packing material

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(on a dry weight basis). The density of the bacterial inocula was determined by serial dilution plating in LB. Bacterial cells were sprayed with a hand sprayer and the treated packing material was thoroughly mixed with a spade prior to biobeds' packing (Photograph 4.3). Biobed 2bioaug, treated with IMZ and DPA, was inoculated with the DPA-degrading bacterium (no IMZ-degrading bacteria were available), while biobed 3bioaug, treated with TBZ and OPP, was amended with the TBZ- and the OPP-degrading bacterial inocula. A photograph of the final pilot biobeds and a schematic diagram of the experimental setup and the organization of the pilot biobeds are shown in Photograph 4.4 and Figure 4.1 respectively.

Photograph 4.3. The application of tailored-made bacterial inocula in the biobed packing material of pilot biobeds "biobed 2bioaug" and "biobed 3bioaug".

Photograph 4.4. The 5 pilot biobeds and the treatment employed in each of them.

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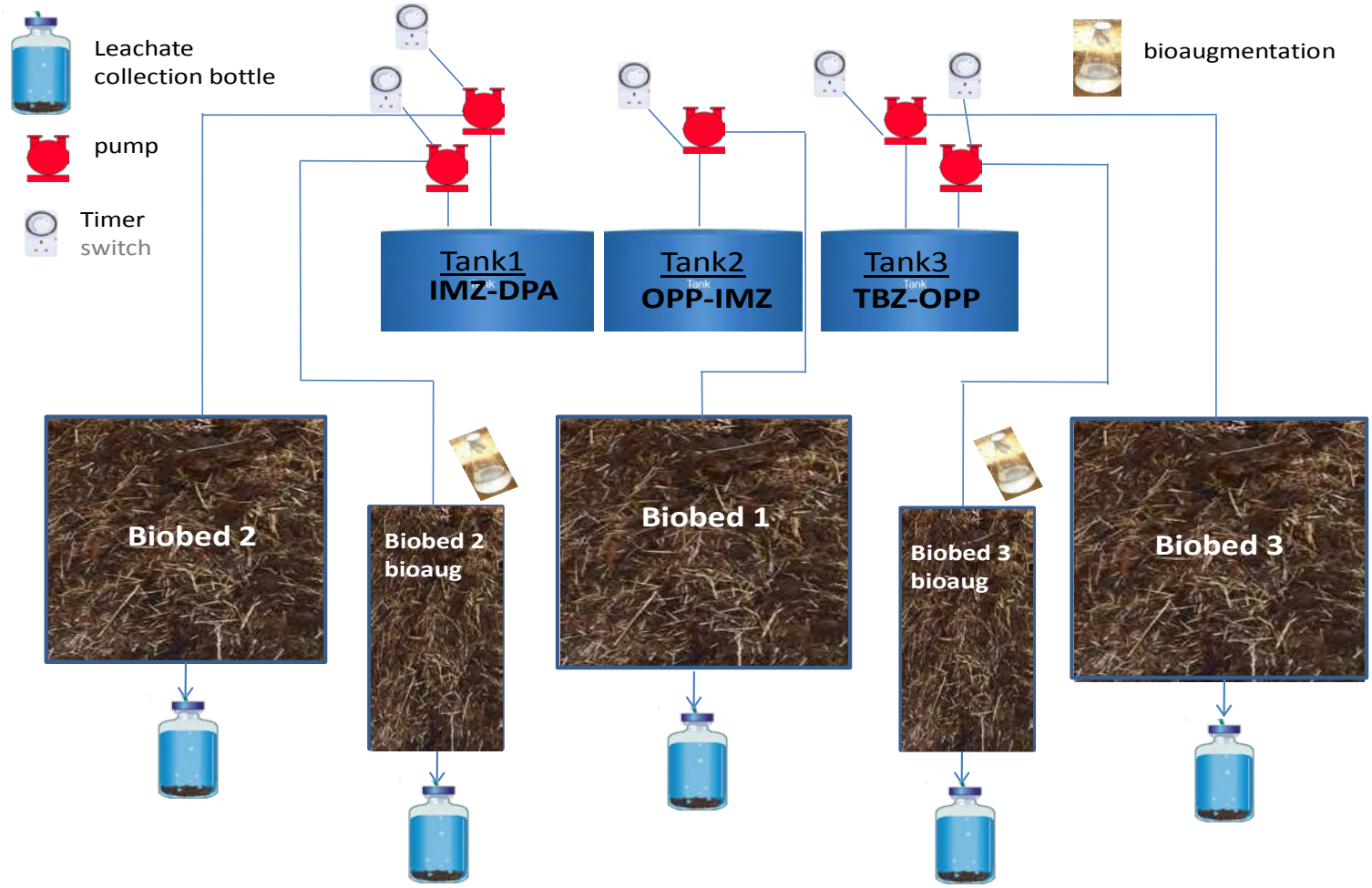

Figure 4.1: A schematic diagram of the experimental setup of the pilot biobeds.

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4.2.5. Risk assessment analysis for the management of biobeds effluents

An assessment of the risk associated with the environmental release of the biobed-depurated wastewater was employed. Two scenarios of practical value simulating the handling of wastewaters produced by a pome (Scenario I) or a citrus (Scenario II) fruit packaging plant were designated. A total volume of 25 m^3 of wastewaters containing DPA, TBZ or IMZ (Scenario I), or a volume of 42 m^3 of OPP- plus 11 m^3 of IMZ- or TBZ-containing wastewaters (Scenario II) were considered to be produced during one operational season. In both scenarios the concentration of pesticides in the wastewaters was 100 mg L^{-1} to align with the pesticide loading scheme in pilot biobeds. The depuration efficiency of our biobeds was used to estimate the total amount of pesticides contained in the treated effluents. Upon treatment the effluents were considered to be uniformely dispersed over a 0.1-ha disposal site (average size of a disposal site). Based on the above scenarios the rates of pesticides reaching the soil of the disposal site were calculated (Table 4.2). Subsequently the exposure levels of the soil in the disposal site (maximum Predicted Environmental Concentration, max PEC_{soil}) and in adjacent surface water systems and sediment (max PEC_{sw} and PEC_{sed}) were calculated using the PEC_{soil} calculator and the STEP 1-2 calculation tool respectively (Focus, 2001). For the calculation of max PEC_{sw} and PEC_{sed} run-off, erosion or drainage were considered as relevant processes while drift was not. The input data in STEP 1-2 calculation tool and the PEC_{soil} calculator were derived from pesticide regulatory documents (Table 4.3). The PECs obtained were used as exposure inputs in risk assessment (Table 4.4).

The risk assessment for aquatic and terrestrial indicator organisms was carried out according to the currently implemented regulatory guidelines (EC, 2002: EFSA, 2013). Regarding aquatic ecotoxicity, the Regulatory Acceptable Concentrations (RACs) were calculated using acute and chronic toxicity data obtained from the pesticides registration documents (Table 4.5). An unacceptable risk was identified when PECs/RACs>1. Regarding terrestrial ecotoxicity, Toxicity Exposure Ratio (TER) or Hazard Quotients (HQ) were calculated using the calculated PECs (Table 4.4) and toxicity data obtained from registration documents (Table 4.5). An unacceptable risk was identified when TER < 10 or HQ > 2 for acute toxicity risk to earthworms and soil-dwelling arthropods respectively. In cases where an unacceptable risk was identified mitigation measures were considered as (a) an increase of the surface of the soil disposal site from 0.1 to 0.2 ha or (b) the use of bioaugmented biobeds for the calculation of PECs (i.e. higher dissipation efficiency for TBZ).

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Table 4.2. Predicted rates of the pesticides in the soil of the disposal site calculated according to Scenario I or II

Scenario		Pesticide in the leachate of biobeds (%)	Pesticide dissipated & retained by biobeds (%)	Pesticide amount in waste before depuration per season (g)	Pesticide amount in waste after depuration per season (g)	Disposal area (ha)	Predicted Pesticide Rate in soil (g/ha)
Scenario I	Diphenylamine	0.05	99.95	2500	1.25	0.1	12.5
	Imazalil	0.19	99.81	2500	4.75	0.1	47.5
	Thiabendazole - Non Bioaug[a]	0.26	99.74	2500	6.5	0.1	65.0
	- Bioaug[b]	<0.001	99.99	2500	0.025	0.1	0.25
	- Disp. Area[c]	0.26[b]	99.74[b]	2500	6.5[b]	0.2	32.5[b]
Scenario II	*Ortho*-phenylphenol	0.04	99.96	4200	0.44	0.1	4.4
	Imazalil	0.19	99.81	1100	2.09	0.1	20.9
	Thiabendazole - Non Bioaug[a]	0.26	99.74	1100	2.86	0.1	28.6
	- Bioaug[b]	<0.001[a]	99.99[a]	1100	0.011	0.1	0.11[a]
	- Disp. Area[c]	0.26[b]	99.74[b]	1100	2.86[b]	0.2	14.3[b]

[a] Rates for TBZ calculated based on non bioaugmented biobed 3
[b] Rates for TBZ calculated based on bioaugmented biobed 3
[c] Rates for TBZ calculated assuming disposal of depurated effluents on a 0.2 ha disposal site instead of the standard 0.1 ha

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Table 4.3. Endpoints used as input data in STEP 1-2 calculation tool and the PEC_{soil} calculator

Pesticides	Water solubility (mg/L)	DT_{50} in sediment/water system (days)	DT_{50} in water (days)	DT_{50} in sediment (days)	DT_{50} in soil (days) (used in PEC_{sw} calculation)	K_{oc} (L/kg)	DT_{50} soil (days) (used in PEC_{soil} calculation)
Ortho-phenylphenol[a]	450	1000[e]	1000[e]	1000[e]	11.1[f]	347[g]	11.1[f]
Diphenylamine[b]	25.8	1000[e]	1000[e]	1000[e]	1000[e]	4104[j]	1000[e]
Imazalil[c]	184	117	1000	1000	474[f]	4753	86.7[h]
Thiabendazole[d]	30	1000	1000	1000	91.3[i]	2090	1000[f]

[a] EFSA, Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217 (2008) 1-67
[b] EFSA, Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine. EFSA J. 10(2012) 2486
[c] EFSA, Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil. EFSA J. 8 (2010) 1526 and EFSA, Outcome of the consultation with Member States, the applicant and EFSA on the pesticide risk assessment of confirmatory data for the active substance imazalil, Supporting Publication EN-674 (2014)
[d] EFSA, Peer review of the pesticide risk assessment of the active substance thiabendazole. EFSA J. 12 (2014) 3880
[e] Endpoint not available; 1000 days used as worst case assumption
[f] Worst-case from field studies
[i] Geometric mean from laboratory studies
[g] Mean K_{foc}
[h] Maximum laboratory (non-normalized)
[j] Source: Pesticide Properties DataBase (http://sitem.herts.ac.uk)

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Table 4.4 Predicted environmental concentrations (PECs) in soil, surface water and sediment calculated by the PEC_{soil} calculator and the STEP 1-2 calculation tool respectively following Scenarios I and II

Scenario	Pesticides	STEP 1 PEC_{sw} (max; μg/L)	STEP 2 PEC_{sw} (max; μg/L)	STEP 1 PEC_{sed} (max; μg/kg)	STEP 2 PEC_{sed} (max; μg/kg)	PEC_{soil} (max; mg/kg)
Scenario I	Diphenylamine	0.64	-	26.42	-	0.073
	Imazalil	2.16	-	102.57	-	0.067
	Thiabendazole	5.06 (0.44)[a]	1.96 (0.17)[a]	105.79 (9.2)	41.05 (3.57)[a]	0.335
		2.53[b]	-	52.9[b]	-	
Scenario II	*Ortho*-phenylphenol	1.0	-	3.48	-	0.006
	Imazalil	3.11	-	149.9	-	0.029
	Thiabendazole	2.23 (0.19)[a]	0.86	46.55 (4.05)	18.06	0.151

[a] PECs calculated using data obtained from bioaugmented biobed (biobed 3bioaug)
[b] PECs calculated based on a mitigation plan considering disposal of biobeds effluents to a 0.2 ha disposal site
[c] not determined

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Table 4.5. Toxicological endpoints used for the calculation of Regulatory Acceptable Concentrations (RACs), PECs/RACs, Toxicity Exposure Ratios (TERs) and Hazard Quotients (HQ) for all pesticides.

Pesticides	Acute effects					Chronic	
	Invertebrates	Fish	Earthworms	Soil-dwelling arthropods	Algae	Fish	Sediment-dwelling invertebrates
	D. magna EC_{50} 48h (μg L^{-1})	*O. mykiss* LC_{50}96h (μg L^{-1})	LC_{50} (mg kg^{-1})	LR_{50} (g a.s. ha^{-1})	*P. subcapitata* E_rC_{50} 72h (μg L^{-1})	*O. mykiss* or other species NOEC (μg L^{-1})	*Chironomus* spp. NOEC 20-28 d (μg L^{-1})
Ortho-phenylphenol[a]	2420	4000	99.1	n.a.	3570	36.0	1850
Diphenylamine[b]	1200	2200	n.a.	n.a.	300	710	n.a.[e]
Imazalil[c]	1580	885	271	88 (NOEC)	200	43.0	181 (27.5 mg kg^{-1})
Thiabendazole[d]	340	550	>224.5	>180	2300	12.0	2000 (3.0 mg kg^{-1})

[a] EFSA, Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217 (2008) 1-67
[b] EFSA, Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine. EFSA J. 10 (2012) 2486
[c] EFSA, Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil EFSA J. 8 (2010) 1526 & EFSA, Outcome of the consultation with Member States, the applicant and EFSA on the pesticide risk assessment of confirmatory data for the active substance imazalil. Supporting Information EN-674 (2014)
[d] EFSA, Peer review of the pesticide risk assessment of the active substance thiabendazole. EFSA J. 12 (2014) 3880
[e] n.a.: not available

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4.2.6. Decontamination of the spent biobed substrate

At the end of the biobeds' operation period, the packing material of the non bioaugmented biobeds (1, 2 and 3) was removed and it was mixed (50% by volume) with appropriate volumes of fresh organic matter (straw 25% and 25% cotton crop residues) (Photograph 4.5a, b and c). Its C/N ratio was optimized (target value of 25) with addition of NH_4NO_3-N (4.5 kg of a fertilizer, 34.4% N by weight). The material was thoroughly mixed and it was divided into two sub-samples of 8.5 kg and two sub-samples of 154.5 kg. The first set of 8.5- and 154.5 kg samples were treated with a suspension of bacteria degrading TBZ, OPP and DPA (described in Section 4.2.4) resulting in an inoculation density of 10^6 cells g^{-1} packing material dry weight. The 8.5 kg sample was then placed in a plastic bag and incubated at ambient temperature ('bioaugmentation' treatment), and the 154.5-kg sample was placed in a compost bucket (85 cm x 85 cm x 75 cm) and was allowed to compost for 160 days ('bioaugmentation & composting' treatment) (Photograph 4.5d). The remaining samples, one of 8.5-kg and one of 154.5-kg, received the same amount of water without bacteria, and they were handled in the same way as the corresponding bioaugmented samples ('control' and 'composting' treatments respectively). Immediately prior to the treatment and 24 (first mixing of the compost) (Photograph 4.5e), 40 (completion of 2nd thermophilic phase) and 160 days later (completion of maturation) triplicates (20 g) were removed and analyzed for pesticide residues.

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Photograph 4.5: Photographs from the cotton residues (a) and the straw (b) that they were used for the preparation of the new mixture which was composed (c), placed in the compost buckets (d) and mixed after the first thermophilic phase (e).

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4.2.7. Pesticides residue analysis

Extraction of pesticides from the leachates and the biobeds packing material was performed as described in Chapters 2 and 3. Pesticides residues remaining in the packing material were extracted initially with water and subsequently with acetonitrile. Pesticides residues extracted with water constitute the fraction which was retained by the biobed packing material, but was still available for further vertical movement and leaching. Whereas pesticides residues extracted by the organic solvent constitutes the fraction retained by the biobed that was less available for leaching. Pesticides residues were analyzed by HPLC-UV as described in Chapters 2 and 3.

4.2.8. Abundance of microbial taxa and catabolic genes

The abundance of total bacteria, total fungi and of different bacterial taxa (α-, β-, γ-proteobacteria, firmicutes and actinobacteria) was determined in the biobed packing material prior to pesticide application and at the end of the treatment period via q-PCR. In addition the abundance of *catA* and *pcaH* genes, encoding catechol 1,2-dioxygenase and protocatechuate dioxygenase respectively, involved in the metabolism of aromatic compounds (Harwood et al. 1986), was determined via q-PCR. Samples collected from the three different layers of the pilot biobeds were homogenized and four subsamples were processed for DNA extraction using the Power Soil DNA Isolation Kit (MoBio Laboratories, Inc.). All q-PCR reactions were performed in a Strategene MX3000P real-time PCR system. Q-PCR conditions and the primers used are shown in Table 4.6. Q-PCRs were carried out in 10μl reaction volume containing 1X KAPA SYBR® FAST qPCR Master Mix (2X) Universal, 1 μM of each primer, 50 nM ROX Low, 400 ng μL^{-1} BSA, and *ca*. 0.2-10 ng DNA. The copy numbers of the target gene in the environmental samples were determined via external standard curves as described by Rousidou et al. (2013). Briefly, the target gene amplified by a given soil sample or target microorganisms was purified, ligated into pGEM-T Easy vector (Promega, Madison, USA) and transformed to competent *Escherichia coli* cells according to manufacturers' instructions. Plasmid DNA was extracted (NucleoSpin Plasmid, MachereyeNagel) and its concentration was determined with Qubit fluorometer. The copy numbers of the target gene was calculated directly from the concentration of the extracted plasmid DNA. Serial ten-fold dilutions of the recombinant plasmid ranging from

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10^1 to 10^7 copies μl^{-1} were subjected in triplicate to q-PCR to construct the standard curves for each target taxa. For all microbial taxa q-PCR efficiencies ranged from 85 to 105% with r^2 values ranging from 0.985 to 0.999.

4.2.9. Statistical Analysis

Mass balance analysis data were subjected to one-way-ANOVA to identify significant differences per pesticide between biobeds in the different fractions accounted (dissipated / leached /retained and extracted with water or acetonitrile). Data regarding the distribution of pesticides in the biobeds horizons were subjected to two-way-ANOVA. In cases where significant interactions between the main factors were observed significant differences were identified by Tukey's post-hoc tests within each factor. Q-PCR data for each taxon were subjected to one-way ANOVA to identify significant differences between biobeds before and after pesticide application. All statistical analysis were performed with the SPSS statistical package.

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Table 4.6. Q-PCR conditions and primers used for the estimation of the abundance of key microbial taxa and functional genes (*catA, pcaH*) relevant to the degradation of aromatics

Microbial Group or Target Gene	Primers	Amplicon size (bp)	Annealing Temperature (°C)	Reference
Total Bacteria	341F/534R	194	60	Lopez-Gutierrez et al., (2004)
Total Fungi	ITS1F-ITS5.8S	300	53	Fierer et al. (2005)
Actinobacteria	Actino235/Eub518	300	60	Fierer et al. (2005)
Firmicutes	Lgc353/Eub518	181	55	Fierer et al. (2005)
α-proteobacteria	Eub338/Alf685	342	60	Fierer et al. (2005)
β-proteobacteria	Eub338/Bet680	360	55	Fierer et al. (2005)
γ-proteobacteria	Gamma395f/Gamma 871r	497	56	Muhling et al. (2008)
catA	CatAf-CatAr	470	58	El Azhari et al. (2010)
pcaH	PcaHf-PcaHr	395	57	El Azhari et al. (2008)

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4.3. RESULTS

4.3.1. Pesticides leaching from pilot biobeds

The temporal pattern of pesticides in the leachate of the pilot biobeds are shown in Figure 4.2. OPP residues were detected in the leachates of all three treated biobeds, although peak amounts were consistently detected in biobed 1 exceeding 20 mg on three occasions (Fig. 4.2a). On the contrary biobed 3bioaug showed the lowest amounts of the fungicide in the leachate with only four positive detections and max amount of 2.4 mg. DPA was detected in the leachate of the two biobeds on a regular basis, but its amount never exceeded 10 mg (Fig. 4.2b).

IMZ was rarely detected in the leachates of the biobeds. High amounts of IMZ were, however, detected on two occasions (90 and 94 d) in the leachates of biobed 1 (Fig. 4.2c). TBZ showed a substantially different leaching pattern in the two biobeds tested. A peak in TBZ leaching appeared early (31 d, 520 mg) in biobed 3 followed by the detection of lower TBZ amounts (<7 mg) from 100 days onwards (Fig. 4.2d). In contrast, residues of TBZ were detected in the leachates of biobed 3bioaug in only three occasions at levels below 1 mg.

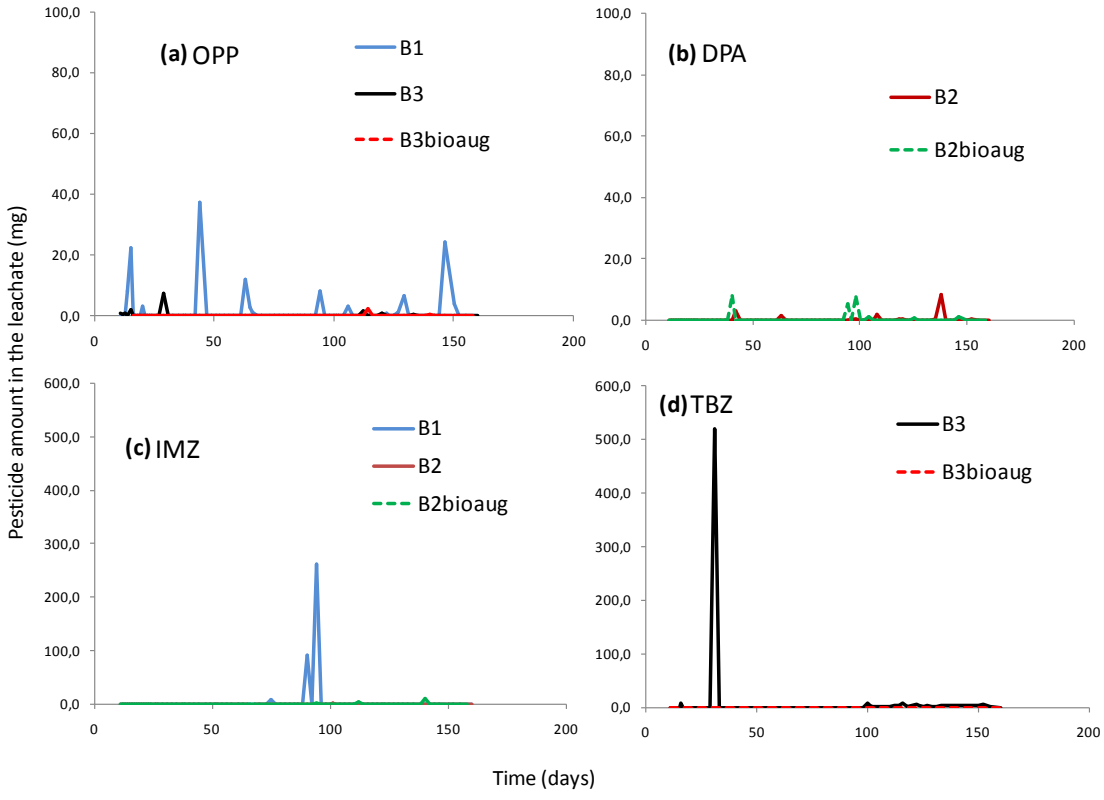

Figure 4.2. Leaching patterns of OPP (a), DPA (b), IMZ (c) and TBZ (d) from the pilot biobeds.

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4.3.2. Mass balance analysis of pesticides in the pilot biobeds

Mass balance analysis was performed upon measurement of pesticide residues in the cores obtained from the pilot biobeds at the end of the operation period (Fig. 4.3). The dissipation of OPP did not significantly differ between biobeds and ranged from 96.8% in biobed 3bioaug to 98.6 and 99.5% in biobeds 1 and 3 respectively (Fig. 4.3a). The amount of OPP in leachate was negligible ranging from 0.1% (biobed 1) to 0.01% (biobed 3 and biobed 3bioaug). The amount of OPP retained by the biobeds was mostly extractable with water, suggesting its availability for biodegradation or further mobility. OPP residues were present in the whole vertical profile of the biobed, however over 80% was found at the top 50 cm (Fig. 4.4a). DPA was nearly fully dissipated in both biobeds (99.9%), regardless of bioaugmentation (Fig. 4.3b).

IMZ showed different behaviour in the three biobeds (Fig. 4.3c). A significantly lower dissipation of IMZ ($p<0.05$) was observed in biobed 1 (72%) compared to biobed 2 and biobed 2bioaug (91 and 95.7% respectively). This was mirrored into the significantly higher amounts ($p<0.05$) of IMZ retained (27.5%) and leached (0.52%) from biobed 1 compared to biobed 2 (8.8 and 0.02% respectively) and biobed 2bioaug (4.25 and 0.03% respectively). Regarding the amount of IMZ retained by biobed 1, no significant differences ($p>0.05$) were observed between the fractions extracted by acetonitrile (15.4%) or water (12.2%). When the distribution of IMZ residues along the profile of the biobeds was investigated over 95% of the fungicide was found in the top layer (0-20 cm) (Fig. 4.4b).

A nearly complete dissipation of TBZ was evident in biobed 3bioaug (Fig. 4.3d) compared to a significantly lower dissipation (86.7%, $p<0.05$) in the corresponding non-bioaugmented biobed 3. The rest of TBZ applied in biobed 3 was retained and it was mostly extractable with water (8.5%) rather than with acetonitrile (4.6%). The significant difference in the dissipation between bioaugmented and non-bioaugmented biobeds was reflected in the overall amount of TBZ leached which ranged from <0.001% in the former to 0.26% in the latter. Regarding the distribution of TBZ residues in the profile of the biobeds, nearly 85% of TBZ was retained in the top layer (0-20 cm) while lower amounts, 14 and 1%, were detected at the 20-50-cm and 50-80-cm layers respectively (Fig. 4.4c).

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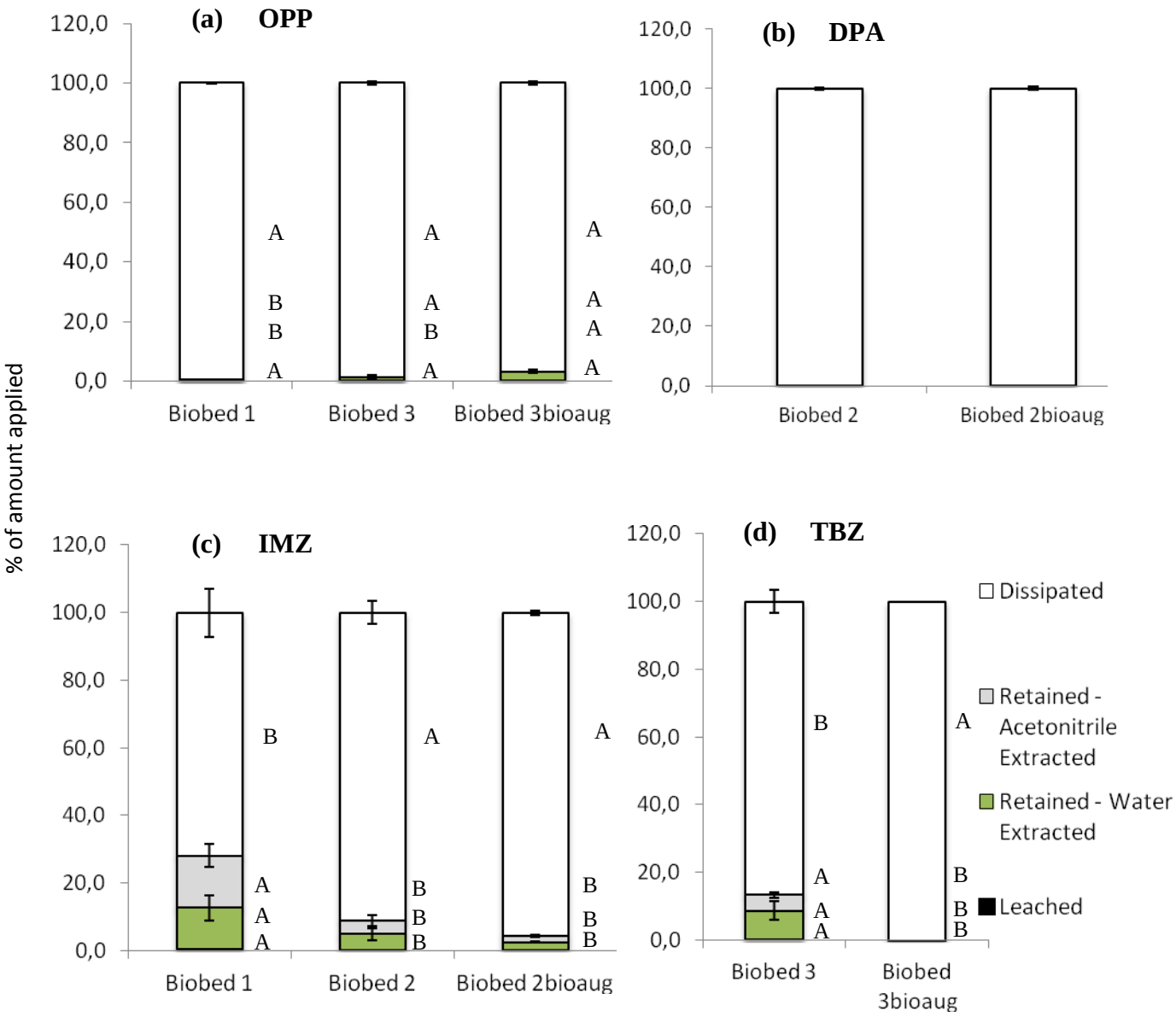

Figure 4.3. Mass balance analysis of OPP (a), DPA (b), IMZ (c) and TBZ (d) in the pilot biobeds. Pesticides amounts retained by the biobeds matrix were estimated by successive extractions with water and acetonitrile (as described in Chapter 3). Stacked bar parts designated by different letters indicate significant differences ($p<0.05$) between biobeds.

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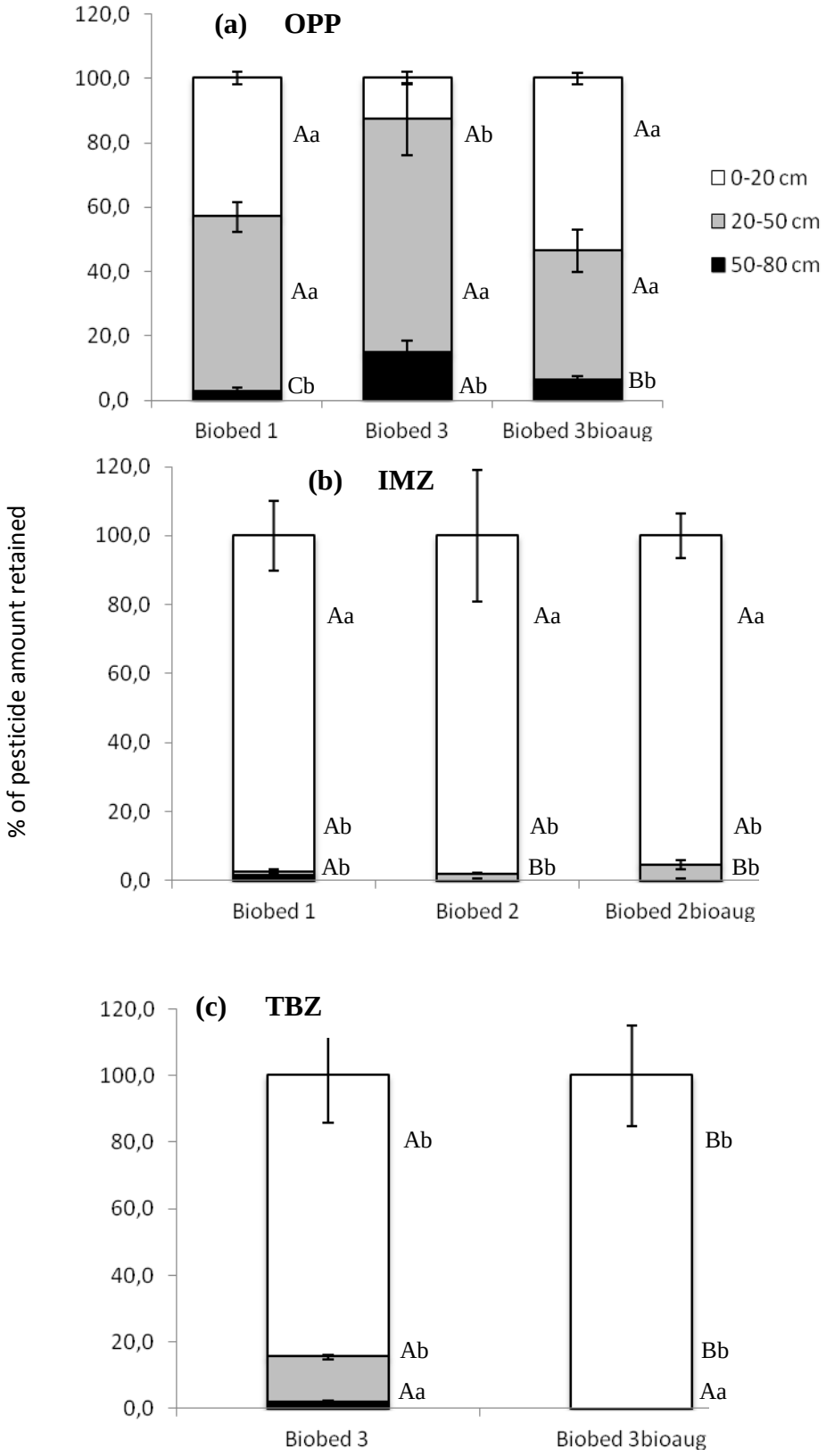

Figure 4.4. The distribution of OPP (a), IMZ (b) and TBZ (c) residues in the three layers of the pilot biobeds at the end of the study. Stacked bar parts designated by different capital letters indicate significant differences ($p<0.05$) between biobeds in the amount of pesticide

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retained in a layer, while different lower case letters indicate significant differences in the amounts of pesticides retained in the different layers within a biobed.

4.3.3. Risk assessment regarding biobed-treated effluents

Risk assessment analysis based on scenario I (pome fruit packaging plant) suggested no risk for aquatic (inverterbrate, fish, algae, sediment-dwelling invertebrates) and terrestrial (earthworms, terrestrial arthropods) non-target organisms by the use of IMZ and DPA (Tables 4.7 and 4.8). Whereas an unacceptable risk for chronic exposure to fishes were identified for TBZ (PEC/RAC = 1.633>1). This risk was alleviated only after the implementation of mitigation measures such as an increase of the surface of the disposal site area from 0.1 to 0.2 ha (PEC/RAC = 0.817<1) (Table 4.7) or when the depuration performance of the bioaugmented biobed 3 was considered for the calculation of PECs in the risk assessment (PEC/RAC = 0.366<1). Regarding Scenario II, no unacceptable risk for aquatic and terrestrial organisms was observed for all pesticides involved (i.e. OPP, TBZ and IMZ) (Tables 4.7 and 4.8).

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Table 4.7. Risk assessment for biobed-treated effluents according to Scenarios I and II for aquatic organisms. Ratios of max PEC_{sw}/RAC >1 indicate unacceptable risk for aquatic organisms (in bold).

Pesticides		Acute Toxicity		Chronic Toxicity		
		Invertebrates	Fish	Algae	Fish	Sediment-Dwelling Invertebrates
		Daphnia magna	*Oncorhynchus mykiss*	*Pseudokirchneriella subcapitata*	*Oncorhynchus mykiss*	*Chironomus sp.*
Ortho-phenylphenol	Scenario II	0.041	0.025	0.003	0.278	0.005
Diphenylamine	Scenario I	0.053	0.029	0.021	0.009	n.d.[d]
Imazalil	Scenario I	0.138	0.244	0.108	0.502	0.119
	Scenario II	0.197	0.351	0.156	0.723	0.172
Thiabendazole	Scenario I - Step1	**1.490**	0.920	0.022	**4.217**	0.025
	Scenario I - Step2	0.576	- [a]	-	**1.633**	-
	Mitigation/Refinement	-	-	-	0.817[b] (0.366)[c]	-
	Scenario II - Step1	0.656	0.405	0.010	**1.858**	0.011
	Scenario II - Step2	-	-	-	0.717	-

[a] not calculated since no unacceptable risk was evident at Step1
[b] calculated based on disposal of biobeds effluents to a 0.2 ha disposal site (mitigation)
[c] calculated based on the depuration efficiency of the bioaugmented biobed (biobed 3bioaug) (refinement)
[d] n.d.: not determined since no toxicity endpoint values were available (see Table 4.5)

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Table 4.8. Risk assessment for biobed-treated effluents according to Scenarios I and II for terrestrial organisms. TER >10 and HQ < 2 indicate low acute risk for earthworms and soil dwelling arthropods respectively.

Pesticides		TER - Earthworms	HQ - Soil-dwelling arthropods
Ortho-phenylphenol	Scenario II	16517	n.d.[a]
Diphenylamine	Scenario I	n.d.[a]	n.d.[a]
Imazalil	Scenario I	4045	0.540
	Scenario II	9310	0.238
Thiabendazole	Scenario I	>335	<0.639
	Scenario II	>743.5	<0.281

[a]n.d.: not determined because no toxicity endpoint values were available (Table 4.5).

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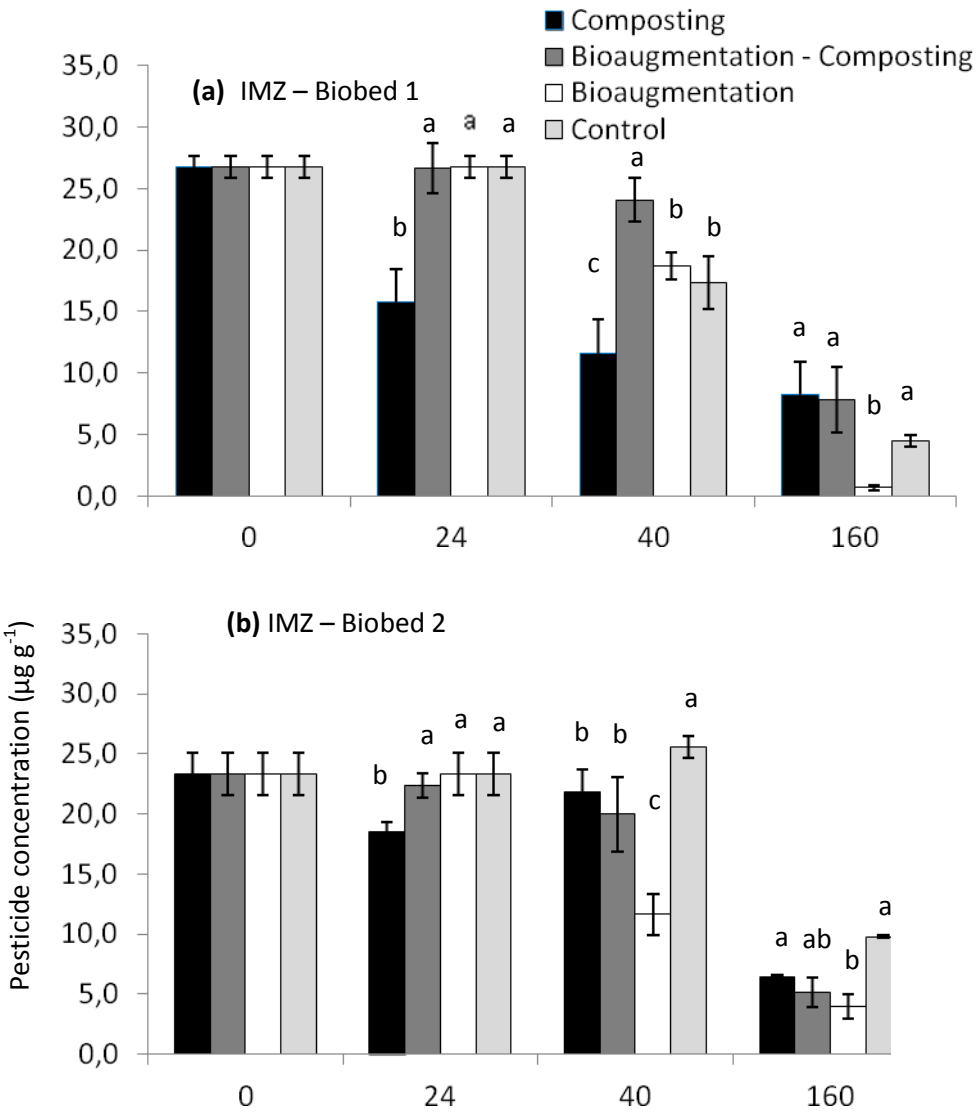
4.3.4. Decontamination of spent biobed packing material

Measurements of pesticide levels in the spent packing material (the biobed packing material at the end of the experimental period) showed that residues of TBZ (29 μg g^{-1}) and IMZ (23 to 26 μg g^{-1}) were still present (Fig. 4.5). Bioaugmentation was the most successful decontamination approach for both IMZ and TBZ resulting in a significantly higher dissipation (83-97%, $p<0.05$) of the former (Fig. 4.5a and 4.5b) and a complete dissipation of the latter (Fig. 4.5c). The spent packing material was successfully composted with the evolution of two thermophilic phases: the first and main one lasting 10 days (days 4 to 14) with a peak temperature of 50°C, and the second milder one which reached a max temperature of 35°C (days 23 to 28) (Figure 4.6). During the active phase (0-40 days) composting significantly accelerated the dissipation of IMZ relatively to the control (Fig. 4.5a and 4.5b), whereas for TBZ a significant acceleration in its dissipation was achieved only when composting was combined with bioaugmentation (Fig. 4.5c).



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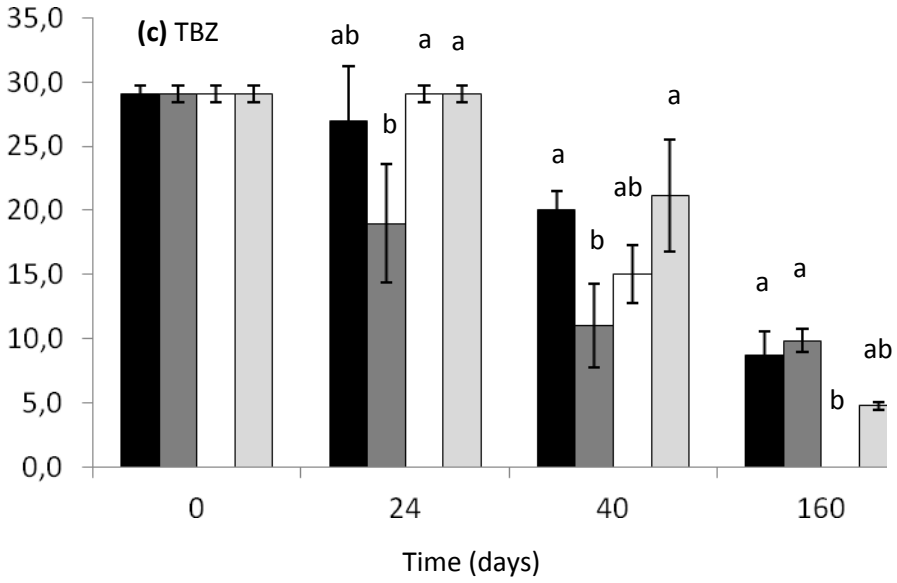

Figure 4.5. The dissipation of IMZ (a & b) and TBZ (c) in the spent packing material from biobeds 1 (a), 2 (b) and 3 (c) subjected to bioaugmentation, bioaugmentation and composting, composting or stored at ambient temperature (control). Within each time, bars followed by the same letter are not significantly different ($p<0.05$)

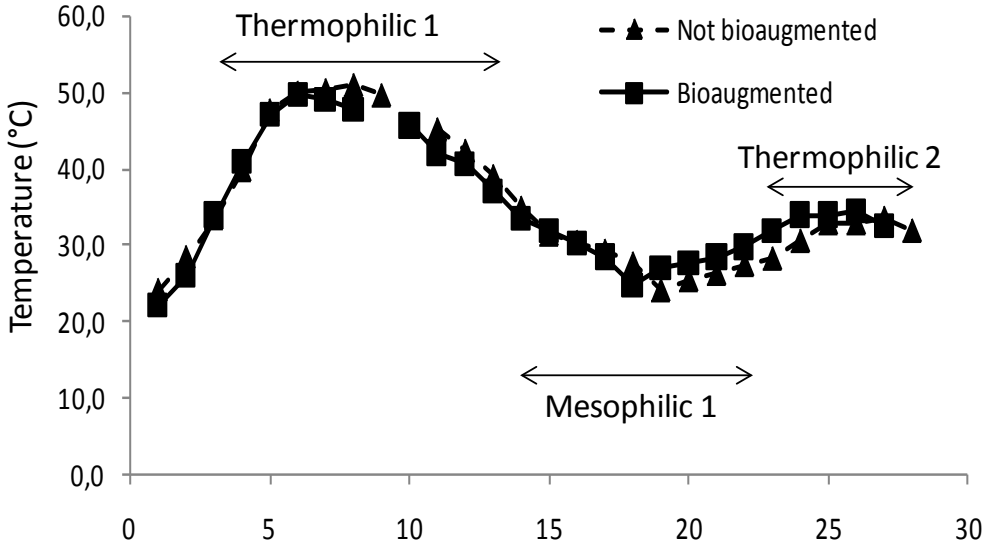

Figure 4.6: The temperature profile during the process of composting of the spent biobed substrate (bioaugmented/not bioaugmented).

4.3.5. Abundance of microbial taxa and catabolic genes

The abundance of total bacteria, total fungi and α-proteobacteria were significantly higher ($p<0.05$) in the biobeds at the end of the study compared to their abundance in the packing

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material prior to pesticides application (Fig. 4.7a). Actinobacteria were the most abundant bacterial taxa, followed by α-proteobacteria, firmicutes and γ-proteobacteria, while β-proteobacteria showed low abundance. Significantly higher copy numbers of the *pcaH* and *catA* genes were detected in the biobeds at the end of the experimental period compared to their corresponding copy numbers in the packing material prior to the initiation of the pesticide application (Fig. 4.7b). On the other hand, no significant difference ($p<0.05$) in the abundance of *pcaH* and *catA* were found between non bioaugmented and their corresponding bioaugmented counterparts.

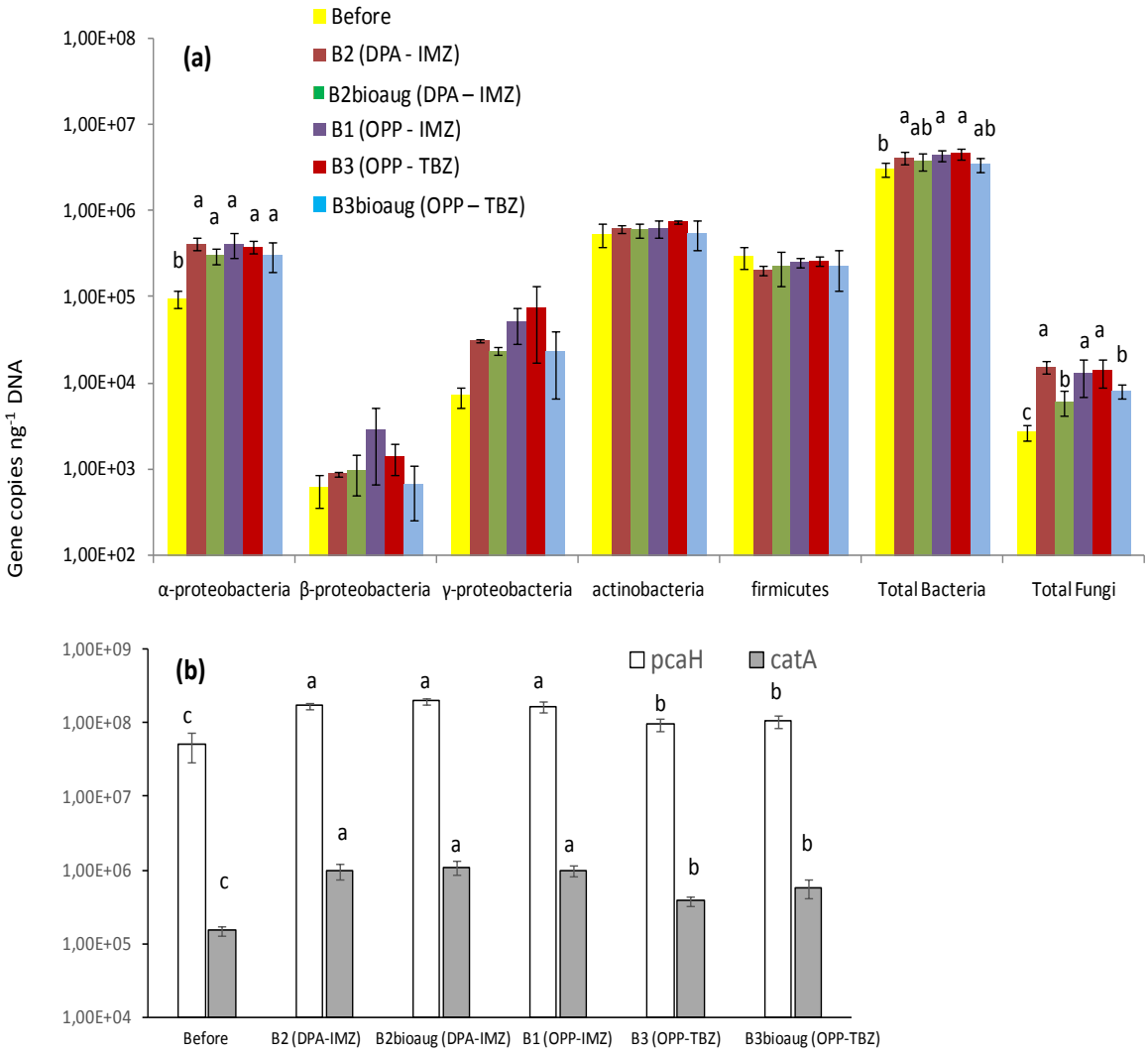

Figure 4.7. The abundance of different bacterial taxa, total bacteria and total fungi (a), and of the catabolic genes *catA* and *pcaH* (b) in the biobed packing material prior to pesticide application (before) and at the end of the study (B1, B2, B3, B2bioaug, B3bioaug). Within

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each microbial group and gene, bars designated by the same letter are not significantly different ($p>0.05$)

4.4. DISCUSSION

Pilot biobeds showed a high depuration efficiency which varied amongst pesticides but exceeded 99.5% in all cases. The higher depuration efficiency against OPP and DPA was attributed to their rapid dissipation in line with the limited persistence of those chemicals in packing materials similar to the one used in the current study (OPP DT_{50s} of 0.34 - 4.7 days; DPA DT_{50s} 1-4.1 days) (see Chapter 2). Regarding TBZ and IMZ although high dissipation levels were achieved, significant amounts were recovered by the biobeds packing material at the end of the study. This is in agreement with the well documented persistence of TBZ and IMZ in soil (Kreuzig et al. 2010: EC,2013) and biobed packing materials demonstrated in Chapter 2 and by Omirou et al. (2012). Residues of TBZ and IMZ retained by the biobed packing material were mostly concentrated at the top biobed layers, in contrast to OPP whose residues were distributed to the whole biobed profile. Similarly Omirou et al. (2012) reported a deeper vertical distribution of OPP residues in the profile of a full-scale biobed compared to IMZ and TBZ which were mostly retained in the top layer (0-20 cm).

The wide acceptance of biobeds relies mainly on their high biodegradation capacity against a broad range of chemical structures found in the different pesticide groups (Castillo et al. 2008). However in cases where biobeds are challenged with mobile (Verhagen et al. 2013) and/or recalcitrant chemicals, like TBZ or IMZ, bioaugmentation could be a useful optimization strategy. A high depuration efficiency of biobeds against OPP and DPA was evident even in the absence of bioaugmentation, in contrast to TBZ for which bioaugmentation significantly advanced their depuration performance. These results suggest that the indigenous microbial community of biobeds has, or develops rapidly, the catabolic capacity to degrade the generally biodegradable OPP and DPA (Karas et al. 2011), whereas it showed a lower capacity to transform the less biodegradable TBZ for which bioaugmentation with a specialized microbial inocula was necessary to maximize depuration. Bioaugmentation of biobeds has been tested in lab-scale experiments via different approaches: the amendment of soil primed for the rapid degradation of one or multiple pesticides (Sniegowski et al. 2014) and inoculation with specific pesticide-degrading bacteria (Verhagen and De Gelder 2013) or white rot fungi (Wiren-Lehr et al. 2001). Our study

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offers the first successful example of bioaugmentation of pilot-scale biobeds with tailored-made bacterial inocula.

Although several studies have verified the high depuration performance of biobeds (De Wilde et al. 2007), the risk associated with the direct environmental disposal of their treated effluents has not been explored. Based on our risk assessment analysis for pome and citrus-fruit packaging plants schemes the disposal of the biobed-treated effluents on an 0.1-ha land area does not entail an unacceptable risk for non-target terrestrial and aquatic organisms. The only exception was shown for TBZ-contaminated effluents produced by pome fruit-packaging plants (Scenario I) where either mitigation measures or bioaugmentation were necessary to alleviate the high risk to fishes which are very sensitive to chronic TBZ exposure (EC, 2013).

One of the main problems hampering the wider implementation of biobeds is the lack of established methods for the decontamination of the spent packing material. These are generally contaminated with considerable pesticide loads and should be depurated prior to their final environmental disposal. TBZ and IMZ residues were recovered in the spent biobed substrate and based on their recalcitrance and their high ecotoxicity (EC, 2009: EC, 2013), decontamination of the spent packing material is essential. We tested different strategies for the decontamination of the spent biobed packing material removed from the pilot biobeds after one operating season. Bioaugmentation was the most effective method for the removal of TBZ and IMZ. It should be noted that no IMZ-degrading bacterial inocula was available and the IMZ-containing spent packing material from biobeds 1 and 2 was inoculated with OPP- and DPA-degrading bacteria since those biobeds had been also treated with OPP and DPA during the study. Our previous studies showed that the OPP and DPA-degrading strains used were not able to degrade IMZ (Perruchon et al. 2015: Perruchon et al. 2016a) so the enhanced dissipation of IMZ in the 'bioaugmentation' treatment cannot be attributed to the inocula used. However bioaugmentation of contaminated soil could induce a general perturbation favoring r-strategists and higher microbial activity (Wenderoth et al. 2003). This in turn might have resulted in a more active co-metabolic biodegradation of IMZ by the non-specialized soil microflora in the bioaugmented composts. Bioaugmentation has not been tested in the past for the decontamination of the spent packing material. This is probably due to the complex pesticides mixture contained in the packing material of on-farm systems, compared to the limited number of chemicals expected to be present in biobeds receiving effluents from fruit packaging plants, making targeted bioaugmentation a feasible approach.

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Composting applied either alone or in combination with bioaugmentation accelerated the dissipation of IMZ and TBZ respectively during the active composting phase. Previous studies by De Wilde et al. (2010) showed that composting resulted in 70% dissipation of bentazon and linuron by a spent biobed substrate. Composting contributes to the dissipation of pesticides via a range of processes with biodegradation being dominant in most cases (Büyüksönmez et al. 2000). Our composting did not lead to the establishment of long thermophilic phases characterized by high temperatures which could have further accelerated pesticides dissipation (Büyüksönmez et al. 1999).

Little is known regarding the composition and the dynamics of the microbial community in biobed systems. An increase in the abundance of total bacteria, α-proteobacteria and fungi was observed at the end of the 160-d operation period suggesting that despite the copious amounts of pesticides applied those systems could support a rich microbial community dominated by actinobacteria, firmicutes and α-proteobacteria. These bacterial taxa are known to be involved in processes relevant to biobed systems such as the decomposition of organic matter coming from plant debris and the degradation of organic pollutants (de Menezes et al. 2015; Wegner and Liesack 2016). Apart from a phylogenetically-rich microbiota, biobeds constitute an artificial ecosystem which support the rapid emergence of novel catabolic traits by the microbial community (Dunon et al., 2013). In line with this we measured a significant increase in the abundance of *catA* and *pcaH* genes at the end of the 160-day period. These genes encode enzymes involved in the transformation of key intermediates produced by the microbial metabolism of natural aromatics and organic pollutants (D'Argenio 1999; Hussain et al. 2011). They have been found in elevated numbers in polluted sites and are considered as indicators of the biodegradation potential of polluted environments (El Azhari et al. 2008). However, bioagumentation did not result in higher copy numbers of these genes at the end of the 160-day period, indicating that the microbial inocula are not key factors for the spread of these genes in the biobed matrix.

4.5. CONCLUSIONS

We explored the biodepuration potential of pilot-scale biobed against effluents from the fruit-packaging industry and addressed practical issues holding back their implementation. Pilot biobeds achieved effective depuration of the relevant agro-industrial wastewater producing treated effluents whose environmental disposal into a pre-defined soil disposal

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area entails no unacceptable environmental risk. The lower depuration efficiency of pilot biobeds for TBZ was alleviated by bioaugmentation with TBZ-degrading bacteria, a method feasible for biobed systems receiving effluents from fruit packaging plants which contain a limited number of specific pesticides. Bioaugmentation was also the most potent method for the decontamination of spent packing material with composting or even long-term storage at ambient temperature being a valuable alternative in the absence of pesticide-degrading inocula.

4.6. REFERENCES

Büyüksönmez F., Rynk R., Hess T.F., Bechinski E., (1999). Occurrence, degradation and fate of pesticides during composting Part I: composting, pesticides, and pesticide degradation. *Comp. Sci. Utiliz.* 7 66-82

Büyüksönmez F., Rynk R., Hess T.F., Bechinski E., (2000). Literature review: Occurrence, degradation and fate of pesticides during composting: Part II: Occurrence and fate of pesticides in compost and composting systems. *Comp. Sci. Utiliz*. 8 61-81.

Campo, J., Masia, A., Blasco, C., Pico, Y., (2013). Occurrence and removal efficiency of pesticides in sewage treatment plants of four Mediterranean River Basins. *J. Haz. Mater*. 263P, 146-157

Castillo L.E., E. Martinez, C. Ruepert, C. Savage, M. Gilek, M. Pinnock, E. Solis, (2006). Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. Sci. Total Environ. 367 418-432

Castillo M.d.P., Torstensson L., Stenstrom J., (2008). Biobeds for environmental protection from pesticide use - A review. *J Agric. Food Chem*. 56 6206-6219

D'Argenio D.A., Vetting M.W., Ohlendorf D.H., Ornston L.N., (1999). Substitution, insertion, deletion, suppression, and altered substrate specificity in functional protocatechuate 3,4- dioxygenase. *J. Bacteriol* 181 6478–6487

de Menezes A.B., Prendergast-Miller M.T., Poonpatana P., Farrell M., Bissett A., Macdonald L.M., (2015). C/N ratio drives soil actinobacterial cellobiohydrolase gene diversity. *Appl. Environ. Microbiol*. 81 3016-3028

De Wilde T., Debaer C., Ryckeboer J., Springael D., Spanoghe P., (2010). The influence of small-and large-scale compostingon the dissipation of pesticide residues in a biopurification matrix. *J. Sci. Food Agric*. 90 1113–1120

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

De Wilde T., Spanoghe P., Debaer C., Ryckeboer J., Springael D., Jaeken P., (2007). Overview of on-farm bioremedation systems to reduce the occurence of point source contamination. *Pest Manag. Sci.* 63 111-128.

Dunon V.S.K., Bers K., Lavigne R., Smalla K., Springael D., (2013). High prevalence of IncP-1 plasmids and IS1071 insertion sequences in on-farm biopurification systems and other pesticide polluted environments. *FEMS Microbiol Ecol* 86 415-431

El Azhari N., Bru D., Sarr A., Martin-Laurent F., (2008). Estimation of the density of the protocatechuate degrading bacterial community in soil by real-time PCR. *Eur. J. Soil Sci.* 59 665-673

El Azhari N., Bru D., Sarr A., Martin-Laurent F., (2008). Estimation of the density of the protocatechuate degrading bacterial community in soil by real-time PCR. *Eur. J. Soil Sci.* 59 665-673

El Azhari N., Devers-Lamrani M., Chatagnier G., Rouard N., Martin-Laurent F., (2010). Molecular analysis of the catechol-degrading bacterial community in a coalwasteland heavily contaminated with PAHs. *J. Haz. Mater*. 177 593-601

European Commission (EC), (2001). Review report for the active substance thiabendazole finalised in the Standing Committee on Plant Health at its meeting on 12 December 2000 in view of the inclusion of thiabendazole in Annex I of Directive 91/414/EEC.7603/VI/97-final

European Commission (EC), (2009). Draft Assessment Report, Initial risk assessment provided by the rapporteur member state the Netherlands for the existing substance imazalil. Volume 1 pp. 1-151

European Commission (EC), (2010). Commission Directive 2010/77/EU of 10 November 2010 amending Council Directive 91/414/EEC as regards the expiry dates for inclusion in Annex I of certain active substances. L293/48

European Commition (EC), (2013). Draft renewal assessment report prepared according to the Commission Regulation (EU) N.1141/2010, Second programme for the renewal of the inclusion of the following active substance under Regulation (EC) 1107/2009, Thiabendazole, Vol. 1, Report and proposed decision

European Commition EC, (2002). Guidance document on terrestrial ecotoxicology. Draft Working Document SANCO/10329/2002 rev.final, October 17

European Food Safety Authority (EFSA) (2012), Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine. EFSA J. 10 1426

European Food Safety Authority (EFSA), (2008). Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217 1-67

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

European Food Safety Authority (EFSA), (2013). PPR Panel, Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA J. 11:3290, 268 pp.

Fierer N., Jackson J.A., Vilgalys R., Jackson R.B., (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* 71 4117-4120

FOCUS (2001), Surface Water Scenarios in the EU Evaluation Process under 91/414/EEC.Report of the FOCUS Working Group on Surface Water Scenarios, EC Document Reference SANCO/4802/2001-rev.2.245 pp.

Garcia Portillo M., Avino E.S., Vicente J.O., De Andres R.L., Jimenez M.A.S., Blanco J.P.L., (2004). Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States Patent, US 6,709,585 B1 p. 9

Harwood C.S., Parales R.E., (1986). β-ketoadipate pathway and the biology of self-identity, *Ann. Rev. Microbiol*. 50 553–590

Hussain S., Devers-Lamrani M., El Azhari N., Martin-Laurent F., (2011). Isolation and characterization of an isoproturon mineralizing *Sphingomonas* sp. strain SH from a French agricultural soil. *Biodegradation* 22 637-650

Karanasios E., Tsiropoulos N., Karpouzas D.G., (2012). On-farm biopurification systems for the depuration of pesticide-wastewaters: recent advances and future perspectives. *Biodegradation* 23 787-802

Karanasios E., Tsiropoulos N., Karpouzas D.G., Menkissoglu-Spiroudi U., (2010). Novel biomixtures based on local Mediterranean ligninocellulosic materials: evaluation for use in biobeds. *Chemosphere* 80 914-921

Karas P., Perucchon C., Exarhou C., Ehaliotis C., Karpouzas D.G., (2011). Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. *Biodegradation 2*2 215-228.

Kreuzig R., Hartmann C., Teigeler J., Höltge S., Cvetkovic B., Schlag P., (2010). Development of a novel concept for fate monitoring of biocides in liquid manure and manured soil taking ^{14}C-imazalil as an example. *Chemosphere* 79 1089-1094

MacRae IC, and Alexander M, (1965). Microbial degradation of selected herbicides in soil. *J. Agric. Food Chem*, 13:72-76.

Magkos, F, Arvaniti, F and Zampelas, A. (2006) Organic Food: Buying more safety or just peace of mind? A critical review of the literature. *Crit. Rev. Food Sci. Nutr., 46*, 23-55

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Mari M., Bertolini P. and Pratella G.C. (2003). Non-conventional methods for the control of post-harvest pear diseases: a review. *Journal of Applied Microbiology*, 94, 761–766.

Maruyama T., Komatsu C., Michizoe J., Sakai S. and Goto M. (2007). Laccase-mediated degradation and reduction of toxicity of the postharvest fungicide imazalil. *Process Biochemistry*, 42, 459–461

Masiá, A., Campo, J., Vázquez-Roig, P., Blasco, C., Picó, Y., (2013). Screening of currently used pesticides in water, sediments and biota of the Guadalquivir River Basin (Spain). *J. Hazard. Mater*. 263 (Part 1), 95–104.

McIntyre T, (2003). Phytoremediation of heavy metals from soils. *Advance Biochemical Engineering Biotechnology*, 78:97–123.

Mokrini A., Oussi D. and Esplugas S., (1981). "Oxidaiton of Aro- matic Compounds with UV Radiation/Ozone/Hydrogen Peroxide," *Water Science and Technology*, Vol. 35, (4), 95-102 65

Monaci E, Coppola L, Casucci C, Perucci P and Vischetti C, (2009). Retention capacity of an organic bio-mixture against different mixtures of fungicides used in vineyards. *J Environ Sci Health B* 44:724–729.

Muto N. Hirai H., Tanaka T., Itoh N. and Tanaka K. (1997). Induction and inhibition of cytochrome P450 isoforms by imazalil, a food contaminant, in mouse small intestine and liver. *Xenobiotica*, 27, 1215-1223.

Omirou M., Dalias P., Costa C., Papastefanou C., Dados A., Ehaliotis C., Karpouzas D.G., (2012). Exploring the potential of biobeds for the depuration of pesticide-contaminated wastewaters from the citrus production chain: laboratory, column and field studies. *Environ. Pollut.* 166 31-39

Perruchon C., Chatzinotas A., Omirou M., Vasileiadis S., Menkissoglou-Spiroudi U., Karpouzas D.G. (2016b). Isolation of a bacterial consortium able to degrade the fungicide thiabendazole: the key role of a *Sphingomonas* phylotype. *Appl. Microbiol. Biotechn*. (submitted)

Perruchon C., Patsioura V., Vasiliadis S., Karpouzas D.G., (2016a). Isolation and characterisation of a *Sphingomonas strain* able to degrade the fungicide ortho-phenylphenol. *Pest Manag. Sci.,* 72:113-124

Perruchon C., Zouborlis S., Batianis C., Papadopoulou E.S., Ntougias S., Vasileiadis S., Karpouzas D.G., (2015). Isolation of a diphenylamine-degrading bacterium and characterization of its metabolic capacities, bioremediation and bioaugmentation potential. *Environ. Sci. Pollut. Res.* 22: 19485-19496

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

Rousidou C., Papadopoulou E.S., Kortsinidou M., Giannakou I.O., Singh B.K., Menkissoglu-Spiroudi U., Karpouzas D.G., (2013). Bio-pesticides: Harmful or harmless to ammonia oxidizing microorganisms? The case of a *Paecilomyces lilacinus*-based nematicide. *Soil Biol. Biochem.* 67, 98-105

Sanchez Perez J.A., Carra A., Sirtori C. , Aguera A., Esteban B., (2014). Fate of thiabendazole through the treatment of a simulated agro-food industrial effluent by combined MBR/Fenton processes at mg/L scale. *Water Res*. 5155-63

Sirtori C., Aguera A., Carra I.,. Sanchez Perez J.A, (2014). Identification and monitoring of thiabendazole transformation products in water during Fenton degradation by LC-QTOF-MS. *Anal. Bioanal. Chem*. 406 5323–5337

Sniegowski K., Springael D., (2014). Establishment of multiple pesticide biodegradation capacities from pesticide-primed materials in on-farm biopurification system microcosms treating complex pesticide-contaminated wastewater. *Pest Manag. Sci.* 71 986-995

Torstensson L., (2000). Experiences of biobeds in practical use in Sweden—a method for elimination of point-source contamination. *Pest. Outl.* 11 206–211

Valero D., Serrano M., (2010). Postharvest biology and Technology for preserving fruit quality. CRC press Taylor & Francis Group, New York, USA

Verhagen P., De Gelder L., Boon N., (2013). Inoculation with a mixed degrading culture improves the pesticide removal of an on-farm biopurification system. *Cur. Microbiol*. 67 466-471

von Wiren-Lehr S., Castillo MdP., Torstensson L., Scheunert I., (2001). Degradation of isoproturon in biobeds. *Biol. Fert. Soils* 33 535–540.

Wegner C.E., Liesack W., (2016). Microbial community dynamics during the early stages of plant polymer breakdown in paddy soil. *Environ Microbiol* doi: 10.1111/1462-2920.12815 (in press)

Wenderoth D.F., Rosenbrock P., Abraham W-R., Pieper D.H., Hofle M.G., (2003). Bacterial community dynamics during biostimulation and bioaugmentation experiments aiming at chlorobenzene degradation in groundwater. *Microb. Ecol*. 46 161-176

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Chapter 5

Final Conclusions and Future Perspectives

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5.1. FINAL CONCLUSIONS

Postharvest treatment of fruits with pesticides guarantees their protection from fungal infestations and physiological disorders during storage. However, it leads to the production of large volumes of pesticide-contaminated effluents whose direct environmental release would compromise the integrity of natural resources (Castillo et al. 2006). This is exemplified by the high aquatic toxicity of the pesticides used in this industrial sector like TBZ (EFSA 2014) IMZ (EFSA 2010a), OPP (EFSA 2008), EQ (EFSA 2010b) and DPA (EFSA 2012. The need for the treatment of those effluents is stressed in the relevant pesticide regulatory documents which state that member-states should ensure *that appropriate waste management practices to handle the waste solution remaining after application are put in place* (EC 2010). In accordance with the regulatory framework implemented by the EC, the present thesis presented a gradually scaled up approach to assess the potential of biobeds for the treatment of effluents from the fruit packaging plants.

Initial laboratory dissipation studies with anaerobically digested sewage sludge and liquid aerobic sewage sludge showed that municipal wastewater treatment plants are expected to effectively remove OPP, DPA, EQ and its oxidation products but not TBZ and IMZ. These first results stressed the need for the implementation of more efficient but still simple and low-cost depuration methods to effectively remove all pesticides contained in those effluents. A first step towards the optimization of the dissipation potential of biobed systems was the selection of a biobed packing material characterised by high dissipation and sorption capacity against the relevant pesticides. SMS was a key component of the packing materials tested, as a by product of mushroom units which is available at large amounts and no cost. Laboratory studies showed that a biobed packing material composed of SMS:Straw:Soil in volumetric rations of 50:25:25 showed the highest dissipation potential and high retention capacity for the pesticides tested, particularly of the recalcitrant TBZ and IMZ, suggesting that biobeds packed with such organic biomixtures could effectively depurate the wastewaters from the fruit-packaging industry.

Based on the results of Chapter 2, a leaching column study was undertaken to evaluate the capacity of the best performing biomixture (in Chapter 2) to retain and dissipate OPP and IMZ under a high hydraulic loading scheme which represents a realistic wastewater production pattern of a citrus fruit packaging plant. OPP and IMZ were selected as pesticides with contrasting characteristics regarding their persistence and mobility. In addition the role of *P. ostreatus* contained in SMS on the depuration capacity of biobeds was assessed by

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incorporating in the leaching study FMS. Our findings suggested that the optimized SMS-rich packing material could effectively retain OPP and IMZ under realistic high hydraulic loadings. Despite its known capacity to degrade organic pollutants, *P. ostreatus* present in the SMS did not seem to actively contribute to the degradation of those fungicides.

Based on the results of the optimization laboratory and column studies described in Chapters 2 and 3, pilot biobeds were constructed and their depuration performance against the pesticides contained in the effluents from fruit packaging plants was evaluated in Chapter 4. Their performance was evaluated under pesticide treatment scenarios relevant to pome and citrus fruit packaging plants, while optimization of their depuration performance was employed with bioaugmentation with tailored made bacterial inocula for OPP, DPA and TBZ. Biobeds showed high depuration performance for all pesticides tested which reached >99.5% for the most persistent chemicals IMZ, TBZ and >99.9% for the less persistent OPP and DPA. The depuration performance of biobeds against TBZ was maximized (>99.9%) upon bioaugmentation with a TBZ-degrading proteobacterial consortium. In the absence of IMZ-degrading inocula, bioaugmentation of biobed systems with tailored-made inocula destined to rapidly degrade recalcitrant pesticides like TBZ is a valuable optimization strategy and feasible in view of the limited number of pesticides contained in those agro-industrial effluents.

To provide a holistic assessment of the implementation potential of biobed systems in fruit packaging plants we seeked in Chapter 4 to address issues which hold back the implementation even of on-farm biobed systems: *(a) Is the quality of biobeds-treated effluents high enough to allow their direct environmental release without imposing unacceptable risks for receiving ecosystems?* and *(b) how could we decontaminate the spent biobed packing material removed from the biobeds at the end of the life span of biobed systems?*. Risk assessment analysis based on the depuration performance of our pilot biobeds provided an answer to the first question. Thus risk assessment based on the current regulatory framework showed that the treatment of wastewaters from citrus (scenario I) and pome (scenario II) packaging plants by biobeds lead to high quality effluents whose direct environmental disposal into a pre-defined soil disposal area entails no environmental risk. The only exception was TBZ-contaminated effluents produced by pome fruit packaging plants where risk mitigation by bioaugmentation or disposal of the effluents in a larger disposal area is required. Spent biobed packing material from the pilot biobeds contained high concentrations of IMZ and TBZ. Different strategies including storage at ambient temperature composting, bioaugmentation and the combination of the last two were tested

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for the decontamination of the spent biobed packing material. Bioaugmentation was the most potent method with composting or storage at ambient temperature for nearly 5 months were valuable alternative in the absence of pesticide-degrading inocula.

Finally the thesis provided pioneering data for the composition of the microbial community in biobeds systems for which still little is known. Molecular analysis revealed that despite the heavy load of pesticides discharged biobeds could support a rich microbial community dominated by actinobacteria, firmicutes, a-proteobacteria and enriched in genes associated with the catabolism of aromatic compounds (*catA* and *pcaH*).

Overall this thesis provides a comprehensive lab-to-pilot scale assessment of the potential of biobeds to depurate pesticide-contaminated effluents from fruit-packaging plants. Our results verified our initial hypothesis that biobeds could be used for the treatment of those wastewaters and provide a viable, effective, cheap and sustainable alternative to the currently followed practices which constitute a significant environmental and economic burden for this agro-industrial sector.

5.2. FUTURE PERSPECTIVES

The current thesis provided compeling evidence for the depuration efficiency of biobeds against wastewaters from the fruit packaging industry. Biobeds is a ready to be implemented solution in fruit packaging plants and the next step towards their market uptake is (a) their certification by relevant authorities as an acceptable wastewater treatment method and (b) the establishment and operation of the first full-scale biobed systems in a fruit packaging plant in Greece. However new research challenges remain to be explored:

a) the further expansion of the uses of biobeds to treat (a) wastewaters from peach and kiwi fruit packaging where alternative fungicides like iprodione and fludioxonil are used and (ii) from other agro-industries dealing with seed-coating and bulb dissinfection where a range of fungicides (metalaxyl-M, fludioxonil, chlorothalonil) and insecticides (cypermethrin, deltamethrin, fluxapyroxad) are utilized.

b) the optimization of bioaugmentation strategies with the inclusion of novel tailored - made inocula targeting persistent molecules like IMZ which is heavily used by most fruit packaging plants in Europe. It should be noted that the current inventory of the Laboratory of the Plant and Environmental Biotechnology, Department of

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Biochemistry and Biotechnology, University of Thessaly, includes bacteria able to degrade TBZ, OPP, DPA and iprodione, all used in fruit packaging plants in Europe.

c) the in-depth investigation of the functional and structural characteristics of the microbial community of biobed systems which drive the high biodegradation capacity of these systems for a range of different pesticide compounds. Within this frame biobeds will be explored as a valuable source of new pesticide catabolic enzymes using functional metagenomic tools.

5.3. REFERENCES

Castillo L.E., Martinez E., Ruepert C., Savage C., Gilek M., Pinnock M., Solis E., (2006). Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. *Sci. Total Environ*. 367 418-432

European Commicion (EC), (2010). Commission Directive 2010/77/EU of 10 November 2010 amending Council Directive 91/414/EEC as regards the expiry dates for inclusion in Annex I of certain active substances. L293/48

European Food Safety Authority (EFSA), (2008). Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. *EFSA Sci. Rep*. 217 1-67

European Food Safety Authority EFSA, (2010a). Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil. *EFSA J*. 8 1526

European Food Safety Authority (EFSA) (2010b). Conclusion on the peer review of the pesticide risk assessment of the active substance ethoxyquin. *EFSA J*. 8 1710

European Food Safety Authority (EFSA), (2012). Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine. *EFSA J*. 10 1426

European Food Safety Authority (EFSA) (2014). Peer review of the pesticide risk assessment of the active substance thiabendazole. *EFSA J*. 12 3880

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The potential of organic substrates based on mushroom substrate and straw to dissipate fungicides contained in effluents from the fruit-packaging industry – Is there a role for *Pleurotus ostreatus*?

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ABSTRACT

Citrus fruit-packaging plants (FPP) produce large wastewater volumes with high loads of fungicides like *ortho*-phenylphenol (OPP) and imazalil (IMZ). No methods are in place for the treatment of those effluents and biobeds appear as a viable alternative. We employed a column study to investigate the potential of spent mushroom substrate (SMS) of *Pleurotus ostreatus*, either alone or in mixture with straw and soil plus a mixture of straw /soil to retain and dissipate IMZ and OPP. The role of *P. ostreatus* on fungicides dissipation was also investigated by studying in parallel the performance of fresh mushroom substrate of *P. ostreatus* (FMS) and measuring lignolytic enzymatic activity in the leachates. All substrates effectively reduced the leaching of OPP and IMZ which corresponded to 0.014–1.1% and 0.120–0.420% of their initial amounts respectively. Mass balance analysis revealed that FMS and SMS/Straw/Soil (50/25/25 by vol) offered the most efficient removal of OPP and IMZ from wastewaters respectively. Regardless of the substrate, OPP was restricted in the top 0–20 cm of the columns and was bioavailable (extractable with water), compared to IMZ which was less bioavailable (extractable with acetonitrile) but diffused at deeper layers (20–50, 50–80 cm) in the SMS- and Straw/Soil-columns. PLFAs showed that fungal abundance was significantly lower in the top layer of all substrates from where the highest pesticide amounts were recovered suggesting an inhibitory effect of fungicides on total fungi in the substrates tested. Our data suggest that biobeds packed with SMS-rich substrates could ensure the efficient removal of IMZ and OPP from wastewaters of citrus FPP.

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1. Introduction

Fruit-packaging plants (FPP) constitute a serious point-source contamination of natural water resources with pesticides like imazalil (IMZ) and *ortho*-phenylphenol (OPP). These are used mostly in citrus FPP for the control of fungal infestations during storage (Kinay et al., 2007). IMZ is toxic to aquatics, persistent in soil with DT50s of 44-137 days (Environmental Protection Agency (EPA) USA, 2003; European Commission (EC), 2009) and of limited mobility in soil (Kreuzig et al., 2010). On the other hand, OPP is non persistent with $DT50_{soil} < 1$ d (European Food Safe Authority (EFSA), 2008), relatively mobile in soil (Zheng et al., 2011), non-toxic to mammals and birds but highly toxic to aquatics (European Food Safe Authority (EFSA), 2008). Monitoring studies in water bodies adjacent to areas where FPP operate reported the presence of high concentrations of IMZ and OPP (Castillo et al., 2006; Jonkers et al., 2010). Considering the environmental risk imposed by the mishandling of pesticides used in FPP, the European Commission (EC) granted authorization for use until 2021 and 2019 for OPP and IMZ respectively under the clause that *member states should pay particular attention to ensure that appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place* (European Commission (EC), 2009; European Commission (EC), 2010). Although several studies have addressed this issue using physicochemical approaches like photocatalysis (Khodja et al., 2001) or adsorption (Garcia-Portillo et al., 2004), their full

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implementation was hampered by their high cost, high engineering needs for operation and maintenance and the risk for production of toxic intermediates which require further treatment.

Biological treatment of those effluents could be a possible solution either in the form of bioreactors inoculated with tailored-made pesticide-degrading inocula (Perruchon et al. 2015) or through biobeds. The latter are simple on-farm systems packed with organic materials like soil/straw/peat (Castillo et al., 2008) or compost (Omirou et al., 2012) or spent mushroom substrate (SMS) (Karanasios et al., 2010). The latter has been proposed as a key component of biobed packing material (biomixtures) that could promote their biodepuration capacity (Gao et al., 2015) and at the same time facilitate the sustainable recycling of this waste produced by mushroom units (Herrero-Hernandez et al., 2011; Phan and Sabaratnam, 2012). Recent studies by our group showed that SMS-rich substrates were the most efficient in dissipating persistent fungicides like thiabendazole and IMZ used in FPP (Karas et al., 2015). The exact mechanism through which SMS accelerates the dissipation of pesticides in biomixtures is not yet known and the direct involvement of the white rot fungi (i.e. *Pleurotus ostreatus* or *Agaricus bisporus*) present in the SMS is not clear. For example, Garcia-Delgado et al. (2015) showed that soil incorporation of sterilized SMS of *A. bisporus* accelerated the degradation of 3-ring PAHs via stimulation of heterotrophic bacteria while incorporation of non sterilized SMS enhanced the removal of 5,6-ring PAHs stressing the involvement of *A. bisporus* in the removal high molecular weight PAHs. Knowledge of the key microbial component of the SMS and of their role in the dissipation of pesticides would allow the directed optimization of SMS application in biobed systems.

Full scale biobeds packed with a compost-based biomixture and modified to cope with the high wastewater volumes produced by citrus FPP were successfully tested by Omirou et al. (2012). However little is known regarding the processes controlling the dissipation of the pesticides contained in these effluents and their interactions with the microbial community of biomixtures. Knowledge of the processes which dominate the depuration of those effluents is essential. Biological systems where degradation predominates over adsorption are preferable (Karanasios et al., 2012) since the opposite might result in the accumulation of high pesticide loads in the biomixture which when replaced will require detoxification increasing the overall implementation cost of biobeds (De Wilde et al., 2010). In turn information on the interactions of pesticides with the microbiota colonizing biobeds will facilitate the optimized operation of those systems through prevention of toxicity effects and maximization of the microbial catabolic activity. Previous studies have reported a clear correlation between phenoloxidase activity and pesticide degradation in low pH biomixtures which favor fungal activity (Castillo and Torstensson, 2007), whereas others did not observe any correlation between microbial indicators and pesticide degradation (Karanasios et al., 2010).

We employed a leaching column study aiming to (a) evaluate the capacity of different biomixtures composed of SMS, straw and soil mixed at different various combinations to depurate effluents containing IMZ and OPP; (b) explore the contribution of *P. ostreatus* from SMS on pesticides dissipation by comparison with the depuration capacity of fresh mushroom substrate (FMS) of *P. ostreatus*; comparison between FMS and SMS depuration efficiencies is essential, as it allows for comparisons at different physiological colonization states of *P. ostreatus* and (c) investigate the interaction of those pesticides with the microbial community in biobed systems.

2. Materials and methods

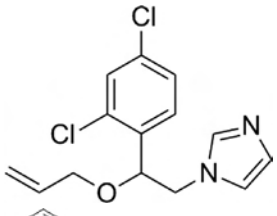
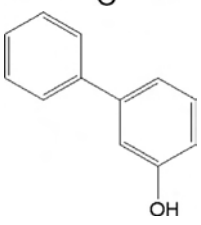
2.1. Pesticides

Analytical standards of IMZ (99.8%, Pestanal®) and OPP (99.9%, Pestanal®) were purchased from Fluka. Pesticides stock solutions in methanol were prepared (1000 mg L^{-1}) from analytical standards and used for analytical purposes. Commercial pesticides formulation like FUNGAZIL® 50EC (IMZ) and FOAMER®20EC (OPP) were used for the preparation of the aqueous pesticides solutions discharged on the leaching columns. The main physicochemical and environmental properties of the two pesticides studied are shown in Table 1.

2.2. Organic substrates

SMS, soil and straw were mixed in different volumetric ratios to prepare two of the four substrates tested in the leaching column study: SMS/Straw/Soil (50/25/25 by volume) and Straw/Soil (75/25

Table 1
The chemical structure, physicochemical properties and environmental fate parameters of imazalil (IMZ) and *ortho*-phenylphenol (OPP).

Pesticides	Chemical structure	Water solubility (mg L^{-1})	Vapour pressure (Pa) at 25 °C	$DT_{50water}$ (d)	DT_{50soil} (d)	K_{foc} (ml g^{-1})
Imazalil[a]		184	1.58×10^{-4}	Stable at pH 5-9[b]	41–135	2080–8150
Ortho-phenylphenol[c]		560	0.906	Stable at pH 4-9[b]	0.11	252–393

[a] European Food Safe Authority (EFSA) 2010 Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil. EFSA Journal 8(3):1526.
[b] < 10% degradation in 5 days.
[c] European Food Safe Authority (EFSA) 2008 Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217: 1–67.

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Table 2
Physicochemical properties of the substrates used to assess the dissipation and sorption of the pesticides studied.

Substrates	pH	Organic matter (%)	Total N (%)	C/N
Soil[a]	7.55	1.05	0.13	8.1
Straw	7.15	38.9	0.80	48.6
SMS	6.83	35.5	1.20	29.6
FMS	5.50	42.0	0.72	58.3
SMS/Straw/Soil (50:25:25)	7.10	8.82	0.54	16.3
Straw/Soil (75:25)	7.35	3.26	0.26	12.5

[a] Soil texture: Sand 37%, Clay 31%, Silt 32% (clay loam).

by volume). The soil used was collected from a farm of the National Agricultural Research Foundation of Greece in Larissa, Greece. It was sieved (2 mm) and stored at 4 °C prior to use. Wheat straw was chopped into small pieces (1–3 cm). SMS was obtained from a *P. ostreatus* edible mushroom unit (Mpoulogeorgos-Meteora, Trikala, Thessaly) after two harvest cycles, while fresh mushroom substrate of *P. ostreatus* (FMS) was obtained from the company DIRFYS, Euvoia, Greece. Mushroom substrates were chopped into small pieces (1–2 cm long) with a blender and stored at 4 °C for a maximum period of 10 days until further use. The physicochemical properties of all materials used are given in Table 2. FMS was more acidic than SMS. The latter was characterized by lower C/N ratio which is attributed to the gradual decomposition of the more easily degradable fractions of its organic matter, mostly hemicellulose and cellulose (Koutrotsios et al., 2014), and straw colonization byprotein-rich fungal biomass

2.3. Leaching column study

The capacity of different organic substrates to remove OPP and IMZ from wastewaters was evaluated in a leaching column study. In total 12 PVC columns of 12.5 cm i.d. and 90 cm long were used. Triplicate columns for each substrate were prepared: SMS, Straw/Soil, SMS/Straw/Soil and FMS. A metal sieve was installed at the bottom of all columns to prevent passage of the packing material in the drainage of the columns. The columns were packed with the following materials from the bottom to the top: a) a 7-cm layer of thoroughly washed gravel (2–3 cm i.d); b) an 80 cm layer of biomixture and c) a 3-cm layer of well washed gravel (2–3 cm i.d.) to ensure uniform wetting and distribution of the pesticide solution into the biomixture. Pesticide solutions applied to the columns were loaded in 2 L separatory funnels with their outlet linked to a plastic tube through which pesticides solutions were discharged at the top of the columns. A flow controller was installed on the plastic tube to adjust solution flow rate and the flow on each column was individually calibrated (flow rates are given below) to ensure uniform delivery of pesticides solution on all columns. A plastic funnel was placed at the bottom of each column to collect the leachates in amber 2.5-L bottles. Right before pesticides application the columns were saturated with water and were left to drain for 4 days.

Columns were treated in a sequential mode with aqueous solutions of OPP (first) and IMZ (secondly). The sequential treatment scheme employed simulated a realistic wastewater production scenario from a citrus FPP (treating annually approximately 15000 tones of citrus fruits) treating oranges with the fungicide OPP for a period of 3 months (January to March) followed by the application of IMZ to tangerines (April). During the application of OPP two types of wastewater are produced: i) a dense OPP aqueous solution (5 g L^{-1}) produced three times per season (approximate total volume 14 m^3) which is expected to be discharged on the biobeds and ii) a diluted wastewater containing 5 mg L^{-1} of OPP (fruits rinsates) which is produced daily at volumes of 25 m^3 and is currently land-filled in nearby field sites. Regarding IMZ, its application later in the season results in the production of approximately 10 m^3 of dense effluent (1.2 g L^{-1}). Based on the above industrial scenario, the dense OPP- and IMZ-containing wastewaters are discharged in a 45 m^2 biobed system of 1 m depth.

In the column experiment employed, columns were initially treated for a period of 60 days (day 1 to day 60) with aqueous solutions of OPP (2.6 g L^{-1}). During this period, OPP solutions were delivered continuously (24 h d^{-1}) onto the columns at a flow rate of 12 ml h^{-1} resulting in a total wastewater and OPP discharge per column of 17.25 L and 44.85 g respectively (4062.5 g of OPP per m^3 of substrate). At the end of the 60-day OPP treatment period, the columns were left to drain for 5 days (days 61 to 65) followed by application of aqueous solutions of IMZ (0.275 g L^{-1}) for a further period of 46 days (days 66 to 112). IMZ aqueous solutions were delivered to the columns every other day at a flow rate of 17 ml/h (24 h/d) resulting in a total wastewater and IMZ discharge per column of 9.4 L and 2.6 g respectively (235.5 g of IMZ per m^3 of substrate). It should be noted that fresh OPP solutions were loaded on the separatory funnels every 4 days, analysis of samples from those solutions at day 1 and at day 4 showed that OPP was stable under the experimental conditions tested (less than 10% losses observed during the storage period). Leachates were collected from the bottom of the columns on 3-day intervals. At each sampling day, the volume of the leachate collected was measured and a 100-ml sub-sample was transferred into plastic bottles which were stored at −20°C until analyzed. A 10-ml fraction from each leachate sample was removed before storage and used for the measurement of enzymatic activities.

Upon completion of the treatment period, the amounts of OPP and IMZ retained in the packing materials of the columns were determined in order to perform a mass balance analysis. Leaching columns were dismantled and their content was divided into three layers (0–20, 20–50 and 50–80 cm). The amounts of pesticides retained in the different layers of the leaching columns were extracted by sequential extractions with water and acetonitrile as described below. The total amount of pesticide recovered by the substrate at the end of the study, plus the amount of pesticide leached were deducted from the total pesticide amount applied on the columns and this amount was considered as 'dissipated'. This was a lump process including degradation and non extractable residues formation (bound residues).

2.4. Pesticide residue analysis

Extraction of IMZ and OPP from water samples was performed by mixing 2 ml of leachate with 8 ml of methanol or acetonitrile respectively. The mixture was vortexed for 1–2 minute and the extract was passed through a 0.45 μm syringe filter (PTFE Syringe Filter, Whatman) prior to analysis. In all cases tests at three fortification levels (0.2, 2 and 20 mg L^{-1}) showed recoveries > 80%.

Extraction of pesticides from the packing material of the columns were sequentially performed initially with water and subsequently with an organic solvent. The water extracted pesticide residues represented the fraction which is readily available, whereas the fraction extracted with the organic solvent constitutes the less available fraction. Based on this, 4 g of biomixture were mixed with 40 ml of ddH_2O and extracted *via* agitation in an orbital shaker at 200 rpm for 30 min. The extract was centrifuged for 5 min at 7000 rpm and the clear supernatant was collected. Aqueous extraction was repeated two more times and the supernatants from each extraction step were combined (120 ml water extract) and subsequently extracted as described above for aqueous samples. Upon the third aqueous extraction cycle the solid substrate remaining in the flasks were extracted with 10 ml of acetonitrile *via* agitation for 90 min in an orbital shaker as

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described above. The extract was subsequently centrifuged as above and the clear supernatant was collected, filtered through a syringe filter (0.45 μm PTFE, Whatman) and stored at −20 °C for subsequent HPLC analysis. Tests at three fortification levels (0.2, 2 and 20 mg kg^{-1}) for the different substrates showed recoveries > 80% in all cases.

Pesticide residues were analyzed in an HPLC-UV Marathon III system equipped with a Grace Smart RP C18 (150 mm × 4.6 mm) column. OPP residues were detected at 254 nm using a mobile phase of 55:44.5:0.5 of acetonitrile:water:25%NH_3 solution (by volume) while IMZ was detected at 204 nm using a mobile phase of 80:20 methanol: 0.25% NH_3 solution (by volume). The flow rate was always 1 ml min^{-1} and the retention times of OPP and IMZ were 3.4 and 5 min respectively. The limit of quantification (LOQ) for the two pesticides in solid substrates and water samples were 0.08 mg kg^{-1} and 0.04 mg L^{-1} respectively.

2.5. Microbial measurements

2.5.1. Enzymatic activity measurements in the leachates

The activity of laccase and manganese peroxidase, commonly produced by *P. ostreatus*, were determined in the leachates of all columns throughout the experimental period. Laccase activity was determined spectrophotometrically at 425 nm by oxidation of 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (Bourbonnais and Paice, 1990). The activity of manganese peroxidase was determined spectrophotometrically at 590 nm by oxidative coupling of 3-methyl-2-benzothiazoline hydrazone and 3-dimethylaminobenzoic acid (Ngo and Lenhoff, 1980).

2.5.2. Phospholipid Fatty Acid Analysis (PLFAs)

Samples leaching study were analyzed for their content in microbial Fatty Acids Methyl Esters (FAME) as described by Papadopoulou et al., (2011). For analysis of the data obtained by the PLFA analysis, FAMEs 15:0, a15:0, i15:0, i16:0, 17:0, i17:0 were used as indicators of Gram positive (GP) bacteria, 18:1ω9*cis/trans*, 16:1ω7, cy17:0, cy19:0 were used as indicators of Gram negative (GN) bacteria, 16:0 was considered as a general microbial indicator, 18:2ω6,9*cis/trans* were considered as indicators of fungi and 10Me16:0, 10Me17:0, and 10Me18:0 were considered as indicators of actinobacteria (Frostegård and Bååth, 1996; Findlay, 2004).

2.6. Statistical analysis

The data obtained from mass balance analysis, pesticides distribution in the column layers and total PLFA yields were subjected to two way ANOVA followed by Tukey's posthoc test to identify significant differences between the substrates studied. Relative abundance data of FAME indicators of GP and GN bacteria, actinobacteria and fungi were subjected to one way ANOVA to identify significant differences in the abundance of those microbial groups in the different column layers for each substrate tested.

3. Results

3.1. Leaching of OPP and IMZ

In all columns the first leaching event of OPP occurred on day 6 with a peak amount observed in the leachate of SMS and SMS/Straw/Soil columns (Fig. 1a). Thereafter significant amounts of OPP (80, 32, 19 35 and 32 mg) were found mostly in the leachate of SMS-columns at days 8, 11, 14, 43 and 57 days respectively. In general, the total amount of OPP found in the leachates of FMS, Straw/Soil and SMS/Straw/Soil columns did not significantly differ ($p > 0.05$) (0.014, 0.017 and 0.120% of the total OPP amount applied

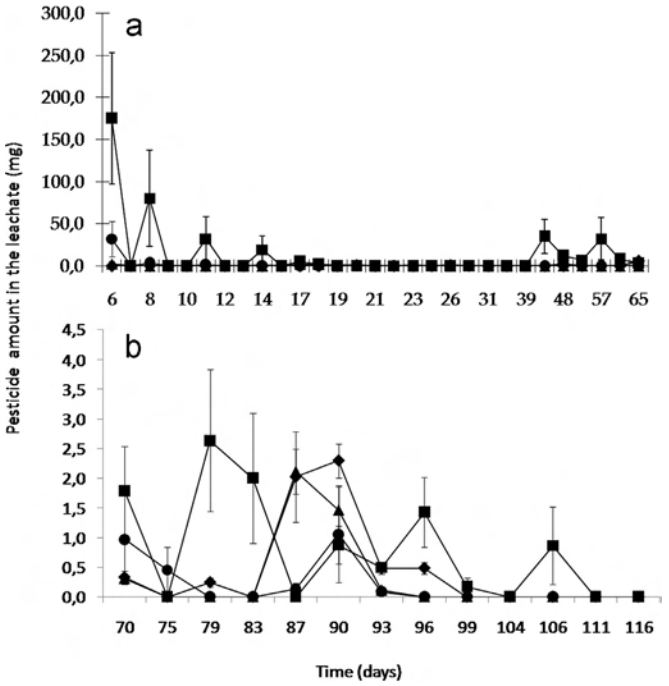

Fig. 1. The temporal pattern of OPP (a) and IMZ (b) amounts (mg) detected in the leachates of the SMS (■), SMS/Straw/Soil (50/25/25 by volume) (●), Straw/Soil (75/25 by volume) (▲) and FMS (◆) columns. Each value is the mean of three replicate columns ± the standard deviation.

respectively) compared to the SMS-columns where significantly higher % leaching ($p < 0.05$) was observed (1.1%).

Regarding the temporal pattern of IMZ leaching, an early peak of IMZ (1.8 and 1 mg) was observed in the leachate of the SMS- and the SMS/Straw/Soil-columns on day 70 (Fig. 1b). Thereafter, IMZ was detected at considerable amounts (1.5-2.5 mg) in the leachates of the SMS columns at 79, 83, 96 and 106 days. Leaching from all the other packing materials peaked between days 87–90 (2.1 mg in Straw/Soil on day 87 and 2.3 mg in FMS on day 90). With the exception of the SMS-columns, the residues of IMZ dropped to levels below the LOQ in the leachates from day 104 onwards. Overall, a tendency for higher % leaching of IMZ was observed in the SMS columns (0.42% of total IMZ amount) followed by FMS (0.322%), Straw/Soil (0.22%) and SMS/Straw/Soil (0.12%) although the differences observed were not significant ($p > 0.05$).

3.2. Mass balance analysis

3.2.1. Ortho-phenylphenol

More than 65% of the total amount of OPP applied to the columns was recovered by the packing materials of the columns at the end of the study (Table 3). The only exception was the FMS-columns where *ca.* 57% of OPP was recovered although this difference was not statistically significant ($p > 0.05$). Furthermore, over 74% of the recovered amount was extracted with water. When the distribution of OPP residues in the different horizons of the columns was examined no significant differences between substrates ($p > 0.05$) were observed with 99-100% of OPP recovered by the top 0–20 cm (Fig. 2a). No significant differences ($p > 0.05$) between substrates in the amounts of OPP considered as dissipated were observed with the highest values, 43%, observed in the SMS/Straw/Soil-columns and the lowest, 30% in the SMS-columns (Table 3).

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Table 3
The mass balance analysis for ortho-phenylphenol (OPP) and imazalil (IMZ) in the columns packed with the different substrates. Within each row, different letters indicate significant differences ($p < 0.05$) in the amount of pesticide leached, dissipated or retained in the different biomixtures. Absence of letters in pesticide fractions indicate that no significant differences ($p > 0.05$) were found.

Pesticides	Fraction (% of initially applied)	Substrates			
		Straw/Soil	FMS	SMS	SMS/Straw/Soil
Ortho-phenylphenol	Leached	0.014b	0.017b	1.100a	0.120b
	Retained-extracted with water	50.4	48.2	51.8	47.8
	Retained-extracted with acetonitrile	17.4	8.8	17.0	17.0
	Dissipated	32.2	42.9	30.1	35.1
Imazalil	Leached	0.220	0.322	0.420	0.120
	Retained-extracted with water	8.1	10.9	19.7	12.5
	Retained-extracted with acetonitrile	45.9	46.4	31.0	29.8
	Dissipated	45.8	42.5	48.9	57.6

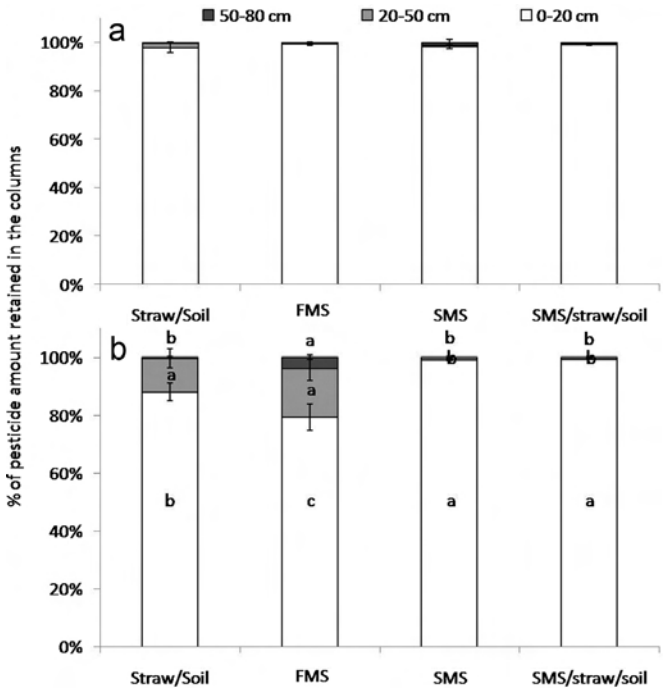

Fig. 2. The distribution of the residues of OPP (a) and IMZ (b) in the three layers (0–20, 20–50 and 50–80 cm) of the Straw/Soil (75/25 by volume), FMS, SMS and SMS/Straw/Soil (50/25/25 by volume) columns. Data are presented as % of the amount of pesticide retained in the columns and extracted by water and acetonitrile (sum is presented). Each value is the mean of three replicate columns ± standard deviation. Different letters indicate significant differences ($p < 0.05$) in the amount of pesticide leached, dissipated or retained in the different biomixtures. The absence of letters in column layers indicates that no significant differences were found.

3.2.2. Imazalil

No significant differences in the amount of IMZ recovered by the different biomixtures were observed ($p > 0.05$). Approximately 57, 54 and 51% of the applied IMZ were recovered from the FMS-, Straw/Soil- and SMS-columns respectively compared to 42% recovered from the SMS/Straw/Soil-columns respectively (Table 3). In contrast to OPP, 72% (SMS/Straw/Soil) to 85% (Straw/Soil) of IMZ recovered by the columns at the end of the study was extractable with acetonitrile. No significant differences ($p > 0.05$) between substrates in the amounts of IMZ considered as dissipated were observed with the highest values 57.6%, observed in the SMS/Straw/Soil-columns and the lowest, 48.9% in the SMS-columns (Table 3).

Significant differences ($p < 0.05$) in the distribution of IMZ residues in the column profiles of the different substrates were observed (Fig. 2b). Thus, in the columns packed with SMS/Straw/Soil and SMS nearly all IMZ was recovered from the top 0–20 cm, whereas significantly lower ($p < 0.05$) amounts (88 and 79%) were detected in the top layer of the Straw/Soil- and FMS-columns respectively. In turn, significantly higher ($p < 0.05$) amounts of IMZ (10 and 25% respectively) were recovered from the 20–50 cm of the Straw/Soil and FMS-columns, while in the latter significant amounts of IMZ (3.6%) were even found at the 50–80 cm layer (Fig. 2b).

3.3. Microbial activity and dynamics in the leaching columns

3.3.1. Peroxidases activity

No manganese peroxidase activity was detected in the leachates of the columns throughout the study. Laccase activity was detected only in the leachates of the columns packed with FMS and SMS, with significantly higher values ($p < 0.05$) observed in the former (Fig. 3). Laccase activity showed a similar temporal pattern in the leachates of both substrates with a peak observed at the first leaching event of OPP (day 6). Thereafter laccase activity decreased to negligible levels in the leachate of both substrates from day 10 onwards.

3.3.2. PLFAs

No significant main effects ($p > 0.05$) of column layer and organic substrate on total PLFA yields were observed, whereas significant interactions between these two factors were observed ($p < 0.05$). Post-hoc tests showed that the total PLFA yields did not significantly differ in the different layers of each of the substrates

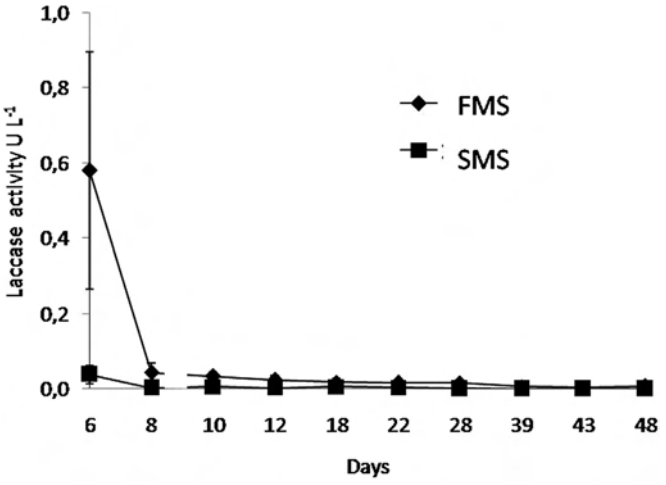

Fig. 3. Laccase activity detected in the leachates of the columns packed with FMS and SMS. Results are presented until day 48 (OPP-treatment period) since no activity of laccase was detected from this day onwards. Each value is the mean of three replicates ± the standard deviation. No laccase activity was detected in the leachates of the columns packed with Straw/Soil and SMS/Straw/Soil.

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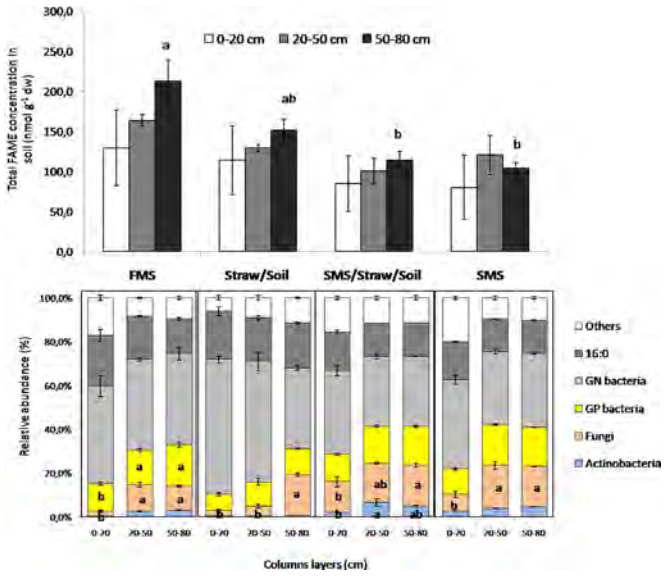

Fig. 4. The changes in the total concentration of (a) fatty acid methyl esters (FAME) and (b) the relative abundance of actinobacteria, fungi, GP bacteria and GN bacteria, and of the general microbial indicator16:0 in the different layers of the substrates tested at the end of the study. Each value is the mean of three replicates ± the standard deviation. Bars designated by the same letters in graph (a) indicate non-significant differences ($p > 0.05$) in the total PLFA yield measured in the same column layer in the different substrates, whereas stacked bars designated by the same letters in graph (b) indicate non significant differences ($p > 0.05$) within each substrate in the relative abundance of the different microbial in the different column layers. Absence of letters indicates non significant differences.

tested (Fig. 4a). However, significantly higher PLFA yields ($p < 0.05$) were observed at the 50–80 cm layer of FMS compared to the PLFAs yields measured at the same layer in SMS/Straw/Soil and SMS (Fig. 4a). Regarding the microbial community structure, GN bacteria were dominant in all substrates, especially in the top layer, whereas fungi showed significantly lower values ($p < 0.05$) in the same layer (Fig. 4b). Apart from the universal reduction in the abundance of fungi in the surface layer of all substrates, GP bacteria and actinobacteria also showed a significantly lower relative abundance ($p < 0.05$) in the surface layer of FMS and SMS/Straw/Soil respectively.

4. Discussion

4.1. Pesticides mass balance analysis

Pesticide leaching was generally low from all columns with OPP showing equal or lower leaching compared to IMZ. This is in contrast to the generally lower adsorption affinity of OPP (K_f 5.01–30.3 g ml^{-1}) compared to IMZ (K_f 183.6–412.4 g ml^{-1}) in similar organic substrates (Karas et al., 2015). In a column study Omirou et al. (2012) observed a higher mobility of OPP over IMZ, however different organic substrates, lower hydraulic and pesticide loadings and a different overall application scheme was employed in their study. The higher % leaching of IMZ compared to OPP observed in our study could be attributed to the realistic disposal scenario employed which took into account the temporal pattern of wastewater production and their pesticide content in a running citrus FFP. Thus the preceding application of large volumes of OPP-contaminated wastewater might have saturated the substrates, limiting their capacity to retain the following application of IMZ despite its higher adsorption affinity.

Regarding the processes that contribute to the capacity of the different substrates to retain OPP, more than half of the amount of the fungicide applied on the columns was recovered from the substrates at the end of the study suggesting a predominance of adsorption over dissipation processes. This is in contrast to the limited persistence of OPP in similar organic biomixtures and soil (Karas et al., 2015). This discrepancy could be attributed to the particularly high amounts of OPP disposed of in the columns in our study compared to previous studies (Karas et al., 2015; Omirou et al., 2012). This combined with the high retention capacity of the tested substrates resulted to the accumulation of high OPP concentrations in the biomixtures and sub-optimal conditions for its degradation. Although more than 70% of the amount of OPP recovered by the columns was extractable with water, in agreement with its generally weak adsorption (Zheng et al. 2011) and high water solubility (European Food Safe Authority (EFSA), 2008), 99% of it remained in the top layer (0–20 cm) of the columns. These results suggest that the mobility of OPP in biobeds packed with those substrates is expected to be limited.

Similarly to OPP, more than 51% of the total IMZ applied was recovered by the different substrates at the end of the study. The only exception was SMS/Straw/Soil in which less than 50% of the totally applied IMZ was recovered suggesting higher contribution of dissipation processes. This is particularly important considering the general recalcitrance of IMZ (Environmental Protection Agency (EPA) USA, 2003; Kreuzig et al., 2010). The more important role of dissipation over adsorption in SMS/Straw/Soil is in line with Karas et al. (2015) who showed a lower persistence of IMZ in SMS/Straw/Soil (DT50=26 d) compared to other biomixtures used in our study like Straw/Soil (50/50) (DT50=58 d). In contrast to OPP, IMZ residues retained in the columns were mostly extractable with acetonitrile, in line with its low water solubility and stronger adsorption affinity (Kreuzig et al., 2010; European Food Safe Authority (EFSA), 2008). Another point which should be noted is the variable distribution of IMZ residues in the different columns at the end of the study. More than 99% of its residues in the SMS/Straw/Soil and SMS columns were retained in the 0–20 cm layer, suggesting a limited potential for further leaching, in contrast to Straw/Soil or FMS where significant levels of IMZ were found below 20 cm. Overall, these results suggest that SMS/Straw/Soil appears as the most desirable substrate for the removal of IMZ from citrus FPP effluents.

It should be noted that our experimental set-up does not allow a distinction between the different dissipation processes contributing to OPP and IMZ loss. However, the overall high degradation rate (Karas et al., 2015), high water solubility (Table 1) and low adsorption affinity of OPP (Zheng et al., 2011) indicate that degradation could be the dominant dissipation process in the substrates studied. Regarding IMZ, its longer persistence (Kreuzing et al. 2010; Karas et al., 2015), lower water solubility (Table 1) and higher adsorption affinity (European Food Safe Authority (EFSA), 2010) imply that the formation of non-extractable residues might have contributed to the pool of pesticide amount considered as dissipated. However, the short period of IMZ application (46 days) which is not known to simultaneously form non extractable residues (European Commission (EC), 2009) and its reported accelerated degradation in substrates like SMS/Straw/Soil (DT50=26 d) in previous studies (Karas et al., 2015) are in support of a significant contribution of degradation in the dissipation of IMZ in the substrates tested.

4.2. The Role of P. ostreatus

Contrasting results for the removal efficiency of FMS vs SMS for the two fungicides were observed. The increasing removal efficiency of FMS over SMS for OPP, was opposed to the generally equal removal efficiency of the two substrates for IMZ. This result provides a first indication that *P. ostreatus* actively growing on a

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fresh substrate (FMS) is more efficient in the degradation of phenolic molecules (OPP) compared to the fungal mycelium still present in the SMS which is depleted of nutrients and energy sources. The higher acidity of FMS over SMS and other substrates tested might have also contributed to the higher enzymatic activity of *P. ostreatus* in the former at the initial phase of the experiment (Castillo et al., 2008). In line with this is the significantly higher activity of laccase in the leachates of the FMS columns compared to SMS in the first 8 days of the experiment and during the application period of OPP (1-60 days). In support of this Karas et al. (2011) showed that *P. ostreatus* actively degraded OPP via its lignolytic enzymatic system whereas it only partially degraded IMZ. Apart from the limited capacity of the fungus to degrade IMZ, the preceding application of OPP might have resulted in the gradual elimination of *P. ostreatus* efficiency which was not enzymatically active in the substrate when the application of IMZ-containing effluents was initiated. In agreement with this is the negligible activity of laccases in the leachates of FMS- and SMS-columns during the IMZ application period (data not shown). Previous studies have verified the limited capacity of white rot fungi such as *P. ostreatus* to survive competition under wastewater treatment conditions (Libra et al., 2003; Gao et al., 2008). In addition, Cordova Juarez et al. (2011) showed that storage of the mushroom substrate leads to a drastic reduction of the enzymatic activity of *Pleurotus pulmonarious* with consequences on its degrading activity against chlorothalonil. However based on the mode of action of IMZ and OPP on fungal cells and the high application rates tested in our study, inhibitory effects on non-target fungi like *P. ostreatus* present in the substrates tested cannot be ruled out, thus limiting its role in the depuration efficiency of biobed systems.

4.3. Interactions of pesticides with the microbial community

Despite that the performance of biobeds relies mostly on their high biodegradation capacity, little is known regarding the interactions of pesticides with the microbial community in those systems. Using PLFA analysis, we observed a rather uniform distribution of the total microbial biomass in the different layers of the substrates tested at the end of the study. In contrast the relative abundance of the different microbial groups in the column layers of the different substrates tested varied and the differences observed were in agreement with the distribution of pesticides residues in the columns. The significantly lower abundance of fungi at the surface layer of all substrates, and of actinobacteria and GP bacteria at the surface layer of SMS/Straw/Soil- and FMS-columns, is in accordance with the accumulation of the studied fungicides in the surface layer of those substrates. So far no studies have investigated the impact of those fungicides on the microbial community and especially on fungi. IMZ acts by inhibiting the biosynthesis of ergosterol, the main sterol of the cellular membranes not only of Ascomycetes, which constitute the main target of IMZ (Guan et al., 1992), but also of Basidiomycetes and Zygomycetes (Wette et al., 2010). Similarly OPP acts by generating active oxygen radicals which destroy components of the fungal membranes in a non-selective mode (Dekker, 1999). Based on their mode of action and their high application rates in the current study, adverse effects on off-target fungi should be expected. Previous studies by Marinozzi et al. (2013) in similar organic substrates showed different responses of fungi upon exposure to fungicides with penconazole inducing higher reductions in the abundance of total fungi compared to cyprodynil and axosystrobin. The differences observed were attributed to differences in the inherent toxicity of the three pesticides to microorganisms.

5. Conclusions

Our study provides evidence that SMS-rich substrates may enhance the depuration capacity of biobeds receiving effluents from citrus FPP containing OPP and IMZ. Despite its known capacity to degrade organic pollutants, *P. ostreatus* present in the SMS did not seem to actively contribute to the degradation of those fungicides. Overall the high depuration capacity of the SMS-rich substrates coupled with the associated benefit of recycling an organic waste of agricultural origin, further stress their potential for application in full-scale systems.

Acknowledgments

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References

Bourbonnais, R., Paice, M.G., 1990. Oxidation of non-phenolic substrates:an expanded role for laccase in lignin biodegradation. FEBS Lett. 267, 99–102.
Castillo, L.E., Martinez, E., Ruepert, C., Savage, C., Gilek, M., Pinnock, M., Solis, E., 2006. Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. Sci. Total Env. 367, 418–432.
Castillo, Md.P., Torstensson, L., 2007. Effect of biobed composition, moisture and temperature on the degradation of pesticides. J. Agric. Food Chem. 55, 5725–5733.
Castillo, Md.P., Torstensson, L., Stenstrom, J., 2008. Biobeds for environmental protection from pesticides use – a review. J. Agric. Food Chem. 56, 6206–6219.
Cordova Juarez, R.A., Gordillo Dorry, L.L., Bello-Mendoza, R., Sanchez, J.E., 2011. Use of spent substrate after *Pleurotus pulmonarius* cultivation for the treatment of chlorothalonil containing wastewater. J. Env. Manag. 92, 948–952.
De Wilde, T., Debaer, C., Ryckeboer, J., Springael, D., Spanoghe, P., 2010. The influence of small-and large-scale composting on the dissipation of pesticide residues in a biopurification matrix. J. Sci. Food Agric. 90, 1113–1120.
Dekker M., 1999. Handbook of pest management. Ruberson J. R. ed., New York.
European Food Safe Authority (EFSA), 2008. Conclusion on pesticide peer review. Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217, 1–67.
European Food Safe Authority (EFSA), 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil. EFSA J. 8 (3), 1526.
Environmental Protection Agency (EPA) USA. 2003. Reregistration eligibility decision for imazalil Case No. 2325, p. 74.
European Commission (EC) 2009. Final Review report for the active substance 2-phenylphenol. Available at: ⟨http://ec.europa.eu/food/plant/protection/evaluation/ existactive/list_2_phenylphenol_en.pdf⟩.
European Commission (EC) 2010. Commission Directive 2010/57/EU of 26 August 2010 amending Annex I to Council Directive 91/414/EEC to renew the inclusion of imazalil as active substance. Official Journal of the European Union L225/5. Available at: ⟨http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010: 225:0005:0009:EN:PDF⟩.
Findlay, R.H., 2004. Determination of microbial community structure using phospholipids fatty acid profiles. Molecular Microbial Ecology Manual, Second Edition 4.08 Kluwer Academic Publishers, Netherlands, pp. 983–1004.
Frostegård, Å., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol. Fert. Soils 22, 59–65.
Garcia-Delgado, C., D'Annibale, A., Pesciaroli, L., Yunta, F., Crognale, S., Petruccioli, M., Eymar, E., 2015. Implications of polluted soil biostimulation and bioaugmentation with spent mushroom substrate (*Agaricus bisporus*) on the microbial community and polycyclic aromatic hydrocarbons biodegradation. Sci. Total Env. 508, 20–28.
Gao, D., Zheng, Y., Wen, X., Qian, Y., 2008. Competition strategies for the incubation of white rot fungi under non-sterile conditions. Proc. Biochem. 43, 937–944.
Gao, W., Liang, J., Pizzul, L., Feng, X.M., Zhang, K., Castillo, Md.P., 2015. Evaluation of spent mushroom substrate as substitute of peat in Chinese biobeds. Int. Biodeter. Biodeg 98, 107–112.
Garcia-Portillo, M., Avino, E.S., Vicente, J.O., De Andres, R.L., Jimenez, MdlAS, Blanco JPL., 2004. Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States

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20/04/2024 10:51:04 EEST - 3.145.162.204

Patent, US 6,709,585 B1, p. 9.
Guan, J., Van Leemput, L., Posthumus, M.A., De Waard, M.A., 1992. Metabolism of imazalil by wild-type and DMI-resistant isolates of *Penicillium italicum*. Neth. J. Pl. Path 98, 161–168.
Herrero-Hernandez, E., Andrades, M.S., Marın-Benito, M.S., Sanchez-Martın, M.J., Rodrıguez-Cruz, M.S., 2011. Field-scale dissipation of tebuconazole in a vineyard soil amended with spent mushroom substrate and its potential environmental impact. Ecotox. Env. Saf. 74, 1480–1488.
Jonkers, N., Sousa, A., Galante-Oliveira, S., Barroso, C.M., Kohler, H.P.E., Giger, W., 2010. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro, Portugal. Environ. Sci. Pollut. Res. 17, 834–843.
Karanasios, E., Tsiropoulos, N., Karpouzas, D.G., Ehaliotis, C., 2010. Degradation and adsorption of pesticides in compost-based biomixtures as potential substrates for biobeds in south Europe. J. Agric. Food Chem. 58, 9147–9156.
Karanasios, E., Papadi-Psyllou, A., Karpouzas, D.G., Tsiropoulos, N., 2012. Optimization of water management and biomixture composition for maximizing the pesticide depuration of peat-free biobed systems. J. Env. Qual. 41, 1787–1795.
Karas, P., Perucchon, C., Exarhou, C., Ehaliotis, C., Karpouzas, D.G., 2011. Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. Biodegradation 22, 215–228.
Karas, P.A., Metsoviti, A., Zisis, V., Ehaliotis, C., Omirou, M., Papadoulou, E.S., Menkissoglu-Spiroudi, U., Manta, S., Komioti, D., Karpouzas, D.G., 2015. Dissipation, metabolism and sorption of pesticides used in fruit-packaging plants: Towards an optimized depuration of their pesticide-contaminated agro-industrial effluents. Sci. Total Env. 530–531, 129–139.
Khodja, A.A., Sehili, T., Pilichowski, J.-F., Boule, P., 2001. Photocatalytic degradation of 2-phenylphenol on TiO_2 and ZnO in aqueous suspensions. J. Photochem. Photobiol. A: Chem. 141, 231–239.
Kinay, P., Mansour, M.F., Gabler, M.F., Margosan, D.A., Smilanick, J.L., 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop. Prot. 26, 647–656.
Koutrotsios, G., Mountzouris, K.C., Chatzipavlidis, I., Zervakis, G.I., 2014. Bioconversion of lignocellulosic residues by *Agrocybe cylindracea* and *Pleurotus ostreatus* mushroom fungi – Assessment of their effect on the final product and spent substrate properties. Food Chem. 161, 127–135.
Kreuzig, R., Hartmann, C., Teigeler, J., Höltge, S., Cvetkovic, B., Schlag, P., 2010. Development of a novel concept for fate monitoring of biocides in liquid manure and manured soil taking ^{14}C-imazalil as an example. Chemosphere 79, 1089–1094.
Marinozzi, M., Coppola, L., Monaci, E., Karpouzas, D.G., Papadopoulou, E., Menkissoglu-Spiroudi, U., Vischetti, C., 2013. The dissipation of three fungicides in a biobed organic substrate and their impact on the structure and activity of the microbial community. Environ. Sci. Pollut. Res. 20, 2546–2555.
Ngo, T.T., Lenhoff, H.M., 1980. A sensitive and versatile assay for peroxidase and peroxidase-coupled reactions. Anal. Biochem. 105, 389–397.
Omirou, M., Dalias, P., Costa, C., Papastefanou, C., Dados, A., Ehaliotis, C., Karpouzas, D.G., 2012. Exploring the potential of biobeds for the depuration of pesticide-contaminated wastewaters from the citrus production chain: laboratory, column and field studies. Environ. Pollut. 166, 31–39.
Papadopoulou, E.S., Karpouzas, D.G., Menkissoglu-Spiroudi, U., 2011. Extraction parameters significantly influence and the quantity and the profile of PLFAs extracted from soil. Microb. Ecol. 62, 704–714.
Perruchon, C., Patsioura, V., Vasiliadis, S., Karpouzas, D.G., 2015. Isolation and characterisation of a *Sphingomonas strain* able to degrade the fungicide ortho-phenylphenol. Pest Manag. Sci. . http://dx.doi.org/10.1002/ps.3970, early view
Phan, C.-W., Sabaratnam, V., 2012. Potential uses of spent mushroom substrate and its associated lignocellulosic enzymes. Appl. Microbiol. Biotechnol. 96, 863–873.
Wette, J.D., Abril, M., Blackwell, M., 2010. Phylogenetic distribution of fungal sterols. PLoS One 5, e10899.
Zheng, C., Focks, A., Ellebrake, K., Eggers, T., Fries, E., 2011. Sorption of ortho-phenylphenol to soils. Clean- Soil Air Water 39, 116–120.

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Dissipation, metabolism and sorption of pesticides used in fruit-packaging plants: Towards an optimized depuration of their pesticide-contaminated agro-industrial effluents

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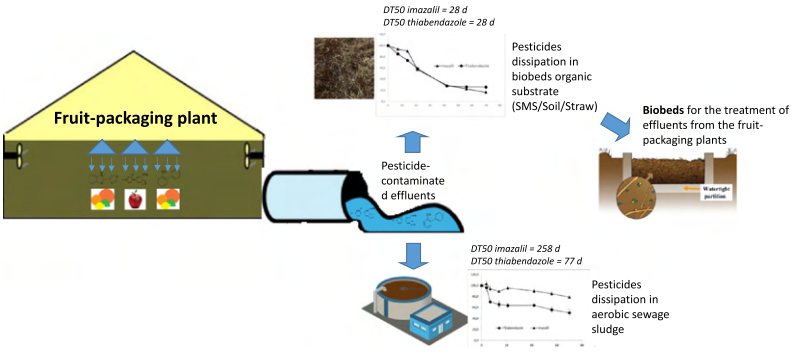
HIGHLIGHTS

- Fruit-packaging effluents are a serious point source contamination with pesticides.
- Pesticide dissipation was determined in sewage-derived substrates and biomixtures.
- *Ortho*-phenylphenol, diphenylamine, ethoxyquin did not persist in substrates tested.
- First evidence for the metabolism of ethoxyquin in soil and other substrates is shown.
- Thiabendazole and imazalil were persistent but dissipated faster in SMS-rich substrates.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewaters from the fruit-packaging industry constitute a serious point source contamination with pesticides. In the absence of effective depuration methods, they are discharged in municipal wastewater treatment plants or spread to land. Modified biobeds could be an applicable solution for their treatment. We studied the dissipation of thiabendazole (TBZ), imazalil (IMZ), *ortho*-phenylphenol (OPP), diphenylamine (DPA) and ethoxyquin (EQ), used by the fruit-packaging industry, in anaerobically digested sewage sludge, liquid aerobic sewage sludge and in various organic substrates (biobeds packing materials) composed of soil, straw and spend mushroom substrate (SMS) in various volumetric ratios. Pesticide sorption was also determined. TBZ and IMZ showed higher persistence especially in the anaerobically digested sewage sludge (DT_{50} = 32.3–257.6 d), in contrast to OPP and DPA which were rapidly dissipated especially in liquid aerobic sewage sludge (DT_{50} = 1.3–9.3 d). EQ was rapidly oxidized mainly to quinone imine (QI) which did not persist and dimethyl ethoxyquinoline (EQNL, minor metabolite) which persisted for longer. Sterilization of liquid aerobic sewage sludge inhibited pesticide decay verifying the microbial nature of pesticide dissipation. Organic substrates rich in SMS showed the highest dissipation capacity with TBZ and IMZ DT_{50s} of ca. 28 d compared to DT_{50s} of >50 d in the other substrates. TBZ and IMZ showed

Abbreviations: TBZ, thiabendazole; IMZ, imazalil; OPP, *ortho*-phenylphenol; DPA, diphenylamine; EQ, ethoxyquin; QI, quinone imine; EQNL, dimethyl ethoxyquinoline; SMS, spent mushroom substrate.

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the highest sorption affinity, whereas OPP and DPA were weakly sorbed. Our findings suggest that current disposal practices could not guarantee an efficient depuration of effluents from the fruit-packaging industry, whereas SMS-rich biobed organic substrates show efficient depuration of effluents from the fruit-packaging industry via accelerated dissipation even of recalcitrant fungicides.

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1. Introduction

Upon their harvest, fruits are transported to fruit-packaging plants where they are treated with fungicides (thiabendazole (TBZ), imazalil (IMZ), *ortho*-phenylphenol (OPP)) or antioxidants (diphenylamine (DPA), ethoxyquin (EQ)) to minimize losses due to fungal infestations or physiological disorders during storage (Smilanick et al., 2008; Jung and Watkins, 2008). Postharvest treatments of fruits result in the production of large wastewater volumes which are characterized by low BOD/COD values but high concentrations of pesticides which should be detoxified prior to environmental release (Santiago et al., 2011). This need has been laid down on the registration documents of all relevant pesticides. For example authorization was granted to IMZ only under the clause that appropriate waste management practices to handle the waste solution remaining after application, including for instance the cleaning water of the drenching system and the discharge of the processing waste are put in place (EC, 2010). The only full-scale treatment system currently in place is based on pesticide sorption onto granular activated carbon (Garcia-Portillo et al., 2004). Although this system is particularly efficient in the removal of TBZ from wastewaters (EC, 2000) its cost is prohibitive for small to medium enterprises which constitute the majority of fruit-packaging plants in the Mediterranean region. Recent semi-pilot studies showed that the combination of membrane bioreactor and advanced oxidation processes could effectively remove TBZ from wastewaters (Sanchez Perez et al., 2014). Similarly, TiO_2 solar photocatalysis showed high depuration efficiency for the removal of IMZ, TBZ (Jimenez et al., 2015) and OPP (Khodja et al., 2001) from wastewaters. However those methods produce several oxidized metabolites which are of unknown toxicity compared to the parent compound plus their full scale implementation is still pending.

In the absence of appropriate and established treatment methods, fruit-packaging plants tend to discharge their wastewater into municipal wastewater treatment plants, abandoned fields or evaporation ponds. Previous studies have provided indirect evidence for the limited removal capacity of municipal wastewater treatment plants for IMZ, TBZ (Campo et al., 2013) and OPP (Jonkers et al., 2010). This, combined with the inappropriate disposal methods currently in place for these wastewaters, has resulted in the frequent detection of these pesticides in receiving water bodies (Castillo et al., 2000, 2006). Thus an efficient, cost-effective and sustainable treatment system for the depuration of those effluents is needed. Omirou et al. (2012) provided first evidence for the potential use of modified biobed systems for the depuration of wastewaters from the fruit-packaging industry. Such modified systems should be packed with organic materials which ensure effective dissipation of the particularly persistent (TBZ (EC, 2013) and IMZ (Kreuzig et al., 2010)) and toxic pesticides (OPP (EFSA, 2008), EQ (EFSA, 2010b) and DPA (EFSA, 2012)) contained in those agro-industrial effluents. The optimum composition of biobed packing material includes a lignocellulosic material like straw, soil and a humified substrate like peat or compost (Castillo et al., 2008). Spent mushroom substrate (SMS) of the fungi *Pleurotus ostreatus* has been found to accelerate the biodegradation potential of on-farm biobed systems (Karanasios et al., 2010a). SMS is produced in large quantities in several areas of the Mediterranean basin and the mushroom production sector is seeking sustainable and environmental-friendly uses for this material (Herrero-Hernandez et al., 2011).

Up to date, little is known regarding the basic processes controlling the dissipation of pesticides contained in the wastewaters from the fruit packaging plants. Only a few studies have investigated the dissipation of IMZ, TBZ and OPP in soil (Kreuzig et al., 2010; Kesavan et al., 1976), municipal wastewater treatment plants (Campo et al., 2013; Korner et al., 2000) and organic substrates (Omirou et al., 2012), while even less are known regarding DPA. In sewage sludge Gardner et al. (1982) reported the metabolism of DPA to aniline, 4-hydroxy-DPA and indole. More recently Shin and Spain (2009) isolated a soil bacterium that metabolized to Krebs cycle intermediates via formation of aniline. In contrast, no information are available regarding the dissipation and metabolism of EQ in the environment. Metabolic studies for EQ are only available on fish feed, fish meals and fruits which identified the formation of several metabolites like a dimer of EQ and quinone imine (QI) (He and Ackman, 2000).

Our study aimed to examine the dissipation of pesticides contained in the wastewaters from the fruit-packaging industry a) in liquid aerobic sewage sludge or anaerobically digested sewage sludge from a municipal wastewater treatment plant where those effluents are discharged and b) in various organic substrates with potential use as packing materials for modified biobeds (with SMS as a key component). Apart from pesticide dissipation, the sorption of these pesticides on the organic substrates was also assessed to evaluate the contribution of the different processes in the removal of pesticides from the wastewaters derived from the fruit-packaging industry. In addition, the metabolism of EQ in all materials was also determined considering that most of the metabolic products of this antioxidant compound are equally active and toxic with the parent compound (Baszczyk et al., 2013).

2. Materials and methods

2.1. Pesticides

Analytical standards of IMZ (99.8% Pestanal®), TBZ (99% Pestanal®), OPP (99.9% Pestanal®), DPA (99.9% Pestanal®) and EQ (99% Pestanal®) were purchased from Fluka, Sigma-Aldrich. For pesticide residue analysis, pesticide stock solutions in methanol were initially prepared (1000 mg l^{-1}) and used for obtaining a series of dilutions at the range of 0.1–50 mg l^{-1} which were used for the construction of calibration curves for quantification of pesticide concentrations by HPLC. Particularly for EQ, preliminary studies indicated a rapid oxidation of EQ (*m/z* 218 $[M + H]^+$, 202 $[M^+\text{-}CH_3]$, 174 $[202\text{-}C_2H_4]$ and retention time (Rt) 9.9 min) in the substrates tested to two metabolites which were tentatively identified via LC–MS/MS analysis as (1) *2,6-dihydro-2,2,4-trimethyl-6-quinone imine* (QI) (*m/z* 188 $[M + 1]^+$, 172 $[M^+\text{-}CH_3]$, 159 $[M^+\text{-}CO]$, 144 $[159\text{-}CH_3]$, Rt 9.2) and (2) *2,4-dimethyl-6-ethoxyquinoline* (EQNL) (*m/z* 202 $[M + 1]^+$, 173 $[M^+\text{-}C_2H_4]$, 144 [173-CHO], Rt 10.1 min) (Fig. 1). Therefore, their concentration along with this of the parent compound were determined in all studies as is described below and will be referred as 'total residues of EQ'. In the absence of commercial analytical standards, the two EQ metabolites were synthesized according to the procedure described by Thorisson et al. (1992) and their structure was verified by NMR analysis (see Supplementary materials). Commercial formulations of the pesticides were used in all fortification experiments described below including TECTO® 50 SC (TBZ), FUNGAZIL® 50 EC (IMZ), FRUITGARD® 20 SL (OPP), NO SCALD® 31.8 EC (DPA) and XEDAQUINE® 50 EC (EQ).

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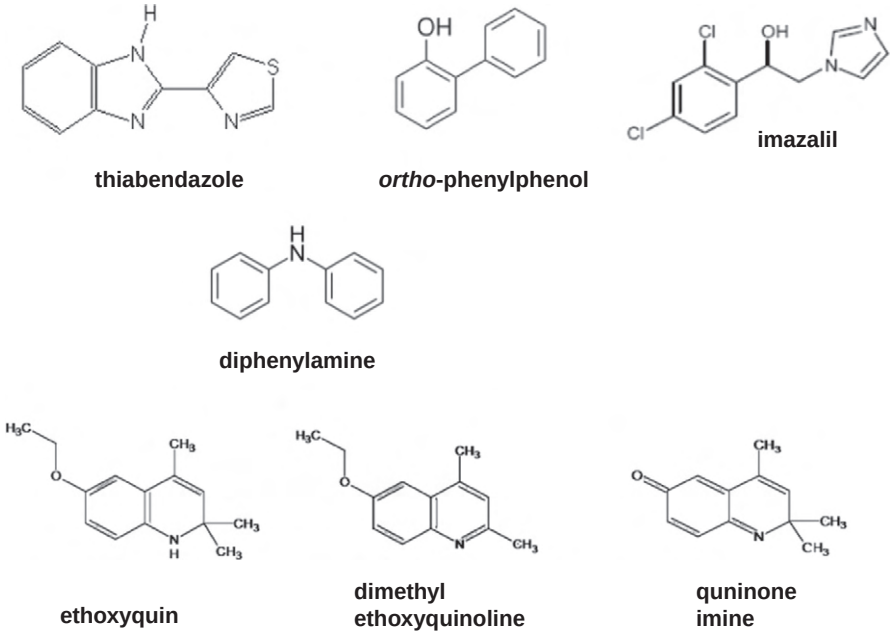

Fig. 1. The chemical structures of the pesticides and their metabolites used in the current study.

2.2. Organic substrates

Anaerobically digested sewage sludge (10 kg) was obtained from the municipal wastewater treatment facility of the city of Larissa, Greece. Anaerobically digested sewage sludge is produced after anaerobic digestion at mesophilic temperatures (35 °C) in anaerobic digesters of continuous flow, allowing complete mixing, and operated at high load rates. The sludge which was digested was collected from the primary settling and mixed with small amounts of sludge from the secondary settling. The sludge produced had a water content of 70% and its properties are shown in Table 1. Upon its production it was stored at aerobic conditions in the municipal wastewater treatment plant prior to its collection. Anaerobically digested sewage sludge was partially dried (to 50% of its water holding capacity) and it was sieved to pass through a 3 mm mesh. LASS was collected from the secondary settlers of the municipal wastewater treatment facility of the city of Larissa, Greece. The liquid aerobic sewage sludge collected was used immediately after its collection to avoid prolonged storage which might suppress the elevated metabolic activities of the microbial biomass.

SMS, soil and straw were mixed in different volumetric ratios to prepare the various organic materials. A soil collected from a farm of the National Agricultural Research Foundation of Greece in Larissa, Greece was used for the preparation of the different organic biomixtures. It was sieved to homogenize (2 mm) and stored at 4 °C prior to use. Straw was chopped into small pieces (1–3 cm) and passed through a 4.75 mm sieve. SMS was obtained from a *P. ostreatus* edible mushroom production unit (Mpoulogeorgos–Meteora, Trikala, Greece). It was chopped into small pieces and stored at 4 °C until further use. The physicochemical properties of the raw materials (soil, straw and SMS), and of their biomixtures produced, are given in Table 1. Total organic C and N contents were determined by wet digestion with concentrated sulfuric acid and the Kjeldahl digestion method respectively. pH was determined in a mixture of 1:2.5–5 air dried solid substrate:water (w:v).

Table 1
Physicochemical properties of the substrates used to assess the dissipation and sorption of the pesticides studied.

Substrates	pH	Organic carbon (%)	Total N (%)	C/N
Soil[a]	7.55	1.05	0.13	8.1
Straw	7.15	79.2	0.80	97.8
SMS	6.83	71.0	1.20	59.2
SMS/soil (50:50)	7.20	16.9	0.33	51.2
SMS/straw/soil (50:25:25)	7.10	29.3	0.30	97.7
Straw/soil (50:50)	7.40	6.6	0.13	50.8
Straw/SMS/soil (50:25:25)	7.20	23.5	0.20	117.5
ADSS	6.95	10.2	2.1	4.8

[a] Soil texture: sand 37%, clay 31%, silt 32% (clay loam).

2.3. Dissipation of pesticides in anaerobically digested sewage sludge

Anaerobically digested sewage sludge was divided into 5 bulk samples (600 g). These were treated with appropriate amounts of aqueous solutions of the pesticides DPA, OPP, IMZ, TBZ and EQ (2000 mg l^{-1}), prepared by their commercial formulations, aiming to a final concentration of 35 mg kg^{-1} for DPA, IMZ, TBZ, EQ and 45 mg kg^{-1} for OPP. The application of OPP generates much higher wastewater volumes and their disposal is expected to result in higher concentrations in the receiving matrices. Upon treatment with pesticides, the moisture content of the anaerobically digested sewage sludge was adjusted to 60% of its water holding capacity with addition of ddH_2O. Subsequently the bulk samples were mixed by hand to ensure uniform distribution of pesticides and were divided into 27 subsamples of 20 g which were placed in airtight plastic bags. All subsamples were incubated in the dark at 25 °C. Immediately after pesticide application, and at regular intervals thereafter, triplicate sub-samples from each treatment were removed from the incubator and stored at −20 °C until analyzed by HPLC-UV.

2.4. Dissipation of pesticides in liquid aerobic sewage sludge

Fifteen 200-ml samples of liquid aerobic sewage sludge were transferred in 500 ml stoppered glass bottles. Triplicate liquid aerobic sewage sludge samples were treated with TBZ, IMZ, EQ, OPP and DPA to give concentrations of 15 mg l^{-1}. Pesticides were added in the form of aqueous pesticide solutions (2000 mg l^{-1}) prepared by their commercial formulations. Upon pesticide treatments, sewage sludge samples were briefly agitated to ensure uniform dissolution of the pesticides and were placed in an orbital shaking platform incubator at 100 rpm and 25 °C. Immediately after pesticide application and at regular intervals

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thereafter subsamples (2 ml) were removed aseptically, extracted with an organic solvent as described below, and analyzed in HPLC-UV.

2.5. Dissipation of pesticides in organic substrates

The dissipation of pesticides in different organic biomixtures was assessed. For each of the five materials; soil, soil + SMS (50:50), SMS + straw + soil (50:25:25), straw + soil (50:50) and straw + SMS + soil (50:25:25) (all volumetric ratios) one bulk sample (1000 gd.w.) was prepared and separated into 27 sub-samples (30 g). These were individually treated with aliquots of aqueous solutions of the pesticides TBZ, IMZ, DPA, EQ and OPP aiming to a final concentration of 35 mg a.i. kg^{-1} d.w for the first four compounds and 45 mg a.i. kg^{-1} d.w for OPP. Those doses were calculated to represent a realistic loading scenario for a biobed system of 30 m^3 which receives in total 22 m^3 of wastewaters containing 10–15 mg l^{-1} of the pesticides studied. Such concentrations have been reported in recycled wastewaters from citrus fruit-packaging plants in Spain (Santiago et al., 2011). Pesticides were evenly mixed into the organic substrates and the moisture content was adjusted to 50% of their water holding capacity. All treatments were incubated in the dark at 25 °C for a period of 70 d. Moisture content was maintained by regular additions of deionized water. Immediately after pesticide application and at fixed intervals thereafter sub-samples from each treatment were removed and stored at −20 °C until analyzed for pesticide residues.

2.6. Pesticide sorption in organic biomixtures

The sorption of TBZ, IMZ, DPA and OPP in the different organic substrates selected as organic biomixtures was determined using the standard batch equilibrium method according to the OECD guideline 106 (OECD, 2000). Preliminary kinetic studies were employed to determine the most appropriate substrate:solution ratios and equilibration times for all pesticides. Thus, the most appropriate solid substrate:solution ratios to achieve 20–80% sorption of TBZ, IMZ, OPP and DPA were 1:50, 1:100, 1:25 and 1:25 respectively. Equilibrium was achieved at 24 h for TBZ, IMZ and at 8 h for OPP and DPA. The shorter equilibration time for OPP and DPA was selected to avoid losses of those two non-persistent pesticides during the equilibration period. All materials tested were prepared, air-dried and stored at room temperature. Individual stock solutions of each pesticide in acetone (10,000 μg ml^{-1}) were prepared. Appropriate amounts of the stock pesticide solutions were dissolved in 0.01 M $CaCl_2$ solution leading to the preparation of four pesticide solutions at concentrations of 10, 20, 40 and 80 μg ml^{-1}. The only exception was OPP for which the concentration levels of the four solutions were 20, 40, 80 and 100 μg ml^{-1} considering the higher exposure expected in biobed systems for this molecule. Triplicate samples (1 to 2 g) were mixed with 50 or 100 ml of each of the above solutions in screw-cupped vials and shaken overnight on an orbital shaker (200 rpm) at room temperature. When equilibrium was reached samples were centrifuged at 4500 rpm for 10 min and the supernatant was collected, extracted and analyzed by HPLC-UV as is described below.

2.7. Pesticide residue analysis

2.7.1. Pesticide extraction from liquid substrates

Extraction of TBZ, IMZ, EQ and its metabolites QI and EQNL was performed by mixing 2 ml from liquid aerobic sewage sludge with 8 ml of methanol in 20-ml screw glass vials. The mixture was shaken vigorously by vortex for a minute and the extract was passed through a 0.45 μm syringe filter (PTFE Syringe Filter). The filtrate was collected in a glass tube and stored at −20 °C until analyzed. Regarding extraction of OPP and DPA the same extraction procedure as above was followed with the only exception that acetonitrile instead of methanol was used.

2.7.2. Pesticide extraction from solid substrates

Regarding TBZ extraction, 10 ml of methanol was mixed with 5 g of solid substrate in a conical flask. The mixture was shaken for an hour in an orbital shaker (200 rpm), centrifuged for 5 min at 11,000 rpm and the clear supernatant was recovered. The remaining soil was re-extracted with further 10 ml of methanol and the clear supernatant from the two extraction cycles were combined. The extract was passed through a 0.45 μm syringe filter (PTFE Syringe Filter) and kept at −20 °C until analyzed. For the extraction of IMZ from the organic substrates, 5 g of substrate were mixed with 1 ml of NaOH 1 N and 10 ml of methanol. Samples were shaken for 30 min and centrifuged at 11,000 rpm for 5 min., the supernatant was collected in a glass bottle, and the soil was re-extracted with another 10 ml of methanol. After 30 min shaking and centrifugation, the clear supernatant from the two extraction cycles were combined and stored at −20 °C. For the extraction of OPP and DPA, 10 g of soil was mixed with 25 ml of acetonitrile. The mixture was agitated for 1.5 h in an orbital shaker at 200 rpm and then centrifuged at 11,000 rpm for 5 min. The supernatant was collected and stored at −20 °C.

The extraction of EQ and its metabolites was performed according to the original buffered QuEChERS method, slightly modified (Anastassiades et al., 2003). Due to the general instability of EQ, special care was taken during the extraction to minimize transformation of EQ to its oxidation derivatives. Thus, all extractions were conducted into a dark cold-room at 4 °C. Briefly, 5 g of solid substrate were mixed in a teflon tube with 5 ml of cold ddH_2O and were agitated manually for a minute. Subsequently, 10 ml of acetonitrile were added and the mixture was vortexed for 1 min. The samples were subsequently amended with a mixture of salts (4 g $MgSO_4$, 1 g NaCl and 1.5 g $C_6H_5Na_3O_7 \cdot 2H_2O$), vortexed for 1 min and centrifuged for 5 min at 7500 rpm. Then 1 ml of the clear supernatant was mixed with 150 mg $MgSO_4$ and 25 mg PSA and the mixture was vortexed for 30 s and centrifuged for 1 min at 3000 rpm. The final extracts for all pesticides were filtered through a syringe filter 0.45 μm (PTFE Syringe Filter) and analyzed by HPLC.

2.7.3. HPLC analysis

Pesticide residues were analyzed in an HPLC-UV system equipped with a Grace Smart RP C18 (150 mm × 4.6 mm). TBZ and OPP residues were detected at 254 nm using a mobile phase of acetonitrile/water/25% NH_3 solution (by volume) with different strengths (39/60.5/0.5 and 55:44.5:0.5 respectively). Under these conditions, the retention time (Rt) of TBZ and OPP were 3.3 and 3.4 min respectively. IMZ residues were detected at 204 nm using a mobile phase of 80:20 methanol:NH_3 solution 0.25% (by volume). Under those conditions the Rt of IMZ was 5 min. DPA residues were determined at 210 nm using a mobile phase of 60:30:10 acetonitrile:water:methanol (by volume) with a Rt of 3.5 min. Residues of EQ and its metabolites were determine at 254 nm using a mobile phase of 69:30:1 acetonitrile:water:NH_3 (by volume). Under these chromatographic conditions EQ, QI and EQNL were eluted at Rt of 5.7, 4.1 and 5.4 min respectively. In all cases a flow rate of 1 ml min^{-1} was used. Methods validation procedures are described in the supplementary material.

2.8. Pesticide dissipation kinetics

The four kinetic models proposed by the FOCUS workgroup on pesticide degradation kinetics (FOCUS, 2006) were used for fitting the dissipation data. The single first order kinetic model and three biphasic models: hockey-stick, first order multi-compartment model and the double first order in parallel model were used. The χ^2 test as well as visual inspection and the distribution of the residuals were used as criteria to assess the agreement between calculated and observed data for a given fit.

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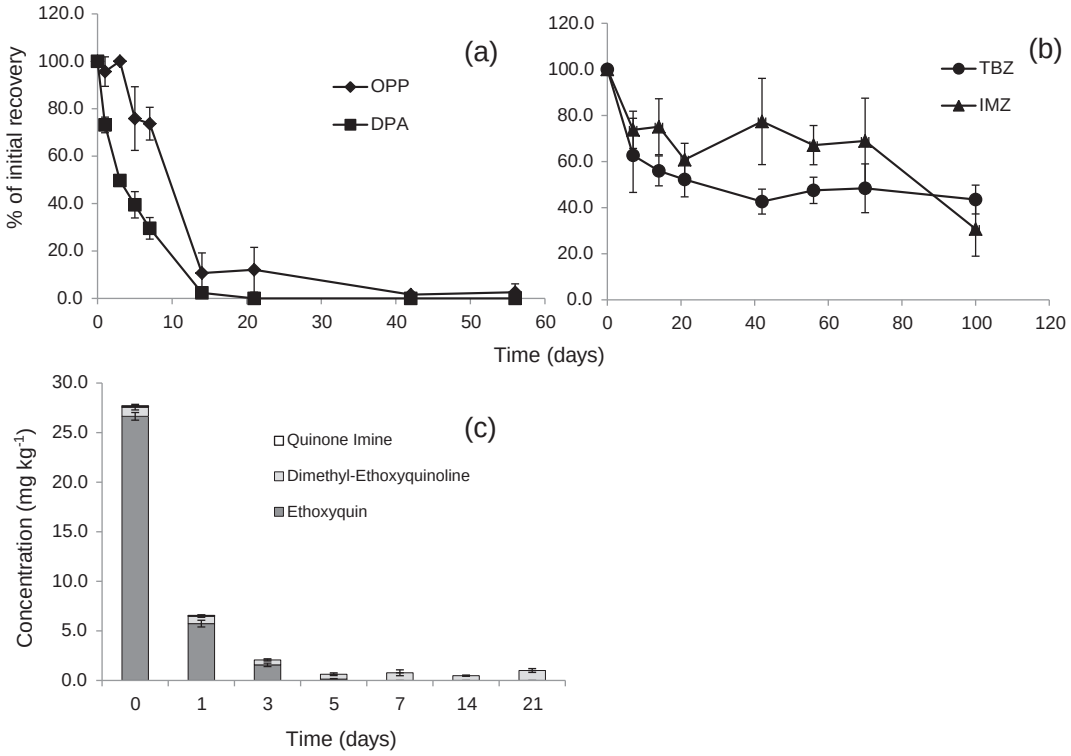

Fig. 2. Dissipation of *ortho*-phenylphenol (OPP) and diphenylamine (DPA) (a), thiabendazole (TBZ) and imazalil (IMZ) (b) and ethoxyquin (EQ) (c) in anaerobically digested sewage sludge. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

3. Results

3.1. The dissipation of pesticides in anaerobically digested sewage sludge

The dissipation patterns of OPP, DPA, TBZ, IMZ and of the total residues of EQ are shown in Fig. 2. A rapid dissipation of OPP and DPA was evident in anaerobically digested sewage sludge with DT50s of 9.3 and 3.6 d respectively (Fig. 2a, Table 2). Similarly, a rapid dissipation of the total residues of EQ was observed with DT50 of <1 d. From the two EQ metabolites, EQNL was formed in low amounts but constituted the only detectable residue from 7 d onwards, while only trace amounts of QI were detected (Fig. 2c). In contrast, TBZ and IMZ showed moderately to high persistence with DT50s of 32.3 and 108.3 d respectively (Fig. 2b, Table 2).

3.2. The dissipation of pesticides in liquid aerobic sewage sludge

The dissipation patterns of the five pesticides in sterilized and non-sterilized liquid aerobic sewage sludge are shown in Fig. 3. A rapid dissipation of OPP, DPA and total residues of EQ was evident with DT50s of 1.3, 1.5 and <1 d respectively (Table 2, Fig. 3a and c). Regarding the metabolism of EQ, no residues of the parent compound were detected from 3 d onwards with QI being the major component of the total residues at day 1, whereas EQNL became the major component from day 3 onwards (Fig. 3c). In contrast, a slow dissipation of TBZ and IMZ was observed with DT50s of 76.9 and 257.6 (extrapolated with the single first order kinetic model) respectively (Fig. 3b). Sterilization of liquid aerobic sewage sludge significantly inhibited the dissipation of all pesticides. This is clearly illustrated with EQ which remained the main component of the total residues of EQ for the first 7 d of the incubation, with the two metabolites, QI and EQNL, becoming the major components of the total residues from day 14 onwards (Fig. 3d).

3.3. The dissipation of pesticides in organic materials

The dissipation of OPP and DPA in the different substrates was very rapid within the first 7 d precluding the calculation of realistic DT50 values (Fig. 4a and c). Thus their dissipation was re-determined in an identical follow up experiment with focus on the first 72 h after application. The results indicated differences in the dissipation rates of the two

Table 2
The half-life values of *ortho*-phenylphenol (OPP), diphenylamine (DPA), imazalil (IMZ), thiabendazole (TBZ) and total residues of ethoxyquin (EQ) in anaerobically digested sewage sludge and in liquid aerobic sewage sludge.

Pesticides	Anaerobically digested sewage sludge			Liquid aerobic sewage sludge					
				Non Sterilized			Sterilized		
	DT50 (d)	χ^2 (%)	Model[a]	DT50 (d)	χ^2 (%)	Model	DT50 (d)	χ^2 (%)	Model
OPP	9.3	16.0	HS	1.3	38.8	SFO	36.0	3.3	SFO
DPA	3.6	9.5	SFO	1.5	5.13	SFO	28.7	5.3	SFO
IMZ	108.3	13.4	SFO	257.6	2.81	SFO	942.0	3.3	SFO
TBZ	32.3	3.6	FOMC	76.9	10.8	SFO	208.7	5.8	SFO
EQ + QI + EQNL[b]	0.46	8.0	SFO	0.18	0.001	SFO	4.7	5.6	SFO

[a] SFO: single first order; HS: hockey-stick; FOMC: first order multi-compartment.
[b] Calculations were made with the sum of residues of ethoxyquin (EQ), quinone imine (QI) and dimethyl ethoxyquinoline (EQNL).

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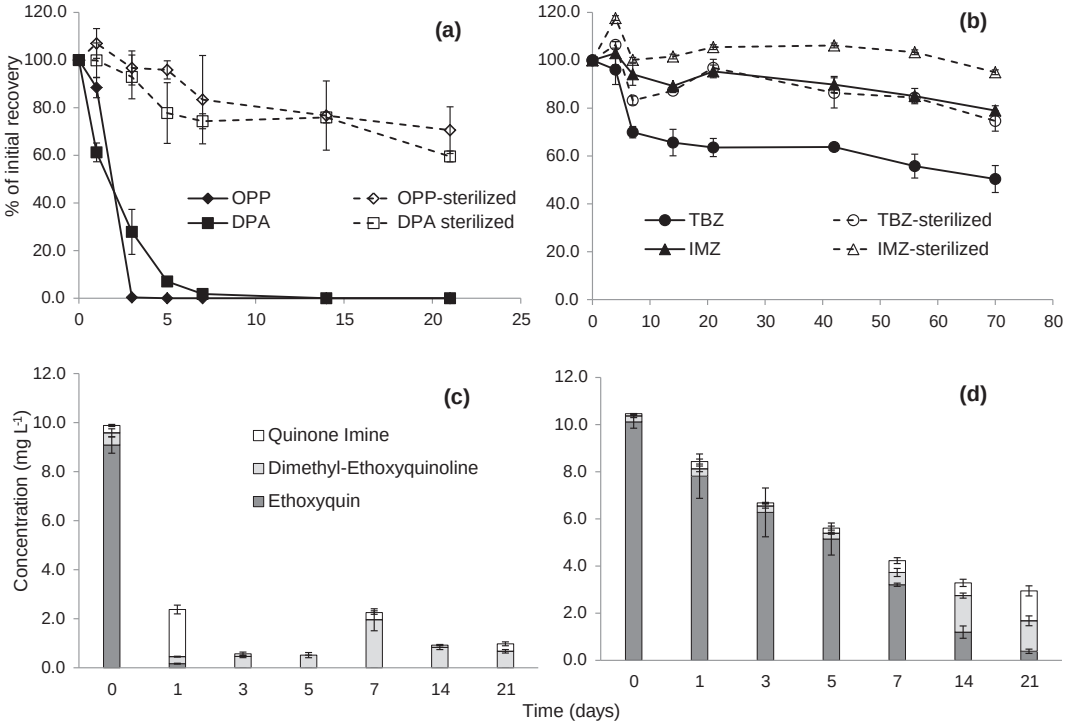

Fig. 3. Dissipation of *ortho*-phenylphenol (OPP) and diphenylamine (DPA) (a), thiabendazole (TBZ) and imazalil (IMZ) (b) and ethoxyquin (EQ) (c and d) by sterilized or non-sterilized liquid aerobic sewage sludge. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

compounds in the different substrates tested (Fig. 4b and d). For both compounds the higher dissipation efficiency was evident in SMS/straw/soil (50:25:25) with DT50s of 0.34 and 1 d for OPP and DPA respectively. On the other hand, the slowest dissipation rates for those two compounds were observed in soil with DT50s > 4 d (Table 3).

In accordance with the dissipation studies in sewage sludge, TBZ and IMZ were again the most persistent chemicals tested (Fig. 4e and f). For both pesticides the most rapid dissipation was evident in the substrates where SMS was the major component (SMS/soil and SMS/straw/soil) (50% by volume) with DT50s of 20–29 d for IMZ and 22.4–28.3 d for TBZ (Table 3). In contrast, the slowest dissipation for those compounds was observed in soil and straw/soil (50:50) for IMZ (DT50 = 79.3 d and 58.3 d) and in straw/soil (50:50) for TBZ (DT50 = 236 d).

The dissipation of EQ and its metabolites was very rapid within the first 7 d after its application with QI being the major component of the total residues of EQ even at 0 d (Fig. 5). This could be attributed to the very rapid transformation of EQ to its metabolites during the 4 h interval between pesticide application and collection and storage of T0 samples. To get a more focused view of the dissipation and metabolism kinetics of EQ, a follow-up study was undertaken to measure the dissipation of EQ and its metabolites during the first 24 h after application. The slowest dissipation of EQ and its metabolites was evident in soil (DT50 = 2.7 d) where the parent compound was immediately transformed to QI which constituted the major component of total residues at 24 h (Fig. 5a). In contrast in the other substrates tested, EQ was more gradually transformed to QI (Fig. 5b to e) with DT50s of less than 0.6 d.

3.4. Pesticide sorption onto organic substrates

The pesticide sorption isotherms are shown in Fig. 6. Pesticide sorption in all cases was well described by the Freundlich equation which was used for calculation of the sorption parameters (K_f, N) (Table 4). OPP and DPA showed weak sorption with the lowest K_f values observed in soil (2.47 and 5.57 g ml^{-1}) and the highest in straw/SMS/soil (50:25:25) (30.3 and 12.02 g ml^{-1}) respectively. IMZ and TBZ showed higher sorption affinity in the organic substrates which were characterized by higher organic matter content compared to soil where, again the lowest K_f values for both pesticides were measured (47.2 and 83.4 g ml^{-1} respectively). In agreement with OPP and DPA, the highest sorption of IMZ was seen in straw/SMS/soil (50:25:25) (Table 4).

4. Discussion

4.1. Pesticide dissipation in sewage sludge

We initially investigated the dissipation of the pesticides contained in the wastewaters from the fruit-packaging industry on anaerobically digested sewage sludge, a by-product of municipal wastewater treatment systems which is increasingly used as soil amendment in agriculture (del la Herras et al., 2005), and in liquid aerobic sewage sludge, which constitutes the metabolically active biomass found in the biological treatment systems of municipal wastewater treatment plants. Both substrates were efficient in rapidly dissipating OPP, DPA, EQ and its metabolites (QI and EQNL) with consistently faster dissipation observed in the latter substrate. It should be noted that the liquid aerobic sewage sludge used in the current study was not acclimated with the tested substances in contrast to several previous studies which found that an acclimation period was essential to achieve effective removal of other pesticides (Gonzalez et al., 2006). Our findings are in agreement with previous studies which reported a DT50 of 1.4 d for DPA in a bioreactor (Christodoulatos et al., 1997) and a rapid metabolism of DPA in sewage sludge with formation of aniline, imine and 4-hydroxy-DPA (Gardner et al., 1982). Similarly previous studies in a municipal wastewater treatment plant in Germany reported the complete removal of OPP although the metabolic pathway of OPP is not reported (Korner et al., 2000). Regarding EQ the only other study that have looked into its behavior in biological wastewater treatment systems showed that it is largely recalcitrant at both anaerobic and aerobic conditions and can induce inhibitory effects on the methanotrophic microbial community at 300 mg l^{-1} (Shah et al., 2005), which are well above the levels tested in our study.

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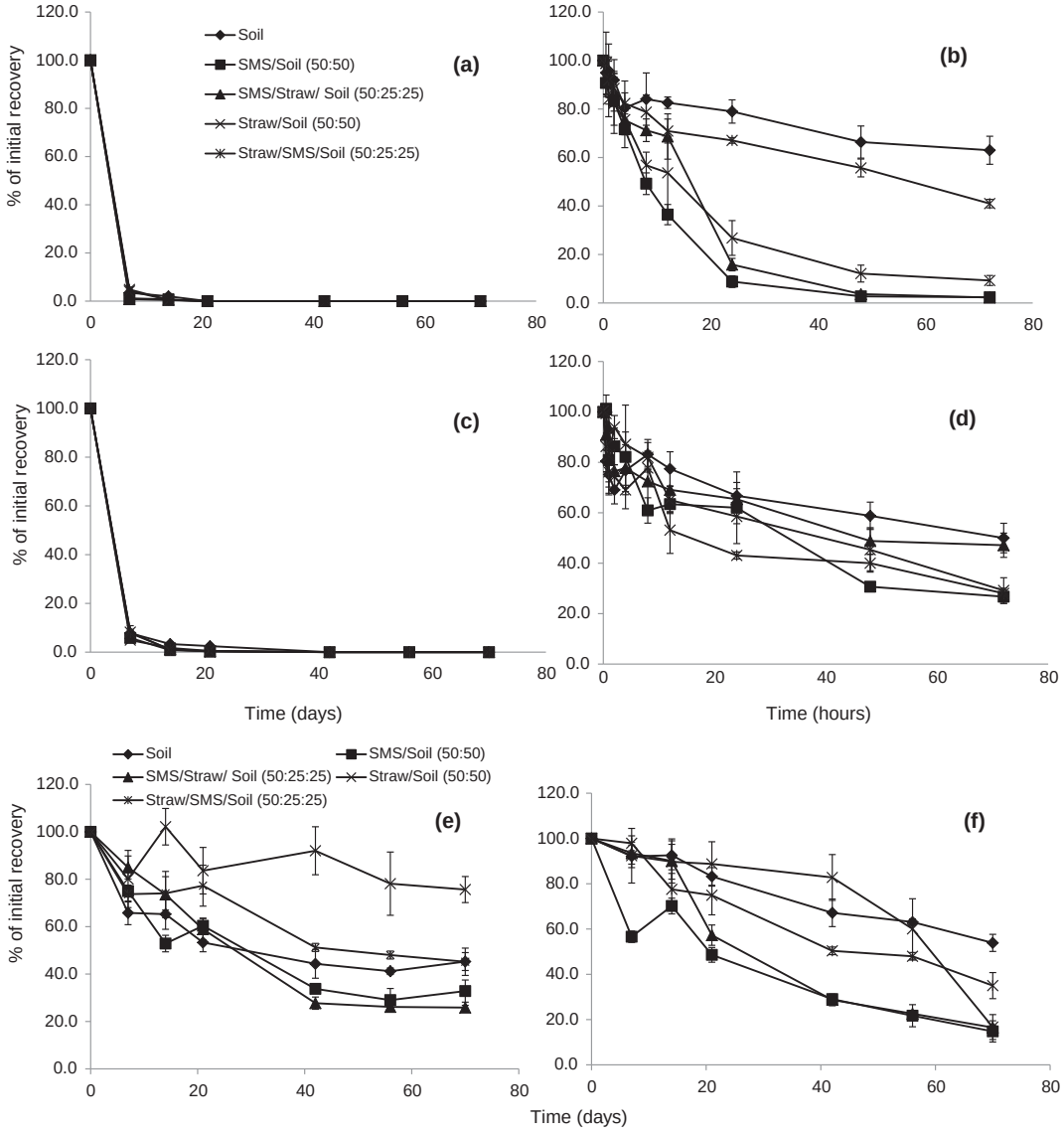

Fig. 4. Dissipation patterns of *ortho*-phenylphenol (OPP) (a & b) diphenylamine (DPA) (c & d) within 70 d or within 72 h after their application in different organic substrates respectively. The dissipation patterns of thiabendazole (TBZ) (e) and imazalil (IMZ) (f) in the same organic substrates are also shown. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

EQ showed a slightly different transformation pattern in anaerobically digested sewage sludge compared to liquid aerobic sewage sludge. This might reflect the different microbial communities in the two substrates: The latter substrate is expected to be dominated by microorganisms accustomed to high metabolic activities and oxidative degradation of organic matter like proteobateria and firmicutes (Yang et al., 2011) compared to the former where anaerobic digestion has drastically altered the microbial community (Pascual et al., 2008). Considering that the main use of EQ is as preservative of fish meal and fruits (indoor uses) the vast majority of studies have looked into its metabolism in animal and

Table 3
The half-life values of *ortho*-phenylphenol (OPP), diphenylamine (DPA), imazalil (IMZ), thiabendazole (TBZ) and total residues of ethoxyquin (EQ) in soil and other organic substrates as they were estimated by fitting the best-fitted kinetic model.

Pesticides	Soil			SMS/soil (50:50)			SMS/straw/soil (50:25:25)			Straw/soil (50:50)			Straw/SMS/soil (50:25:25)		
	DT50 (d)	χ^2 (%)	Model[a]	DT50 (d)	χ^2 (%)	Model	DT50 (d)	χ^2 (%)	Model	DT50 (d)	χ^2 (%)	Model	DT50 (d)	χ^2 (%)	Model
OPP	4.65	4.98	HS	0.57	9.48	SFO	0.34	3.16	SFO	2.5	3.46	FOMC	0.56	5.17	FOMC
DPA	4.08	8.11	SFO	3.16	4.52	FOMC	1.01	8.43	FOMC	1.04	8.92	FOMC	1.46	5.57	FOMC
IMZ	79.3	2.02	SFO	19.9	12.26	HS	28.6	8.89	HS	58.3	1.97	HS	46.0	4.32	SFO
TBZ	31.7	4.49	FOMC	22.4	7.81	FOMC	28.3	6.14	SFO	236.5	7.61	FOMC	54.8	6.04	HS
EQ + QI + EQNL[b]	2.7	7.4	SFO	0.6	10.7	SFO	0.2	4.4	DFOP	0.1	6.9	HS	0.1	12.6	HS

[a] SFO: Single First Order; HS: Hockey-Stick; FOMC: First Order Multi-Compartment.
[b] Calculations were made with the sum of residues of ethoxyquin, quinone imine (QI) and dimethyl ethoxyquinoline (EQNL).

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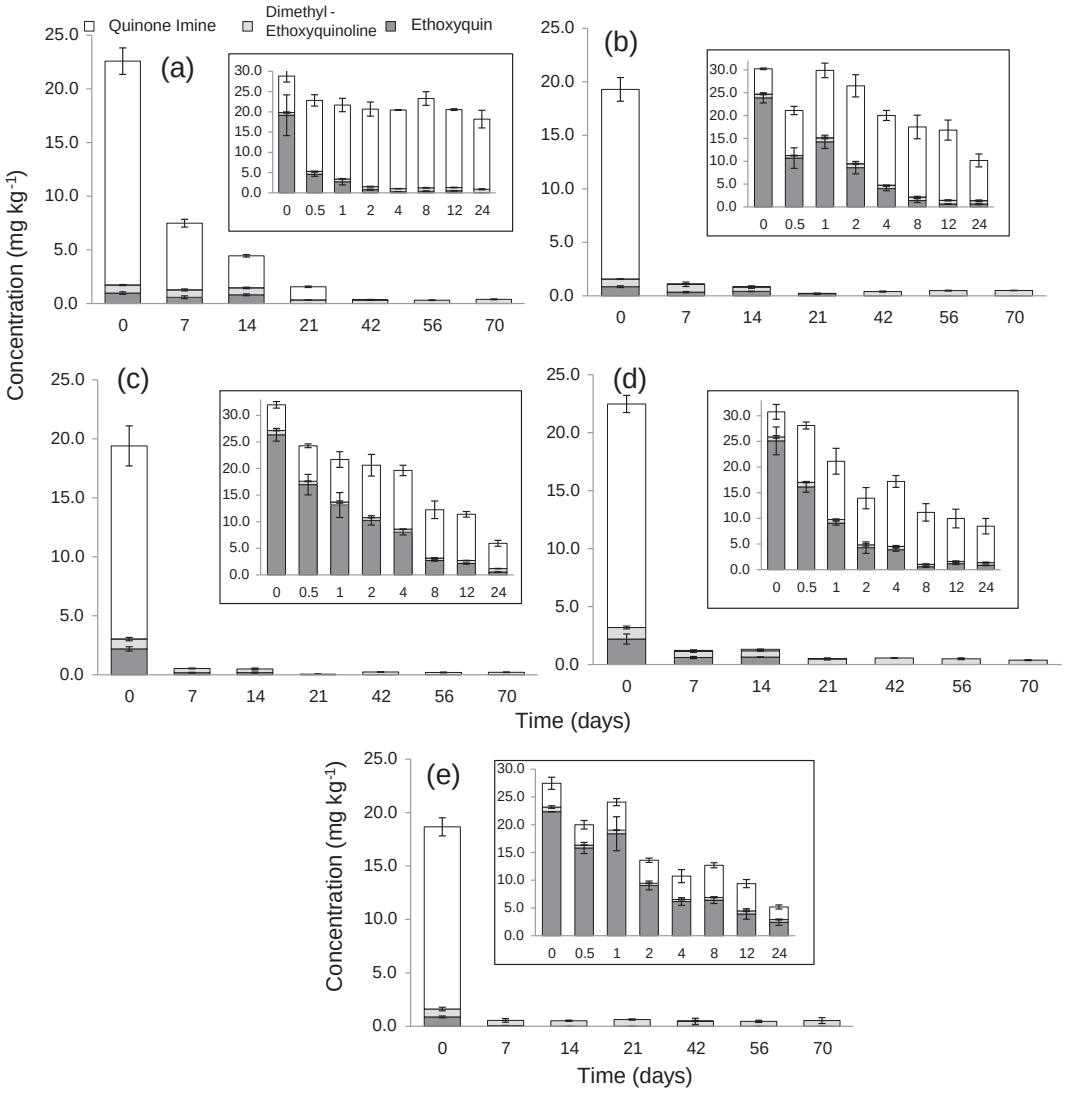

Fig. 5. Dissipation and metabolism of ethoxyquin (EQ) at different time frames, 70 d and 24 h (inserts), after their laboratory application into soil (a) and into various organic substrates including SMS/Soil (50:50) (b), SMS/straw/soil (50:25:25) (c), straw/soil (50:50) (d) and straw/SMS/soil (50:25:25) (e) (all ratios are by volume). Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

plant tissues and a range of metabolites detected including QI, dimeric EQ, methyl-EQ, EQNL, dihydro-EQ and demethyl-EQ (Gupta and Boobis, 2005; JMPR, 2005). On the other hand there is lack of knowledge regarding its metabolism in municipal wastewater treatment plants and environmental compartments. Although QI and EQNL were detected in our study, no residues of the other metabolites reported above were observed. Our study provides first evidence for the metabolism of EQ and its oxidation products in liquid aerobic sewage sludge.

Both types of sewage sludge showed a limited capacity to dissipate TBZ and IMZ. This is in agreement with previous findings which showed that municipal wastewater treatment plants acted as point sources for the contamination of surface water bodies (Campo et al., 2013; Masia et al., 2013). However, the possibility that higher dissipation efficiency for TBZ and IMZ could be achieved through acclimation of the liquid aerobic sewage sludge cannot be ruled out and should be tested in future studies. During our study no metabolites of TBZ and IMZ were measured. The metabolism of these two fungicides in sewage sludge is largely unknown. Recent studies showed that TBZ could be transformed via oxidation (Fenton/ PhotoFenton process) to OH-TBZ, thiazole-4-carboxamidine and other derivatives produced upon fusion of the benzyl ring (Sanchez Perez et al., 2014; Sirtori et al., 2014).

Sterilization of liquid aerobic sewage sludge resulted in drastic inhibition of pesticide dissipation stressing the microbial nature of the decay observed. It should be noted that EQ oxidation to QI and EQNL was also hampered in the sterilized LASS suggesting that those transformation steps are mostly biologically-driven, an information which was largely unknown. Overall our data suggest that the direct discharge of wastewaters from fruit-packaging industry onto municipal wastewater treatment plants are expected to effectively remove DPA, OPP, EQ and its derivatives but not the persistent fungicides TBZ and IMZ which entail a risk for the ecological integrity of receiving ecosystems.

4.2. Pesticide dissipation in organic biomixtures

The recalcitrance of TBZ and IMZ in sewage sludge suggests that other treatment methods should be applied to effectively eliminate those fungicides from those agro-industrial effluents. Previous studies by Omirou et al. (2012) showed that biobeds could be an efficient method for the depuration of wastewaters from the citrus fruit-packaging plants. To further exploit this, we investigated not only the dissipation of TBZ, IMZ, OPP, previously studied by Omirou et al. (2012) but also of preservatives like DPA and EQ which are heavily used in packaging plants of pears and apples. In addition we introduced SMS as a

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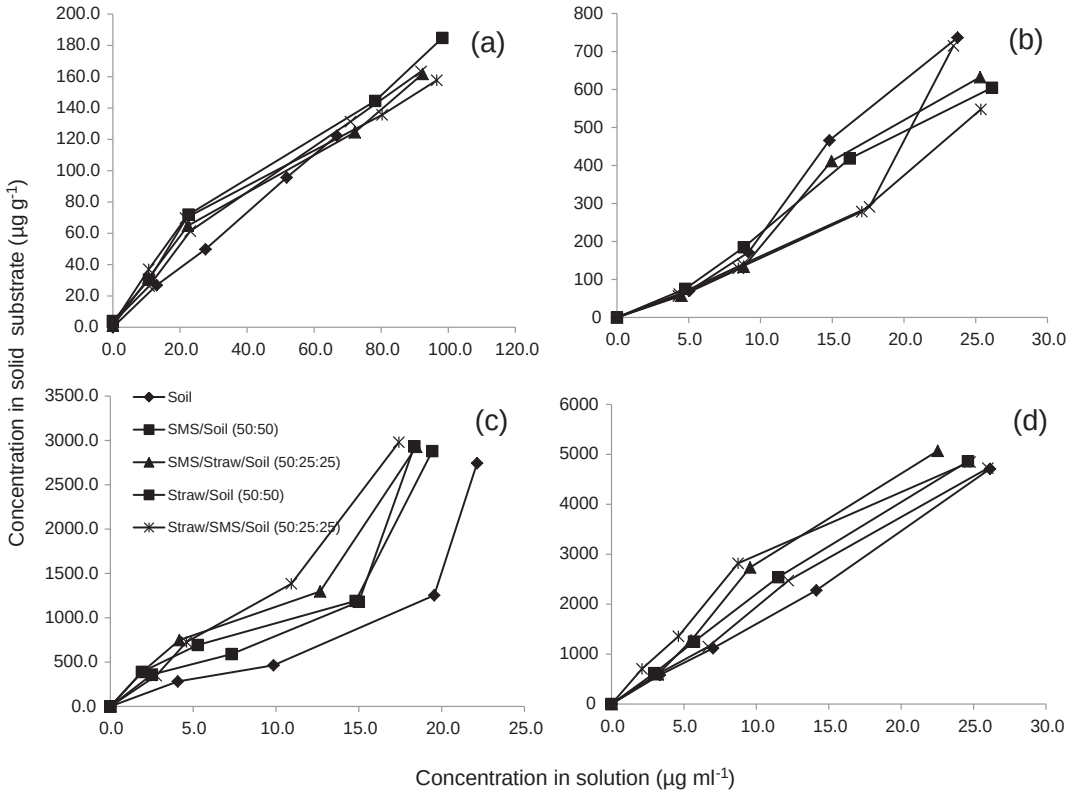

Fig. 6. Sorption isotherms of *ortho*-phenylphenol (OPP) (a), diphenylamine (DPA) (b), thiabendazole (TBZ) (c) and imazalil (IMZ) (d) in soil and in various organic substrates. Each value is the mean of three replicates.

potentially effective organic substrate to ameliorate the depuration efficiency of biobed systems receiving wastewaters from the fruit packaging industry.

Overall, the persistence of pesticides in soil and in the different organic biomixtures increased in the following order EQ < DPA < OPP < TBZ = IMZ in accordance with the sewage sludge dissipation patterns. The long persistence of both IMZ (US EPA, 2003; Kreuzig et al., 2010) and TBZ (Kesavan et al., 1976; EC, 2013) in soil is well documented. Regarding organic biomixtures, our results are in agreement with Omirou et al. (2012) who identified OPP and IMZ as the least persistent and the most persistent chemicals respectively, in different organic biomixtures derived from by-products of the winery and olive oil agro-industries compared to our SMS-rich biomixtures. No possible metabolites of TBZ, IMZ, OPP and DPA were found in the substrates tested although a more sensitive and high-resolution analytical approach is needed to verify this. Little is known regarding the metabolism of those fungicides in soil and they are mostly coming from regulatory documents. TBZ dissipation in soil was followed by formation of negligible amounts of benzimidazole and 5-OH-TBZ (EC, 2013), while for IMZ its main metabolite was IMZ-ethanol which was detected at low amounts (EFSA, 2010a).

Organic biomixtures showed a higher dissipation capacity compared to soil for all pesticides tested. Substrates with the highest % of SMS such as SMS/straw/soil (50:25:25) and SMS/soil (50:50) showed the highest dissipation potential for all pesticides tested. In particular, the DT50s obtained for TBZ and IMZ in those organic biomixtures were amongst the lowest ever reported verifying their enhanced dissipation efficiency (Kesavan et al., 1976; Kreuzig et al., 2010; Omirou et al., 2012). Our findings are in accordance with the positive correlation between % of SMS in biobed substrates and pesticide biodegradation observed by Karanasios et al. (2010a). This substrate is generally rich in complex and partly degraded organic C macromolecules (cellulose, hemicellulose, lignin) and N substrates which could support the growth of a particularly active microbial community able to degrade pesticides (Marin-Benito et al., 2009). The contribution of *P. ostreatus* by the SMS on the higher dissipation capacity of the SMS-augmented materials is not clear. Previous studies have shown that the role of this fungus on the degradation of pesticides in similar organic biomixtures is negligible (Karanasios et al., 2010b) and its mycelium is progressively surpassed by other fast-growing microorganisms when mixed with soil (Tuomela et al., 2002). The significant role of white rot fungi like *P. ostreatus* on the degradation of pesticides is mostly documented in peat-based organic biomixtures where their survival and activity is favored by the acidic pH of those materials (Castillo et al., 2008), whereas the neutral to alkaline pH of the organic biomixture used in our study are not expected to favor their survival. However, the indirect role of *P. ostreatus* in partly degrading and modifying the properties of the raw MS material leading

Table 4
Sorption parameters K_f (g ml^{-1}), K_{foc} (g ml^{-1}) and N for the pesticides *ortho*-phenylphenol (OPP), diphenylamine (DPA), imazalil (IMZ) and thiabendazole (TBZ) in soil and organic substrates used in the study.

Pesticides	Soil			SMS/soil (50:50)			SMS/straw/soil (50:25:25)			Straw/soil (50:50)			Straw/SMS/soil (50:25:25)		
	K_f	K_{foc}	N	K_f	K_{foc}	N	K_f	K_{foc}	N	K_f	K_{foc}	N	K_f	K_{foc}	N
OPP	2.47	235.2	1.065	5.01	29.6	1.195	11.67	39.8	1.374	8.01	120.0	1.273	30.3	128.9	1.573
DPA	5.57	530.5	1.574	11.57	68.5	1.245	6.37	21.7	1.456	7.82	117.9	1.353	12.02	51.1	1.245
TBZ	47.2	4965.7	1.141	217.2	1285.2	0.755	226.8	774.1	0.800	128.4	1936.7	0.926	120.3	511.9	1.091
IMZ	83.4	7942.9	1.013	222.3	1315.4	0.976	186.2	635.5	1.100	183.6	2769.2	1.005	412.4	1754.9	0.798

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to an optimized co-substrate for pesticide degradation may be critical for the observed SMS performance. Overall, the beneficial effect of SMS on the dissipation efficiency of biobeds packing material provides an option to the mushroom units in the Mediterranean basin for the sustainable, environmental-friendly and effective exploitation of this waste.

The inclusion of high proportions of straw (50%) in the organic substrates substantially increased the persistence of most pesticides with TBZ showing the most prominent increase. Despite the well documented beneficial effect of straw on pesticides dissipation in biobed systems (Castillo et al., 2008), its high organic C content could enhance the sorption affinity of lipophilic substances like TBZ and IMZ resulting in higher persistence due to reduced bioavailability. The higher K_f values reported for straw-rich packaging materials in our sorption isotherm results are in line with this.

EQ showed similar metabolic patterns in soil and organic substrates with rapid conversion to QI which constituted the major component of the total residues of EQ 24 h after application. On the other hand EQNL was a minor metabolite which was formed at low amounts but persisted until 70 d post-application. This metabolic pattern deviates from the metabolic pattern observed in anaerobically digested sewage sludge. This could be attributed to the different compositions of those materials which are expected to support microbial communities with different metabolic capacities: anaerobically digested sewage sludge is mostly composed of hydrocarbons, amino acids and lipids compared to biobed materials which are mostly composed of cellulose, hemicellulose and lignin (Rodriguez-Cruz et al., 2012) and may favor aerobic oxidation processes. This is the first study providing data for the fate and the transformation of EQ in soil and biobed packing material.

4.3. Pesticide sorption onto organic biomixtures

Sorption of pesticides in soil and organic biomixtures provided explanations for the dissipation patterns observed. In particular, OPP and DPA showed a weak sorption in agreement with previous studies which also showed moderate sorption for those pesticides with soil K_{foc} values of 894–1793 ml g^{-1} (Zheng et al., 2011) and 1212–6593 ml g^{-1} (US EPA, 1998) respectively. On the other hand, TBZ and IMZ were strongly sorbed onto soil and organic biomixtures which is in accordance with previous soil sorption studies with K_{foc} values of 4059 (Kreuzig et al., 2010) to 4357 ml g^{-1} for IMZ (EFSA, 2010a) and of 1104 to 22,467 ml g^{-1} for TBZ (EC, 2001). The strong sorption of IMZ and TBZ combined with their limited biodegradability explain the general recalcitrance of those chemicals. Omirou et al. (2012) also showed in column and full-scale biobeds that OPP was mobile but dissipated rapidly compared to TBZ and IMZ which remained in the top layers of the biobed (an indication of high sorption affinity) and dissipated at low rates.

Regarding the impact of substrate on the sorption behavior of pesticides, soil showed a substantially lower sorption affinity compared to the organic substrates tested. This is in agreement with previous studies which have attributed this to the higher organic C content of the latter providing more sorption sites for non-polar pesticides (De Wilde et al., 2009). Amongst the organic biomixtures tested, straw/SMS/soil (50:25:25) showed the highest sorption capacity for OPP, DPA and IMZ and SMS/straw/soil (50:25:25) for TBZ. This is in accordance with the higher organic C content of those two substrates compared to the rest of the substrates tested (Table 1). Pesticides desorption was not measured in the current study. Previous studies have suggested a limited reversibility of pesticides adsorption in soil amended with SMS (Marin-Benito et al., 2009) and various biobeds packing materials (Karanasios et al., 2010b) compared to soil. On the one hand, this might favor the efficient removal of pesticides from the agro-industrial effluents but on the other hand it might result in limited bioavailability and retardation of the degradation of the retained pesticide residues.

5. Conclusions

Wastewaters from the fruit packaging industry constitute a serious point source contamination of natural water resources with pesticides. Our findings suggest that municipal wastewater treatment plants are expected to effectively remove OPP, DPA, EQ and its oxidation products but not TBZ and IMZ stressing the need for the implementation of more efficient but still simple and low-cost depuration methods. SMS-rich organic biomixtures accelerated the dissipation of all pesticides, particularly of the recalcitrant TBZ and IMZ, suggesting that biobeds packed with such organic biomixtures could effectively depurate the wastewaters from the fruit-packaging industry.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.05.086.

References

Anastassiades, M., Lehotay, S.J., Stajnbaher, D., Schenck, F.J., 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J. AOAC Int. 86, 412–431.

Baszczyk, A.B., Augustyniak, A., Skolimowski, J., 2013. Ethoxyquin: an antioxidant used in animal feed. Int. J. Food Sci. http://dx.doi.org/10.1155/2013/585931 (Article ID 585931).

Campo, J., Masia, A., Blasco, C., Pico, Y., 2013. Occurrence and removal efficiency of pesticides in sewage treatment plants of four Mediterranean River Basins. J. Hazard. Mater. 263P, 146–157.

Castillo, L.E., Ruepert, C., Solis, F., 2000. Pesticide residues in the aquatic environment of banana plantation areas in the North Atlantic Zone of Costa Rica. Environ. Toxicol. Chem. 19, 1942–1950.

Castillo, L.E., Martinez, E., Ruepert, C., Savage, C., Gilek, M., Pinnock, M., Solis, E., 2006. Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. Sci. Total Environ. 367, 418–432.

Castillo, M.d.P., Torstensson, L., Stenstrom, J., 2008. Biobeds for environmental protection from pesticides use — a review. J. Agric. Food Chem. 56, 6206–6219.

Christodoulatos, C., Koutsospyros, A.B., Brodman, B.W., Korfiatis, G.P., 1997. Biodegradation of diphenylamine by selected microbial cultures. J. Environ. Sci. Health A32, 15–30.

De Wilde, T., Spanoghe, P., Ryckeboer, J., Jaeken, P., Springael, D., 2009. Sorption characteristics of pesticides on matrix substrates used in biopurification systems. Chemosphere 75, 100–108.

del la Herras, J., Manas, P., Labrador, J., 2005. Effects of several applications of digested sewage sludge on soil and plants. J. Environ. Sci. Health A40, 437–451.

Environmental Protection Agency (EPA) USA, 1998. R.E.D. Facts—Diphenylamine. EPA-738-F-97-010.

Environmental Protection Agency (EPA) USA, 2003. Reregistration Eligibility Decision for Imazalil Case No. 2325. p. 74.

European Commission (EC), 2000. Opinion of the scientific committee on plants regarding the evaluation of thiabendazole in the context of council directive 91/414/EEC concerning the placing of plant protection products on the market — The Scientific Committee on Plants. SCP/THIABEN/002—Final, p. 7.

European Commission (EC), 2001. Review report for the active substance thiabendazole finalised in the Standing Committee on Plant Health at its meeting on 12 December 2000 in view of the inclusion of thiabendazole in Annex I of Directive 91/414/EEC. 7603/VI/97—Final.

European commission (EC), 2010. Draft Assessment Report, Initial Risk Assessment Provided by the Rapporteur Member State the Netherlands for the Existing Substance Imazalil volume 1, pp. 1–151.

Institutional Repository - Library & Information Centre - University of Thessaly
20/04/2024 10:51:04 EEST - 3.145.162.204

European Commission (EC), 2013. Draft renewal assessment report prepared according to the commission regulation (EU) N.1141/2010. Second Programme for the Renewal of the Inclusion of the Following Active Substance under Regulation (EC) 1107/2009. Thiabendazole vol. 1 (Report and proposed decision).
European Food Safe Authority (EFSA), 2008. Conclusion on pesticide peer review. Peer review of the pesticide risk assessment of the active substance 2-phenylphenol. EFSA Sci. Rep. 217, 1–67.
European Food Safety Authority (EFSA), 2010a. Conclusion on the peer review of the pesticide risk assessment of the active substance imazalil. EFSA J. 8 (3), 1526.
European Food Safety Authority (EFSA), 2010b. Conclusion on the peer review of the pesticide risk assessment of the active substance ethoxyquin. EFSA J. 8 (9), 1710.
European Food Safety Authority (EFSA), 2012. Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine. EFSA J. 10 (1), 2486–2545.
FOCUS, 2006. Guidance document on estimating persistence and degradation kinetics from environmental fate studies on pesticides in EU registration. Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference Sanco/10058/2005 version, 2.0, 2006 (434 pp.).
Garcia-Portillo, M., Avino, E.S., Vicente, J.O., De Andres, R.L., Jimenez, M.d.l.A.S., Blanco J.P.L., 2004. Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States Patent, US 6,709,585 B1, pp. 9.
Gardner, A.M., Alvarez, G.H., Ku, Y., 1982. Microbial degradation of ^{14}C-diphenylamine in a laboratory model sewage sludge system. Bull. Environ. Contam. Toxicol. 28, 91–96.
Gonzalez, S., Muller, J., Petrovic, M., Barcelo, D., Knepper, T.D., 2006. Biodegradation studies of selected priority acidic pesticides and diclofenac in different bioreactors. Sci. Total Environ. 144, 926–932.
Gupta, P.K., Boobis, A., 2005. Ethoxyquin (addendum). http://www.inchem.org/documents/jmpr/jmpmono/v2005pr-10.pdf.
He, P., Ackman, R.G., 2000. HPLC determination of ethoxyquin and its major oxidation products in fresh and stored fish meals and fish feeds. J. Sci. Food Agric. 80, 10–16.
Herrero-Hernandez, E., Andrades, M.S., Marın-Benito, M.S., Sanchez-Martın, M.J., Rodrıguez-Cruz, M.S., 2011. Field-scale dissipation of tebuconazole in a vineyard soil amended with spent mushroom substrate and its potential environmental impact. Ecotoxicol. Environ. Saf. 74, 1480–1488.
Jimenez, M., Maldonado, M.I., Rodriguez, E.M., Hernandez-Ramirez, A., Saggioro, E., Carrae, I., Sanchez, J.A., 2015. Supported TiO_2 solar photocatalysis at semi-pilot scale: degradation of pesticides found in citrus processing industry wastewater, reactivity and influence of photogenerated species. J. Chem. Technol. Biotechnol. 90, 149–157.
JMPR, 2005. Pesticide residues in food—2005. Report of the joint meeting of the FAO panel of experts on pesticide residues in food and the environment and the WHO core assessment group on pesticide residues. JMPR Report, Ethoxyquin 035 (http://www.fao.org/ag/AGP/AGPP/Pesticid/JMPR/Download/99eva/14Ethoxyquin.pdf).
Jonkers, N., Sousa, A., Galante-Oliveira, S., Barroso, C.M., Kohler, H.P.E., Giger, W., 2010. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro, Portugal. Environ. Sci. Pollut. Res. 17, 834–843.
Jung, S.-K., Watkins, C.B., 2008. Superficial scald control after delayed treatment of apple fruit with diphenylamine (DPA) and 1-methylcyclopropene (1-MCP). Postharvest Biol. Technol. 50, 45–52.
Karanasios, E., Tsiropoulos, N., Karpouzas, D.G., Menkissoglu-Spiroudi, U., 2010a. Novel biomixtures based on local Mediterranean ligninocellulosic materials: evaluation for use in biobeds. Chemosphere 80, 914–921.
Karanasios, E., Tsiropoulos, N., Karpouzas, D.G., Ehaliotis, C., 2010b. Degradation and adsorption of pesticides in compost-based biomixtures as potential substrates for biobeds in south Europe. J. Agric. Food Chem. 58, 9147–9156.
Kesavan, R., Van Wambeke, E., Van Assche, C., 1976. The effects of certain factors on the persistence of thiabendazole, 2-(4-thiazolyl) benzimidazole in the soil environment. Meded. Fak. Landbouwwet. R. U. Gent 41 (2), II.
Khodja, A.A., Sehili, T., Pilichowski, J.-F., Boule, P., 2001. Photocatalytic degradation of 2-phenylphenol on TiO_2 and ZnO in aqueous suspensions. J. Photochem. Photobiol. A Chem. 141, 231–239.
Korner, W., Bolz, U., Submuth, W., Hiller, G., Schuller, W., Hanf, V., Hagenmaier, H., 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 40, 1141–1142.
Kreuzig, R., Hartmann, C., Teigeler, J., Höltge, S., Cvetkovic, B., Schlag, P., 2010. Development of a novel concept for fate monitoring of biocides in liquid manure and manured soil taking ^{14}C-imazalil as an example. Chemosphere 79, 1089–1094.
Marin-Benito, J.M., Sanchez-Martin, M.J., Andrades, M.S., Periz-Clavijo, M., Rodriguez-Cruz, 2009. Effect of spent mushroom substrate amendment of vineyard soils on the behavior of fungicides: 1. Adsorption–desorption of penconazole and metalaxyl by soils and subsoils. J. Agric. Food Chem. 57, 9634–9642.
Masia, A., Campo, J., Vazquez-Roig, P., Blasco, C., Pico, Y., 2013. Screening of currently used pesticides in water, sediments and biota of the Guadalquivir River Basin (Spain). J. Hazard. Mater. 263P, 95–104.
OECD 106, 2000. OECD Guideline for the Testing of Chemicals: Adsorption–desorption using a Batch Equilibrium Method.
Omirou, M., Dalias, P., Costa, C., Papastefanou, C., Dados, A., Ehaliotis, C., Karpouzas, D.G., 2012. Exploring the potential of biobeds for the depuration of pesticide-contaminated wastewaters from the citrus production chain: laboratory, column and field studies. Environ. Pollut. 166, 31–39.
Pascual, I., Aviles, M., Aguirreola, J., Sanchez-Diaz, M., 2008. Effect of sanitized and non-sanitized sewage sludge on soil microbial community and the physiology of pepper plants. Plant Soil 310, 41–53.
Rodriguez-Cruz, M.S., Herrero-Hernandez, E., Ordaz, J.M., Marin-Benito, J.M., Draoui, K., Sanchez-Martin, M.J., 2012. Adsorption of pesticides by sewage sludge, grape marc, spent mushroom substrate and by amended soils. Int. J. Environ. Anal. Chem. 92, 933–948.
Sanchez Perez, J.A., Carra, A., Sirtori, C., Aguera, A., Esteban, B., 2014. Fate of thiabendazole through the treatment of a simulated agro-food industrial effluent by combined MBR/Fenton processes at mg/L scale. Water Res. 51, 55–63.
Santiago, D.E., Melian, E.P., Rodriguez, C.F., Mendez, J.A.O., Perez-Baez, S.O., Dona-Rodriguez, J.M., 2011. Degradation and detoxification of banana postharvest treatment water using advanced oxidation techniques. Green Sustain. Chem. 1, 39–46.
Shah, A.G., Pierson, J.A., Pavlostathis, S.G., 2005. Fate and effect of the antioxidant ethoxyquin on a mixed methanogenic culture. Water Res. 39, 4251–4263.
Shin, K.A., Spain, J.C., 2009. Pathway and evolutionary implications of diphenylamine biodegradation by *Burkholderia* sp. strain JS667. Appl. Environ. Microbiol. 75, 2694–2704.
Sirtori, C., Aguera, A., Carra, I., Sanchez Perez, J.A., 2014. Identification and monitoring of thiabendazole transformation products in water during Fenton degradation by LC-QTOF-MS. Anal. Bioanal. Chem. 406, 5323–5337.
Smilanick, J.L., Mansour, M.F., Gabler, F.M., Sorenson, D., 2008. Control of citrus postharvest green mold and sour rot by potassium sorbate combined with heat and fungicides. Postharvest Biol. Technol. 47, 226–238.
Thorisson, S., Gunstone, F.D., Hardy, R., 1992. Some oxidation products of ethoxyquin including those found in autoxidising systems. Chem. Phys. Lipids 60, 263–271.
Tuomela, M., Oivanen, P., Hatakka, A., 2002. Degradation of synthetic ^{14}C-lignin by various white-rot fungi in soil. Soil Biol. Biochem. 34, 1613–1620.
Yang, C., Zhang, W., Liu, R., Li, Q., Li, B., Wang, S., Song, C., Qiao, C., Mulchandani, A., 2011. Phylogenetic diversity and metabolic potential of activated sludge microbial communities in full-scale wastewater treatment plants. Environ. Sci. Technol. Lett. 4, 7408–7415.
Zheng, C., Focks, A., Ellebrake, K., Eggers, T., Fries, E., 2011. Sorption of ortho-phenylphenol to soils. Clean Soil Air Water 39, 116–120.

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Integrated biodepuration of pesticide-contaminated wastewaters from the fruit-packaging industry using biobeds: Bioaugmentation, risk assessment and optimized management

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HIGHLIGHTS

- Fruit-packaging plants wastewaters constitute a serious point source contamination.
- Biodepuration with pilot biobeds achieved >99.5% removal of the pesticides tested.
- Bioaugmentation with tailored-made inocula maximized depuration for thiabendazole.
- Risk assessment suggested no unacceptable risk by the release of biobed effluents.
- Bioaugmentation or composting could decontaminate the spent biobed packing material.

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ABSTRACT

Wastewaters from fruit-packaging plants contain high loads of toxic and persistent pesticides and should be treated *on site*. We evaluated the depuration performance of five pilot biobeds against those effluents. In addition we tested bioaugmentation with bacterial inocula as a strategy for optimization of their depuration capacity. Finally we determined the composition and functional dynamics of the microbial community via q-PCR. Practical issues were also addressed including the risk associated with the direct environmental disposal of biobed-treated effluents and decontamination methods for the spent packing material. Biobeds showed high depuration capacity (>99.5%) against all pesticides with bioaugmentation maximizing their depuration performance against the persistent fungicide thiabendazole (TBZ). This was followed by a significant increase in the abundance of bacteria, fungi and of catabolic genes of aromatic compounds *catA* and *pcaH*. Bioaugmentation was the most potent decontamination method for spent packing material with composting being an effective alternative. Risk assessment based on practical scenarios (pome and citrus fruit-packaging plants) and the depuration performance of the pilot biobeds showed that discharge of the treated effluents into an 0.1-ha disposal site did not entail an environmental risk, except for TBZ-containing effluents where a larger disposal area (0.2 ha) or bioaugmentation alleviated the risk.

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1. Introduction

Postharvest treatment of fruits with pesticides guarantees their protection from fungal infestations and physiological disorders during storage. However, it leads to the production of large volumes of pesticide-contaminated effluents whose discharge without prior treatment entails serious environmental risks [1]. This is exempli-

Abbreviations: TBZ, thiabendazole; IMZ, imazalil; OPP, ortho-phenylphenol; DPA, diphenylamine; SMS, spent mushroom substrate; RACs, regulatory acceptable concentrations; TER, toxicity exposure ratio; PECs, predicted environmental concentrations; HQ, hazard quotient; EFSA, European food safety authority; EC, European Commission.

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fied by the high aquatic toxicity of the pesticides used in this sector like thiabendazole (TBZ) [2] imazalil (IMZ) [3], *ortho*-phenylphenol (OPP) [4] and diphenylamine (DPA) [5].

The need for treatment of those effluents is stressed in the relevant pesticide regulatory documents which state that member-states should ensure *that appropriate waste management practices to handle the waste solution remaining after application are put in place* [6]. Several methods have been tested for the treatment of those effluents but integrated full scale implementation has not been achieved yet. Garcia-Portilo et al. [7] patented a treatment system based on activated carbon which showed high removal efficiency for TBZ. However its high cost have prevented its wide implementation in fruit-packaging plants. Recent studies by Sanchez Perez et al. [8] proposed a combined membrane biological reactor/Fenton-Photo Fenton process for the dissipation of TBZ. However this study was performed at pesticide levels (0.1 mg L^{-1}) which are multi-fold lower than the levels found in the effluents. In addition, those methods lead to the formation of oxidation products of unknown toxicity [9]. In the absence of treatment systems industries dispose their effluents in municipal sewage treatment plants which are not effective in the removal of those pesticides transferring the contamination to receiving water systems [10].

Biological treatment systems like biobeds could be a possible solution for the treatment of those effluents. They are simple and efficient systems used up to now for the depuration of pesticide-contaminated effluents at on farm level [11]. In their simplest form they are composed of a pit packed with a mixture of bioorganic material [12]. Omirou et al. [13] first tested biobeds for the depuration of wastewater produced by the citrus fruit production chain, thus pesticides like DPA used in pome fruit-packaging plants were not considered. In their study, TBZ and IMZ were retained by the biobed packing material leading to a potential build-up of high pesticide residues stressing the need for decontamination of the spent packing material. This constitutes a key regulatory issue withholding the wider adoption of biobeds [14]. However only a few studies have addressed it [15,16].

Little attention has been given also to the post-treatment handling of biobeds-treated effluents. Despite the high depuration performance of biobeds [12], pesticide residues are still present in their effluents and their environmental release should be allowed pending risk assessment. This is feasible for biobeds receiving wastewaters from the fruit-packaging industry where a limited number of pesticides is used, in contrast to on-farm systems which receive a much wider pesticide range and thus complex risk assessment approaches are required.

Biodegradation has been identified as the key process controlling biobeds performance [11]. Despite that little is known about the composition of the microbial community in biobeds and the microbial dynamics driving the biodegradation process. Good knowledge of the microbiology of biobeds will facilitate their optimization. Bioaugmentation has been explored as a strategy for optimization of biobeds performance. Karanasios et al. [17] showed that the use of spent mushroom substrate (SMS) from the edible fungus *Pleurotus ostreatus* in biobeds accelerated pesticide dissipation. Sniegowski and Springael [18] showed that the use of soil carrying a microbial community adapted to the rapid degradation of specific pesticides as a component of the packing material could ameliorate the depuration capacity of biobeds. This strategy or bioaugmentation with tailored-made microbial inocula could be ideal in cases where biobeds receive effluents containing a few known pesticides like in fruit-packaging plants.

The main aims of this study were to a) evaluate the depuration performance of pilot biobeds against pesticides used in fruit-packaging plants, and assess bioaugmentation as an optimization strategy, b) identify the key microbial groups, phylogenetically and functionally relevant to biobed systems, c) estimate the risk associated with the environmental disposal of the biobed-treated effluents and d) assess methods for the decontamination of the spent biobed packing material.

2. Materials and methods

2.1. Pesticides

Analytical standards of IMZ (99.8%), TBZ (99%) OPP (99.9%) and DPA (99.9%) (Pestanal®, Sigma-Aldrich) were used for residue analysis. Commercial formulations of TBZ (TECTO® 50% SC), IMZ (FUNGAZIL® 50% EC), OPP (FRUITGARD® 20%SL) and DPA (NO SCALD® 31.8%EC) were utilized for the preparation of the aqueous solutions applied on biobeds.

2.2. Biobed packing material

Following earlier optimization studies [19,20], a mixture of SMS, soil and straw (50:25:25 by volume) was used for the packing of the pilot biobeds. The soil used was collected from a field site in Larissa, Greece. It was sieved to homogenize (4 mm) prior to mixing with organic materials. Wheat straw was chopped into small pieces (1–3 cm) and passed through a 4.75 mm sieve. SMS was obtained from a *P. ostreatus* mushroom production unit (Mpoulogeorgos, Trikala, Greece) and it was chopped into small pieces. Soil, straw and SMS were mixed thoroughly and were left to mature for a month. Properties of the materials used are listed in Supplementary Table 1. Total organic C and N content were determined by the wet digestion [21] and the Kjeldahl digestion method [22] respectively. pH was determined in a mixture of 1:2.5-5 air dried solid substrate:water (w:v). Soil texture was determined with the Bouyoucos hydrometer method [23].

2.3. Set up of pilot biobeds

Five pilot biobeds composed of plastic containers of 1.1 m^3 (3 biobeds) or 0.24 m^3 (2 biobeds) volume were set up. The bottom of the biobeds was covered with a metal wire mesh and on top of this a 5-cm layer of well-washed gravel (2–3 cm diameter) was placed. The remaining volume was filled with the packing material described above. A 10-cm diameter hole was made at the bottom of the biobeds to allow collection of the draining effluent. A plastic funnel was positioned under the outer side of the hole and it was connected to a plastic tube (15 mm i.d.) leading to a 2.5-L amber glass bottle where effluents were collected.

The pesticide solutions applied on the biobeds were prepared in three 100-L tanks each containing an aqueous solution of two pesticides: IMZ + DPA (Tank 1), OPP + IMZ (Tank 2) and TBZ + OPP (Tank 3). The concentration of all pesticides in the aqueous solutions was 100 mg L^{-1} assuming a 10-fold dilution of their concentration in the water during the treatment process and considering the pesticides recommended dose rates (0.6 g L^{-1} for OPP, 1.2 g L^{-1} for TBZ, 1 g L^{-1} for IMZ and 2 g L^{-1} for DPA) [5,24–26]. Pesticides combinations were established according to their use patterns: IMZ + DPA and OPP + IMZ or TBZ are used in pome and citrus fruit-packaging plants respectively. In total 1080 and 252 L of pesticide solutions were discharged into the large and the small pilot biobeds respectively within a period of 160-d corresponding to the average operation period of a fruit-packaging plant [27]. Pesticide solutions were pumped (max capacity 10 L h^{-1}) into the biobeds daily (Three 10-min application periods per day). This resulted in a daily discharge of 7.5 and 2.0 L in the large and the small pilot biobeds respectively. Pesticide solutions were pumped at the top of the pilot biobeds via a drip irrigation system ensuring their uniform dispersion onto the biobeds surface. A schematic diagram of the experimental setup is

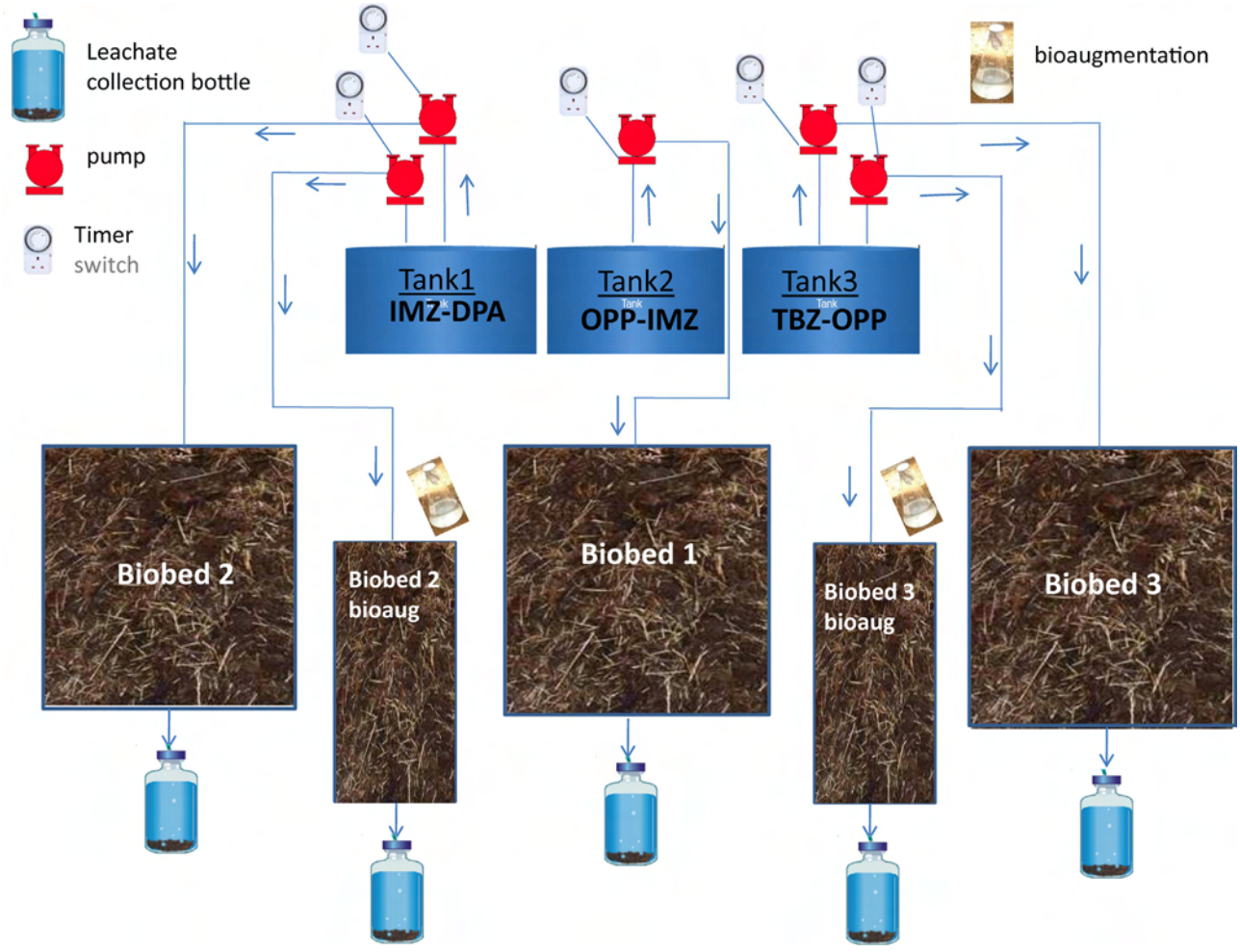

Fig. 1. A schematic diagram of the setup of the pilot biobeds. Arrows indicate the direction of the wastewater flow.

shown in Fig. 1. Prior to pesticides application, biobeds were irrigated for three days with clean water and were left to drain for a week to allow for equilibration. Biobeds leachates were collected every two days. Each time, the volume of the leachate collected was recorded and a subsample (0.5 L) was stored at −20 °C for analysis.

At the end of the 160-d period three cores were collected from each pilot biobed using a 90-cm long PVC plastic tube (8 cm i.d.). The packing material cores were sectioned into three layers: 0–20, 20–50 and 50–80 cm and stored at −20 °C until analyzed. Pesticides amounts retained in the different layers of the biobeds were determined by sequential extractions with water and acetonitrile as described below. The total amount of pesticides recovered by the substrate at the end of the study, plus the amount of pesticide leached were deducted from the total pesticide amount applied and it was considered as 'dissipated'. This was a lump process including degradation and non-extractable residues formation.

2.4. Bioaugmentation of pilot biobeds

We evaluated bioaugmentation with OPP- (*Sphingomonas haloaromaticamans*) [28], DPA- (*Pseudomonas putida*) [29] and TBZ-degrading bacteria (consortium comprised of different proteobacteria) [30] as a strategy for ameliorating the depuration performance of biobeds. Briefly, the pesticide-degrading bacteria were grown in mineral salts media where the pesticide constituted the sole C (OPP) or the sole C and N source (TBZ, DPA) [29]. Bacterial inocula were harvested at the mid-logarithmic phase, cells were washed three times with sterile ddH_2O and they were resuspended to ddH_2O which was applied to the packing material of the two small pilot biobeds (biobed 2bioaug and biobed 3bioaug) aiming to a final inoculum density of 10^6 cells g^{-1} of packing material (on a dry weight basis). The density of the bacterial inocula was determined by serial dilution plating in LB. Bacterial cells were sprayed with a hand sprayer and the treated packing material was thoroughly mixed with a spade prior to biobeds' packing. Biobed 2bioaug, treated with IMZ and DPA, was inoculated with the DPA-degrading bacterium (no IMZ-degrading bacteria were available), while biobed 3bioaug, treated with TBZ and OPP, was amended with the TBZ- and the OPP-degrading bacterial inocula.

2.5. Risk assessment analysis for the management of biobeds effluents

An assessment of the risk associated with the environmental release of the biobed-depurated wastewater was employed. Two scenarios simulating the handling of wastewaters produced by a pome (Scenario I) or a citrus (Scenario II) fruit-packaging plant were designated. A total volume of 25 m^3 of wastewaters containing DPA, TBZ or IMZ (Scenario I), or a volume of 42 m^3 of OPP-plus 11 m^3 of IMZ- or TBZ-containing wastewaters (Scenario II) were considered to be produced during one operational season. In both scenarios the concentration of pesticides in the wastewaters was 100 mg L^{-1} to align with the pesticide loading scheme in pilot biobeds. The depuration efficiency of our pilot biobeds was used to estimate the

total amount of pesticides contained in the treated effluents. Upon treatment the effluents were considered to be uniformely dispersed over a 0.1 ha disposal site (approximate size of fields next to fruit-packaging plants potentially used as disposal sites). Based on these scenarios the rates of pesticides reaching the soil of the disposal site were calculated (Supplementary data Table 2). Subsequently the exposure levels of the soil in the disposal site (maximum Predicted Environmental Concentration, max PEC_{soil}) and in adjacent surface water systems and sediment (max PEC_{sw} and PEC_{sed}) were calculated using the PEC_{soil} calculator and the STEP1-2 calculation tool respectively [31]. For the calculation of max PEC_{sw} and PEC_{sed} run-off, erosion or drainage were considered as relevant processes while drift was not. The input data in STEP 1–2 calculation tool and the PEC_{soil} calculator were derived from pesticide regulatory documents (Supplementary data Table 3). The PECs obtained were used as exposure inputs in risk assessment (Supplementary data Table 4).

The risk assessment for aquatic and terrestrial indicator organisms was carried out according to the currently implemented regulatory guidelines [32,33]. Regarding aquatic ecotoxicity, the Regulatory Acceptable Concentrations (RACs) were calculated using acute and chronic toxicity data obtained from the pesticides registration documents (Supplementary data Table 5). An unacceptable risk was identified when PECs/RACs > 1. Regarding terrestrial ecotoxicity, Toxicity Exposure Ratio (TER) or Hazard Quotients (HQ) were calculated using the calculated PECs (Supplementary data Table 4) and toxicity data obtained from registration documents (Supplementary data Table 5). An unacceptable risk was identified when TER < 10 or HQ > 2 for acute toxicity risk to earthworms and soil-dwelling arthropods respectively. In cases where an unacceptable risk was identified mitigation measures were considered including an increase of the surface of the soil disposal site from 0.1 to 0.2 ha or refinement *via* the use of the depuration efficiency of bioaugmented biobeds for the calculation of PECs.

2.6. Decontamination of the spent biobed substrate

At the end of the biobeds' operation period, the packing material of the non bioaugmented biobeds (1–3) was removed and thoroughly mixed (50% by volume) with appropriate volumes of fresh organic matter (straw 25% and 25% cotton crop residues). Its C/N ratio was optimized (target value of 25) with addition of NH_4NO_3-N (4.5 kg of a fertilizer, 34.4% N by weight). The mixture was then divided into two sub-samples of 8.5 kg and two sub-samples of 154.5 kg. The first set of 8.5- and 154.5-kg samples were treated with a suspension of bacteria degrading TBZ, OPP and DPA (described above) resulting in an inoculation density of 10^6 cells g^{-1} packing material dry weight. The 8.5-kg sample was then placed in a plastic bag and incubated at ambient temperature ('bioaugmentation' treatment), and the 154.5-kg sample was placed in a compost bucket (85 cm x 85 cm x 75 cm) and was allowed to compost for 160 days ('bioaugmentation & composting' treatment). The remaining samples, one of 8.5-kg and one of 154.5-kg, received the same amount of water without bacteria, and they were handled in the same way as the corresponding bioaugmented samples ('control' and 'composting' treatments respectively). Immediately prior to the treatment and 24 (first mixing of the compost), 40 (completion of second thermophilic phase) and 160 days later (completion of maturation) triplicates (20 g) were removed and analyzed for pesticide residues.

2.7. Pesticides residue analysis

Extraction of pesticides from leachates and biobeds packing material was performed according to Karas et al. [20]. Pesticides residues in the packing material were extracted initially with water and subsequently with acetonitrile. Pesticides residues extracted with water constitute the fraction which was retained by the packing material, but was still available for leaching. Whereas pesticides residues extracted by the organic solvent constitutes the fraction retained by the biobed that was less available for leaching. Pesticides residues were analyzed by HPLC-UV as described by Karas et al. [19].

2.8. Abundance of microbial taxa and catabolic genes

The abundance of total bacteria, total fungi and different bacterial taxa (α-, β-, γ-proteobacteria, firmicutes and actinobacteria) was determined prior to pesticide application and at the end of the treatment period via q-PCR. In addition the abundance of *catA* and *pcaH* genes, encoding catechol 1,2-dioxygenase and protocatechuate dioxygenase respectively, involved in the metabolism of aromatic compounds [34], was determined via q-PCR. Samples collected from the three different layers of the pilot biobeds were homogenized and four subsamples were processed for DNA extraction using the Power Soil DNA Isolation Kit (MoBio Laboratories, Inc.). Q-PCR conditions and the primers used are shown in Supplementary data Table 6. Q-PCRs were carried out in 10 μL reaction volume containing 1X KAPA SYBR® FAST qPCR Master Mix (2X) Universal, 1 μM of each primer, 50 nM ROX Low, 400 ng μL^{-1} BSA, and *ca.* 0.2–10 ng DNA. The copy numbers of the target gene were determined via external standard curves [35] and q-PCR efficiencies ranged from 85 to 105%.

2.9. Statistical analysis

Mass balance analysis data were subjected to one-way-ANOVA to identify significant differences per pesticide between biobeds in the different fractions accounted (dissipated/leached/retained and extracted with water or acetonitrile). Data regarding the distribution of pesticides in the biobeds horizons were subjected to two-way-ANOVA. In cases where significant interactions between the main factors were observed significant differences were identified by Tukey's post-hoc tests within each factor. Q-PCR data were subjected to one-way ANOVA to identify significant differences between biobeds before and after pesticide application. All statistical analysis were performed with the SPSS statistical package.

3. Results

3.1. Pesticides leaching from pilot biobeds

The temporal patterns of pesticides in the leachate of the pilot biobeds are shown in Fig. 2. OPP residues were detected in the leachates of all three treated biobeds, and peak amounts were consistently detected in biobed1 exceeding 20 mg on three occasions (Fig. 2a). On the contrary biobed 3bioaug showed the lowest amounts of the fungicide in the leachate with only four positive detections and max amount of 2.4 mg. DPA was detected in the leachate of the two biobeds on a regular basis, but its amount never exceeded 10 mg (Fig. 2b).

IMZ was rarely detected in the leachates of the biobeds. High amounts of IMZ were, however, detected on two occasions (90 and 94 d) in the leachates of biobed 1 (Fig. 2c). TBZ showed a substantially different leaching pattern in the two biobeds tested. A peak in TBZ leaching appeared early (31 d, 520 mg) in biobed 3 followed by the detection of lower TBZ amounts (<7 mg) from 100 days onwards (Fig. 2d). In contrast, residues of TBZ were detected in the leachates of biobed 3bioaug in only three occasions at levels below 1 mg.

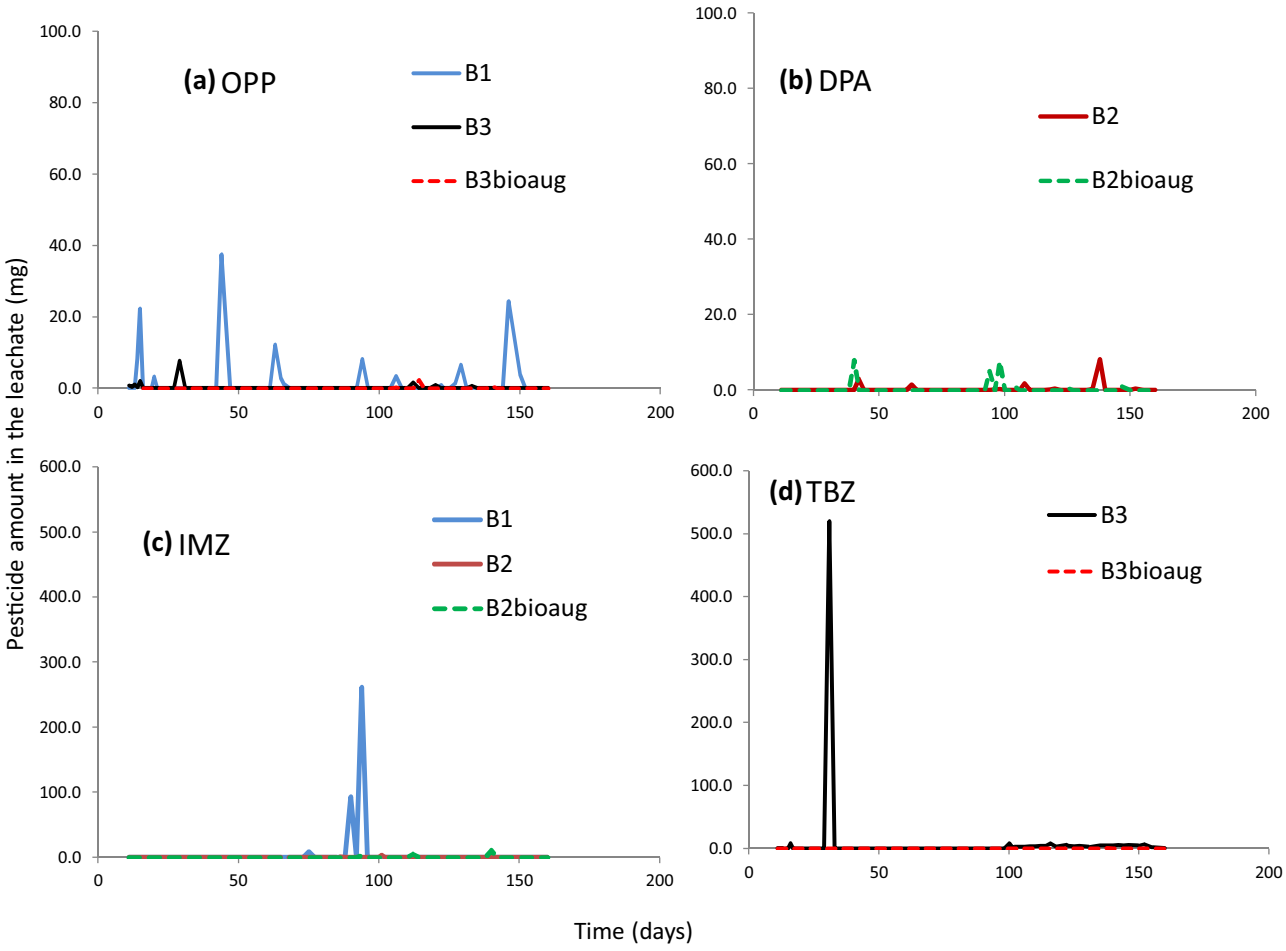

Fig. 2. Leaching patterns of OPP (a), DPA (b), IMZ (c) and TBZ (d) from the pilot biobeds.

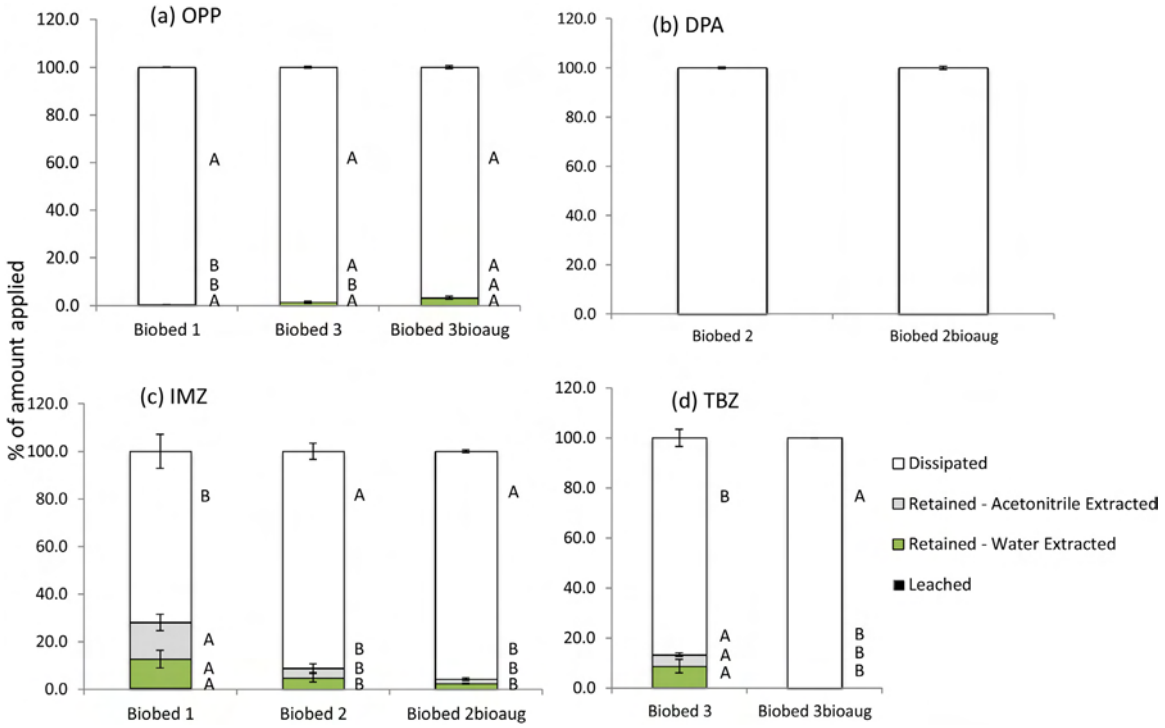

Fig. 3. Mass balance analysis of OPP (a), DPA (b), IMZ (c) and TBZ (d) in the pilot biobeds. Pesticides amounts retained by the biobeds matrix were estimated by successive extractions with water and acetonitrile. Letters following each stacked bar fraction indicate statistical differences between biobeds. Within each pesticide fraction (dissipated, retained—acetonitrile or water extracted, leached) stacked bar fractions followed by different capital letters significantly differ ($p < 0.05$) between biobeds.

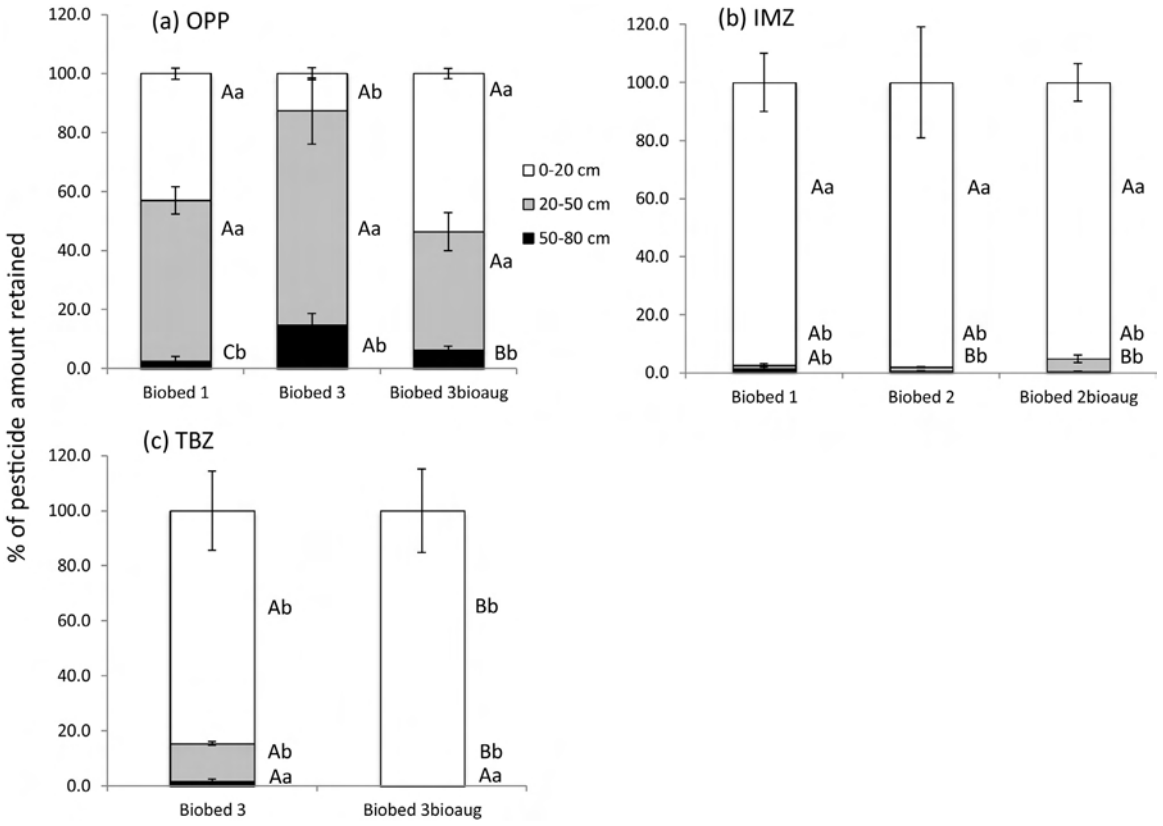

Fig. 4. The distribution of OPP (a), IMZ (b) and TBZ (c) residues in the three layers (0–20, 20–50 and 50–80 cm) of the pilot biobeds at the end of the study. Letters following each stacked bar indicate statistical differences within or between biobeds. Within each biobed stacked bar fractions designated by different lower case letters significantly differ ($p < 0.05$) in the amount of pesticide retained in the three layers. Whereas, within each layer stacked bar fractions designated by different capital letters significantly differ ($p < 0.05$) in the amount of pesticides retained in the different biobeds.

3.2. Mass balance analysis of pesticides in the pilot biobeds

Mass balance analysis of pesticide residues in the individual biobeds was performed (Fig. 3). The dissipation of OPP did not significantly differ between biobeds and ranged from 96.8% in biobed 3bioaug to 98.6 and 99.5% in biobeds 1 and 3 respectively (Fig. 3a). The amount of OPP in leachate was negligible ranging from 0.1% (biobed 1) to 0.01% (biobed 3 and biobed 3bioaug). The amount of OPP retained by the biobeds was mostly extractable with water, suggesting its availability for biodegradation or further mobility. OPP residues were distributed in the whole vertical profile of the biobeds with the higher fractions (39.9–72.4%) found at the 20–50-cm layer (Fig. 4a). DPA was nearly fully dissipated in both biobeds (99.9%), regardless of bioaugmentation (Fig. 3b).

IMZ showed different allocation patterns in the three biobeds (Fig. 3c). A significantly lower dissipation of IMZ ($p < 0.05$) was observed in biobed 1(72%) compared to biobed 2 and biobed 2bioaug (91 and 95.7% respectively). This was mirrored into the significantly higher amounts ($p < 0.05$) of IMZ retained (27.5%) and leached (0.52%) from biobed 1 compared to biobed 2 (8.8 and 0.02% respectively) and biobed 2bioaug (4.25 and 0.03% respectively). Regarding the amount of IMZ retained by biobed 1, no significant differences ($p > 0.05$) were observed between the fractions extracted by acetonitrile (15.4%) or water (12.2%). When the distribution of IMZ residues along the profile of the biobeds was investigated over 95% of the fungicide was found in the top layer (0–20 cm) (Fig. 4b).

A nearly complete dissipation of TBZ was evident in biobed 3bioaug (Fig. 3d) compared to a significantly lower dissipation (86.7%, $p < 0.05$) in the corresponding non-bioaugmented biobed 3. The rest of TBZ applied in biobed 3 was retained and it was mostly extractable with water (8.5%) rather than with acetonitrile (4.6%). The significant difference in the dissipation between bioaugmented and non-bioaugmented biobeds was reflected in the overall amount of TBZ leached which ranged from <0.001% in the former to 0.26% in the latter. Regarding the distribution of TBZ residues in the profile of the biobeds, nearly 85% of TBZ was retained in the top layer (0–20 cm) while lower amounts, 14 and 1%, were detected at the 20–50-cm and 50–80-cm layers respectively (Fig. 4c).

3.3. Risk assessment regarding biobed-treated effluents

Risk assessment analysis based on scenario I (pome fruit-packaging plant) suggested no risk for non-target organisms for IMZ and DPA. Whereas an unacceptable risk for chronic exposure to fishes was identified for TBZ (PEC/RAC = 1.633). This risk was alleviated only after the implementation of mitigation measures such as an increase of the surface of the disposal site from 0.1 to 0.2 ha (PEC/RAC = 0.817) (Table 1) or the use of the depuration performance of the bioaugmented biobed 3 for the calculation of PECs (PEC/RAC = 0.366). Regarding Scenario II, no unacceptable risk for aquatic and terrestrial organisms was observed for all pesticides (Tables 1 and 2).

3.4. Decontamination of spent biobed packing material

TBZ (29 $\mu g\,g^{-1}$) and IMZ (23–26 $\mu g\,g^{-1}$) residues persisted in the biobed packing material (Fig. 5). Bioaugmentation was the most successful decontamination approach for both IMZ and TBZ resulting in a significantly higher dissipation (83–97%, $p < 0.05$) of the former (Fig. 5a and b) and a complete dissipation of the latter. The spent packing material was successfully composted with the evo-

Table 1
Risk assessment for biobed-treated effluents according to Scenarios I and II for aquatic organisms. Ratios of max $PEC_{sw}/RAC > 1$ indicate unacceptable risk (in bold).

Pesticides		Acute Toxicity		Chronic Toxicity		
		Invertebrates	Fish	Algae	Fish	Sediment-Dwelling Invertebrates
		Daphnia magna	*Oncorhynchus mykiss*	*Pseudokirchneriella subcapitata*	*Oncorhynchus mykiss*	*Chironomus* sp.
Ortho-phenylphenol	Scenario II	0.041	0.025	0.003	0.278	0.005
Diphenylamine	Scenario I	0.053	0.029	0.021	0.009	n.d.[d]
Imazalil	Scenario I	0.138	0.244	0.108	0.502	0.119
	Scenario II	0.197	0.351	0.156	0.723	0.172
Thiabendazole	Scenario I–Step1 Scenario I–Step2 Mitigation/Refinement	**1.490** 0.576 –	0.920 –[a] –	0.022 – –	**4.217** **1.633** 0.817[b] (0.366)[c]	0.025 – –
	Scenario II–Step1 Scenario II–Step2	0.656 –	0.405 –	0.010 –	**1.858** 0.717	0.011 –

[a] Not calculated since no unacceptable risk was evident at Step1.
[b] Calculated based on disposal of biobeds effluents to a 0.2 ha disposal site (mitigation).
[c] Calculated based on the depuration efficiency of the bioaugmented biobed (biobed 3bioaug) (refinement).
[d] n.d.: not determined since no toxicity endpoint values were available (see Supplementary data Table 5).

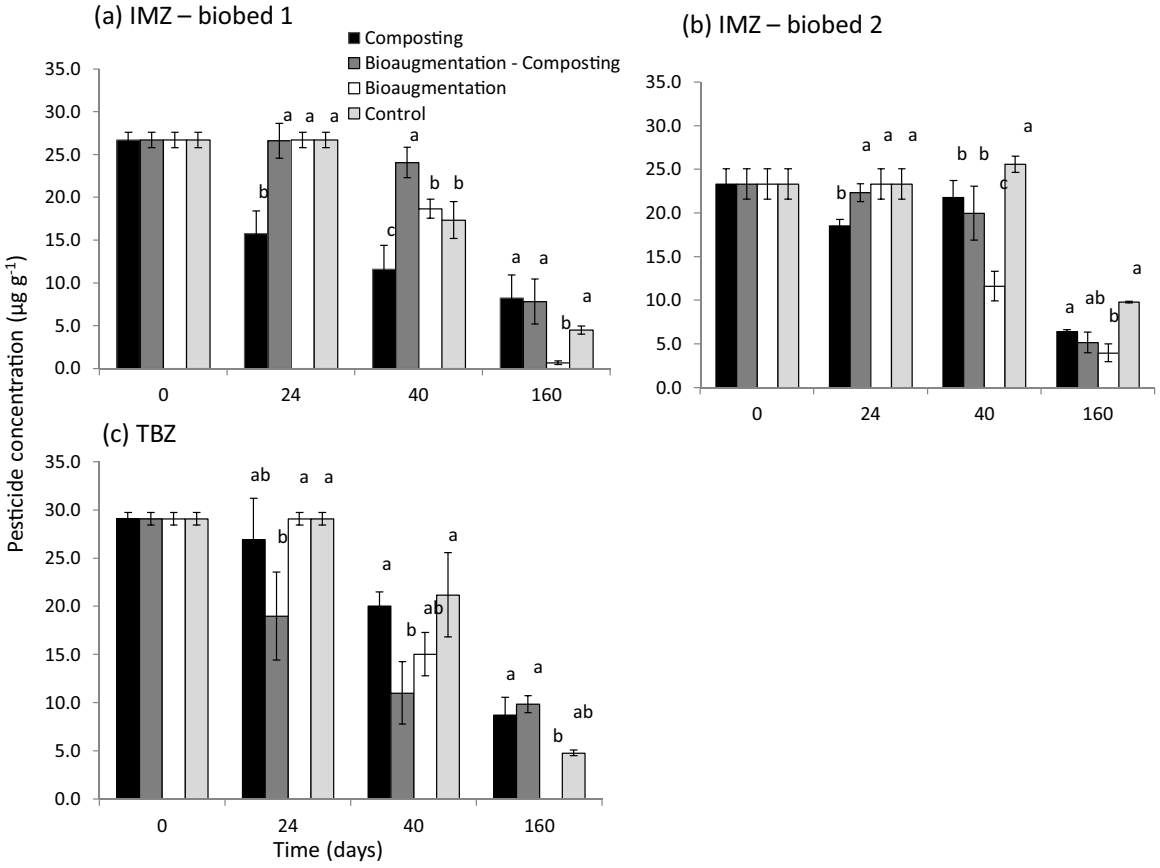

Fig. 5. The dissipation of IMZ (a & b) and TBZ (c) in the spent packing material from biobeds 1 (a), 2 (b) and 3 (c) subjected to bioaugmentation, bioaugmentation and composting, composting or stored at ambient temperature (control). Letters above bars indicate statistical differences between treatments at each time point. In each time point, bars designated by the same letters are not significantly different ($p < 0.05$).

lution of two thermophilic phases: the first and main one lasting 10 days (days 4–14) with a peak temperature of 50 °C, and the second milder one which reached a max temperature of 35 °C (days 23–28) (Supplementary data Fig. 1). During the active phase (0–40 days) composting significantly accelerated the dissipation of IMZ relatively to the control (Fig. 5a and b), whereas for TBZ a significant acceleration in its dissipation was achieved only when composting was combined with bioaugmentation (Fig. 5c).

3.5. Abundance of microbial taxa and catabolic genes

The abundance of total bacteria, fungi and α-proteobacteria were significantly higher ($p < 0.05$) in the biobeds at the end of the study compared to their abundance in the packing material prior to pesticides application (Fig. 6a). Actinobacteria were the most abundant bacterial taxa, followed by α-proteobacteria, firmicutes and γ-proteobacteria, while β-proteobacteria showed low abundance. Significantly higher copy numbers of the *pcaH* and *catA*

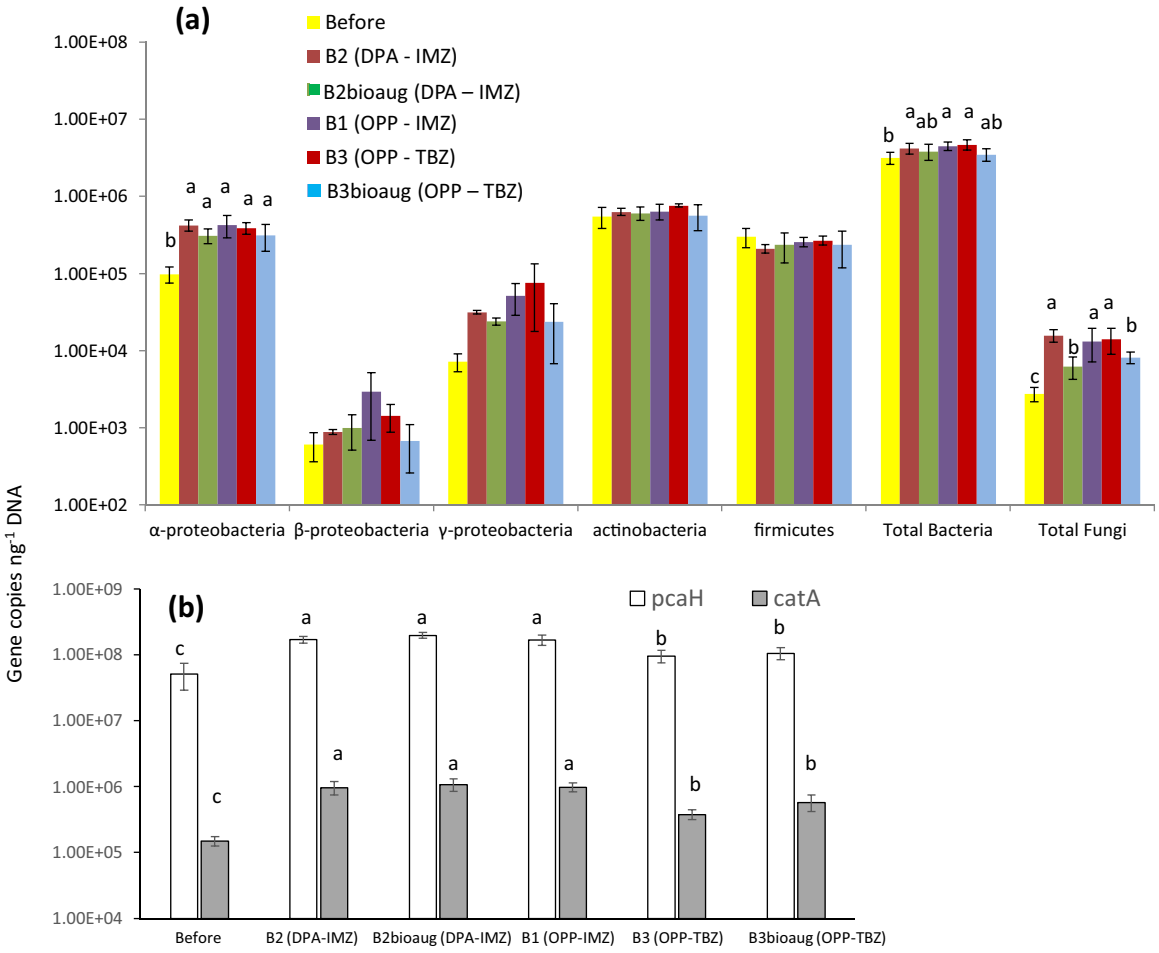

Fig. 6. The abundance of different bacterial taxa, total bacteria and fungi (a), and of the catabolic genes *catA* and *pcaH* (b) in the biobed packing material prior to pesticide application (before) and at the end of the study (B1, B2, B3, B2bioaug, B3bioaug). Letters above bars indicate statistically significant differences between biobeds for each microbial group studied. For each microbial group studied, bars designated by the same letters are not significantly different ($p > 0.05$).

Table 2
Risk assessment for biobed-treated effluents according to Scenarios I and II for terrestrial organisms. TER > 10 and HQ < 2 indicate low acute risk for earthworms and soil dwelling arthropods respectively.

Pesticides		TER–Earthworms	HQ–Soil-dwelling arthropods
Ortho-phenylphenol	Scenario II	16517	n.d.[a]
Diphenylamine	Scenario I	n.d.[a]	n.d.[a]
Imazalil	Scenario I	4045	0.540
	Scenario II	9310	0.238
Thiabendazole	Scenario I	>335	<0.639
	Scenario II	>743.5	<0.281

[a] n.d.: not determined because no toxicity endpoint values were available (see Supplementary data Table 5).

genes were detected in the biobeds at the end of the experiment compared to their copy numbers in the packing material before pesticide application (Fig. 6b). On the other hand, no significant difference in the abundance of *pcaH* and *catA* were found between non bioaugmented and their corresponding bioaugmented counterparts.

4. Discussion

Biobeds showed a high depuration efficiency which varied amongst pesticides but exceeded 99.5% in all cases. The higher depuration efficiency against OPP and DPA was attributed to their rapid dissipation, in line with their limited persistence in the biobed packing materials similar to the one used in the current study (OPP DT_{50s} of 0.34–4.7 days; DPA DT_{50s} 1–4.1 days) [19]. Regarding TBZ and IMZ although high dissipation was achieved, significant amounts were recovered by the biobeds packing material at the end of the study. This is in agreement with the high persistence of TBZ and IMZ in soil [36,37] and biobed packing materials [13,19]. Residues of TBZ and IMZ retained by the biobed packing material were mostly concentrated at the top biobed layers, in contrast to OPP whose residues were distributed to the whole biobed profile. Omirou et al. [13] reported a deeper vertical distribution of OPP residues in the profile of a full-scale biobed compared to IMZ and TBZ which were mostly retained in the top layer (0–20 cm). The higher mobility of OPP is in accordance with its weak adsorption in the biobed packing material ($K_f = 11.67\ g\ ml^{-1}$) which facilitates its vertical translocation in the biobed, compared to IMZ ($K_f = 186.2\ g\ ml^{-1}$) and TBZ ($K_f = 226.8\ g\ ml^{-1}$) which are strongly adsorbed [19].

The wide acceptance of biobeds relies mainly on their high biodegradation capacity against a broad range of chemical structures found in the different pesticide groups [11]. However in cases where biobeds are challenged with mobile [38] and/or recalcitrant chemicals, like TBZ or IMZ, bioaugmentation could be a useful optimization strategy. A high depuration efficiency of biobeds against OPP and DPA was evident even in the absence of bioaugmentation, in contrast to TBZ for which bioaugmentation significantly

advanced the depuration performance. These results suggest that the indigenous microbial community of biobeds has, or develops rapidly, the capacity to degrade the generally biodegradable OPP and DPA [39], whereas it showed a lower capacity to transform the less biodegradable TBZ for which bioaugmentation with a specialized microbial inocula was necessary to maximize depuration. Bioaugmentation of biobeds has been tested at lab scale via different approaches: the amendment of soil primed for the rapid degradation of one or multiple pesticides [18] and inoculation with bacteria [38] or white rot fungi [40].

Although several studies have verified the high depuration performance of biobeds [12], the risk associated with the direct environmental disposal of the biobed-treated effluents has not been explored. Based on our risk assessment analysis for pome and citrus-fruit packaging plants the disposal of the biobed-treated effluents on an 0.1-ha land area does not entail an unacceptable risk for terrestrial and aquatic organisms. The only exception was associated with TBZ-contaminated effluents produced by pome fruit-packaging plants where either mitigation measures or bioaugmentation were necessary to alleviate the high risk for fishes.

One of the main problems hampering the wider implementation of biobeds is the lack of established decontamination methods for the spent packing material. This is usually contaminated with high pesticide loads and should be depurated prior to their final environmental disposal. TBZ and IMZ residues were recovered in the spent biobed substrate and based on their recalcitrance and their high ecotoxicity [3,37], decontamination of the spent packing material is essential. Different decontamination strategies were evaluated with bioaugmentation being the most effective for the removal of TBZ and IMZ residues. It should be noted that no IMZ-degrading bacterial inocula was available and the IMZ-containing spent packing material from biobeds 1 and 2 were inoculated with OPP- and DPA-degrading bacteria since those biobeds had been also treated with OPP and DPA during the study. The OPP and DPA-degrading strains used in our study were not able to degrade IMZ [28,29]. Previous studies have shown that bioaugmentation of contaminated soil could induce a general perturbation favoring r-strategists and higher microbial activity [41]. This in turn might have resulted in a more active co-metabolic biodegradation of IMZ by the non-specialized soil microflora in the bioaugmented composts. Bioaugmentation has not been tested in the past for the decontamination of the spent packing material. This is possibly due to the complex pesticides mixture contained in the packing material of on-farm systems, compared to the limited number of pesticides expected to be present in biobeds receiving effluents from fruit-packaging plants.

Composting applied either alone or in combination with bioaugmentation accelerated the dissipation of IMZ and TBZ respectively during the active composting phase. Previous studies by De Wilde et al. [16] showed that composting resulted in 70% dissipation of bentazon and linuron by a spent biobed substrate. Composting contributes to the dissipation of pesticides via a range of processes with biodegradation being dominant [42]. In our study composting did not lead to the establishment of long thermophilic phases characterized by high temperatures which could have further accelerated pesticides dissipation [43].

Little is known regarding the composition and the dynamics of the microbial community in biobed systems. An increase in the abundance of total bacteria, α-proteobacteria and fungi was observed at the end of the 160-d operation period suggesting that despite the copious amounts of pesticides applied those systems could support a rich microbial community dominated by actinobacteria, firmicutes and α-proteobacteria. These bacterial taxa are known to be involved in processes relevant to biobed systems such as the decomposition of organic matter coming from plant debris and the degradation of organic pollutants [44,45]. Apart from a phylogenetically-rich microbiota, biobeds constitute an artificial ecosystem which support the rapid emergence of novel catabolic traits by the microbial community [46]. In line with this we measured a significant increase in the abundance of *catA* and *pcaH* genes at the end of the 160-day period. These genes encode enzymes involved in the transformation of key intermediates produced by the microbial metabolism of natural aromatics and organic pollutants [47,48] have been found in elevated numbers in polluted sites and have been considered as indicators of the biodegradation potential of polluted environments [49]. However, bioagumentation did not result in higher copy numbers of these genes at the end of the 160-day period, indicating that the microbial inocula are not key factors for the spread of these genes in the biobed matrix.

5. Conclusions

Our study explored the application of biobeds for the depuration of effluents from the fruit-packaging industry and addressed practical issues hampering their wider implementation. Biobeds showed high depuration efficiency and produced treated effluents whose environmental disposal into a pre-defined soil disposal area entails no environmental risk. The lower depuration efficiency of pilot biobeds for TBZ was alleviated by bioaugmentation with tailored-made inoculum. Bioaugmentation was also the most potent method for the decontamination of spent packing material with composting being a valuable alternative when no microbial inocula are available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.07.071.

References

[1] L.E. Castillo, E. Martinez, C. Ruepert, C. Savage, M. Gilek, M. Pinnock, E. Solis, Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica, Sci. Total Environ. 367 (2006) 418–432.
[2] European Commission (EC), Review report for the active substance thiabendazole finalised in the Standing Committee on Plant Health at its meeting on 12 December 2000 in view of the inclusion of thiabendazole in Annex I of Directive 91/414/EEC.7603/VI/97-final (2001).
[3] EC, Draft Assessment Report, Initial risk assessment provided by the rapporteur member state the Netherlands for the existing substance imazalil. Volume 1 (2009) pp. 1–151.
[4] European Food Safety Authority (EFSA), Peer review of the pesticide risk assessment of the active substance 2-phenylphenol, EFSA Sci. Rep. 217 (2008) 1–67.
[5] EFSA, Conclusion on the peer review of the pesticide risk assessment of the active substance diphenylamine, EFSA J. 10 (2012) 1426.
[6] EC, Commission Directive 2010/77/EU of 10 November 2010 amending Council Directive 91/414/EEC as regards the expiry dates for inclusion in Annex I of certain active substances. L293/48 (2010).
[7] M. Garcia Portillo, E.S. Avino, J.O. Vicente, R.L. De Andres, M.A.S. Jimenez, J.P.L. Blanco, Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States Patent, US 6,709,585 B1 (2004) p. 9.
[8] J.A. Sanchez Perez, A. Carra, C. Sirtori, A. Aguera, B. Esteban, Fate of thiabendazole through the treatment of a simulated agro-food industrial effluent by combined MBR/Fenton processes at mg/L scale, Water Res. 51 (2014) 55–63.

[9] C. Sirtori, A. Aguera, I. Carra, J.A. Sanchez Perez, Identification and monitoring of thiabendazole transformation products in water during Fenton degradation by LC-QTOF-MS, Anal. Bioanal. Chem. 406 (2014) 5323–5337.
[10] A. Masia, J. Campo, P. Vazquez-Roig, C. Blasco, Y. Pico, Screening of currently used pesticides in water, sediments and biota of the Guadalquivir River Basin (Spain), J. Hazard. Mater. 263 P (2013) 95–104.
[11] M.d.P. Castillo, L. Torstensson, J. Stenstrom, Biobeds for environmental protection from pesticide use—a review, J. Agric. Food Chem. 56 (2008) 6206–6219.
[12] T. De Wilde, P. Spanoghe, C. Debaer, J. Ryckeboer, D. Springael, P. Jaeken, Overview of on-farm bioremedation systems to reduce the occurence of point source contamination, Pest Manag. Sci. 63 (2007) 111–128.
[13] M. Omirou, P. Dalias, C. Costa, C. Papastefanou, A. Dados, C. Ehaliotis, D.G. Karpouzas, Exploring the potential of biobeds for the depuration of pesticide-contaminated wastewaters from the citrus production chain: laboratory: column and field studies, Environ. Pollut. 166 (2012) 31–39.
[14] E. Karanasios, N. Tsiropoulos, D.G. Karpouzas, On-farm biopurification systems for the depuration of pesticide-wastewaters: recent advances and future perspectives, Biodegradation 23 (2012) 787–802.
[15] L. Torstensson, Experiences of biobeds in practical use in Sweden—a method for elimination of point-source contamination, Pest. Outl. 11 (2000) 206–211.
[16] T. De Wilde, C. Debaer, J. Ryckeboer, D. Springael, P. Spanoghe, The influence of small-and large-scale compostingon the dissipation of pesticide residues in a biopurification matrix, J. Sci. Food Agric. 90 (2010) 1113–1120.
[17] E. Karanasios, N. Tsiropoulos, D.G. Karpouzas, U. Menkissoglu-Spiroudi, Novel biomixtures based on local Mediterranean ligninocellulosic materials: evaluation for use in biobeds, Chemosphere 80 (2010) 914–921.
[18] K. Sniegowski, D. Springael, Establishment of multiple pesticide biodegradation capacities from pesticide-primed materials in on-farm biopurification system microcosms treating complex pesticide-contaminated wastewater, Pest Manag. Sci. 71 (2014) 986–995.
[19] P. Karas, A. Metsoviti, V. Zisis, C. Ehaliotis, M. Omirou, E. Papadopoulou, U. Menkissoglu-Spiroudi, D. Komioti, S. Manta, D.G. Karpouzas, Dissipation, metabolism and sorption of pesticides used in fruit-packaging plants: towards an optimized depuration of their pesticide-contaminated agro-industrial effluents, Sci. Total Environ. 530–531 (2015) 129–139.
[20] P. Karas, S. Makri, E.S. Papadopoulou, C. Ehaliotis, U. Menkissoglu-Spiroudi, D.G. Karpouzas, The potential of organic substrates based on mushroom substrate and straw to dissipate fungicides contained in effluents from the fruit-packaging industry—Is there a role for *Pleurotus ostreatus*? Ecotoxicol. Environ. Saf. 124 (2016) 447–454.
[21] A.J. Walkley, I.A. Black, Estimation of soil organic carbon by chromic acid titration method, Soil Sci. 37 (1934) 29–38.
[22] H.A. McKenzie, The Kjeldahl determination of nitrogen: retrospect and prospect, Trends Anal. Chem. 13 (1994) 138.
[23] B.H. Sheldrick, C. Wang, in: M.R. Carter (Ed.), Particle Size Distribution: Soil Sampling and Methods of Analysis, Canadian Society of Soil Science, Lewis Publishers, Ann Arbor, MI, 1993, pp. 477–512.
[24] European Commission (EC), Draft Assessment Report – Initial risk assessment provided by the rapporteur member-state Spain for the existing active substance 2-phenylphenol. Volume 1 (2008).
[25] European Commission (EC) Draft renewal assessment report prepared according to the Commission Regulation (EU) N.1141/2010, Second programme for the renewal of the inclusion of the following active substance under Regulation (EC) 1107/2009, Thiabendazole, Vol. 3, Annex B.3 (2013).
[26] European Commission (EC), Draft Assessment Report, Initial risk assessment provided by the rapporteur member state the Netherlands for the existing substance imazalil. Volume 1 (2015): 1–151.
[27] D. Valero, M. Serrano, Postharvest Biology and Technology for Preserving Fruit Quality, CRC Press Taylor & Francis Group, New York, USA, 2010.
[28] C. Perruchon, V. Patsioura, S. Vasiliadis, D.G. Karpouzas, Isolation and characterisation of a *Sphingomonas strain* able to degrade the fungicide *ortho*-phenylphenol, Pest Manag. Sci. 72 (2016) 1040–1050.
[29] C. Perruchon, S. Zouborlis, C. Batianis, E.S. Papadopoulou, S. Ntougias, S. Vasileiadis, D.G. Karpouzas, Isolation of a diphenylamine-degrading bacterium and characterization of its metabolic capacities, bioremediation and bioaugmentation potential, Environ. Sci. Pollut. Res. 22 (2015) 19485–19496.
[30] C. Perruchon, A. Pantoleon, A. Chatzinotas, S. Vasileiadis, D.G. Karpouzas, Isolation of bacterial consortia degrading the fungicide thiabendazole and identification of the role of their members via SIP-DGGE and q-PCR analysis, in: 6th Symposium of the Society of Mikrobiokosmos, Athens, Greece, 2015.
[31] FOCUS, Surface Water Scenarios in the EU Evaluation Process under 91/414/EEC. Report of the FOCUS Working Group on Surface Water Scenarios, EC Document Reference SANCO/4802/2001-rev.2.245 pp (2001).
[32] EC, Guidance document on terrestrial ecotoxicology. Draft Working Document SANCO/10329/2002 rev. final, October 17(2002).
[33] EFSA PPR Panel, Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters, EFSA J. 11 (2013) 3290, 268 pp.
[34] C.S. Harwood, R.E. Parales, β-ketoadipate pathway and the biology of self-identity, Ann. Rev. Microbiol. 50 (1986) 553–590.
[35] C. Rousidou, E.S. Papadopoulou, M. Kortsinidou, I.O. Giannakou, B.K. Singh, U. Menkissoglu-Spiroudi, D.G. Karpouzas, Bio-pesticides: harmful or harmless to ammonia oxidizing microorganisms? The case of a *Paecilomyces lilacinus*-based nematicide, Soil Biol. Biochem. 67 (2013) 98–105.
[36] R. Kreuzig, C. Hartmann, J. Teigeler, S. Höltge, B. Cvetkovic, P. Schlag, Development of a novel concept for fate monitoring of biocides in liquid manure and manured soil taking ^{14}C-imazalil as an example, Chemosphere 79 (2010) 1089–1094.
[37] EC, Draft renewal assessment report prepared according to the Commission Regulation (EU) N.1141/2010, Second programme for the renewal of the inclusion of the following active substance under Regulation (EC) 1107/2009, Thiabendazole, Vol. 1, Report and proposed decision (2013).
[38] P. Verhagen, L. De Gelder, N. Boon, Inoculation with a mixed degrading culture improves the pesticide removal of an on-farm biopurification system, Cur. Microbiol. 67 (2013) 466–471.
[39] P. Karas, C. Perucchon, C. Exarhou, C. Ehaliotis, D.G. Karpouzas, Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi, Biodegradation 22 (2011) 215–228.
[40] S. von Wiren-Lehr, MdP. Castillo, L. Torstensson, I. Scheunert, Degradation of isoproturon in biobeds, Biol. Fert. Soils 33 (2001) 535–540.
[41] D.F. Wenderoth, P. Rosenbrock, W.-R. Abraham, D.H. Pieper, M.G. Hofle, Bacterial community dynamics during biostimulation and bioaugmentation experiments aiming at chlorobenzene degradation in groundwater, Microb. Ecol. 46 (2003) 161–176.
[42] F. Büyüksönmez, R. Rynk, T.F. Hess, E. Bechinski, Literature review: occurrence: degradation and fate of pesticides during composting: part II: occurrence and fate of pesticides in compost and composting systems, Comp. Sci. Utiliz. 8 (2000) 61–81.
[43] F. Büyüksönmez, R. Rynk, T.F. Hess, E. Bechinski, Occurrence, degradation and fate of pesticides during composting Part I: composting pesticides, and pesticide degradation, Comp. Sci. Utiliz. 7 (1999) 66–82.
[44] A.B. de Menezes, M.T. Prendergast-Miller, P. Poonpatana, M. Farrell, A. Bissett, L.M. Macdonald, et al., C/N ratio drives soil actinobacterial cellobiohydrolase gene diversity, Appl. Environ. Microbiol. 81 (2015) 3016–3028.
[45] C.E. Wegner, W. Liesack, Microbial community dynamics during the early stages of plant polymer breakdown in paddy soil, Environ. Microbiol. (2016), http://dx.doi.org/10.1111/1462-2920.12815, in press.
[46] V.S.K. Dunon, K. Bers, R. Lavigne, K. Smalla, D. Springael, High prevalence of IncP-1 plasmids and IS1071 insertion sequences in on-farm biopurification systems and other pesticide polluted environments, FEMS Microbiol. Ecol. 86 (2013) 415–431.
[47] D.A. D'Argenio, M.W. Vetting, D.H. Ohlendorf, L.N. Ornston, Substitution insertion, deletion, suppression, and altered substrate specificity in functional protocatechuate 3,4- dioxygenase, J. Bacteriol. 181 (1999) 6478–6487.
[48] S. Hussain, M. Devers-Lamrani, N. El Azhari, F. Martin-Laurent, Isolation and characterization of an isoproturon mineralizing *Sphingomonas* sp strain SH from a French agricultural soil, Biodegradation 22 (2011) 637–650.
[49] N. El Azhari, D. Bru, A. Sarr, F. Martin-Laurent, Estimation of the density of the protocatechuate degrading bacterial community in soil by real-time PCR, Eur. J. Soil Sci. 59 (2008) 665–673.