Bioaugmentation Strategies Development for Pesticide Bioremediation

Master Thesis. Sustainable Biotechnology Section

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Abstract

The increasingly rapid growth of both, population- and food demands has driven a research race in food production efficiency through the last century, which has made the pesticide use seemingly unavoidable. However, many pesticides are very persistent and tend to accumulate in the ecosystem, often threatening biodiversity and public health. From the remediation technologies to reduce pesticide pollution, and bioremediation approaches, such as bioaugmentation and biostimulation, are the most cost effective and environmentally friendly.

This project, framed in the AAU-FMC corporation colaboration, aimed to reduce the persistance of five given pesticides, with the development of an efficient bioaugmentation strategy. For this matter, three potentially pesticide-degraders rich environmental samples were used as inoculum in an eleven weeks enrichment with the five given pesticides. After the enrichment 42 strains were isolated whith a potential ability to degrade pesticides, and 6 were screened in three of the pesticides (along with 12 screened in a parallel project), to find one strain degrading the pesticide dicamba. Different consortia were constructed with the efficient degraders found in this and the parallel study, but no degradation was found. Degradation tests were performed with *P. putida* and *S. marcescens* to find out that *S. marcescens* was only capable of degrading dicamba in the presence of surfactants.

Basing on the results of the metagenomic studies, the HPLC tests, and the degradation experiments, it was concluded that more experiments should be performed to validate the HPLC methods used to further screen and study the strains isolated, and that some tests could be performed to optimize the obtaining of efficient degraders from the enrichment. However, if the sequencing results of the three potential pesticide degraders confirm these results, the enrichment strategy would be proven to be effective to obtain pesticide degrading strains for pesticide bioremediation.

1 Introduction

1.1 History and role of pesticides in the society

Pesticides have become an ineluctable component of modern society, as they are widely used in different sectors, including agriculture, household and public health, which has determined their extensive distribution throughout most ecosystems. A study conducted in 2008 in the city of New York revealed detectable levels of organophosphate (OP) and organochlorines pesticides (OCPs) in 100% and 47-78% of the pregnant women analyzed respectively [1]. OCP include some of the most toxic compounds released to the environment, and among their effects on humans, we find neurologic deficits, cancer, and developmental and reproductive impairment [2]. Not only humans, but also the natural environments are seriously threatened by pesticides. Sprayed herbicides, for example, evaporate into the air, damaging non-targeted plants; water soluble pesticides move to groundwaters and streams; and fat-soluble pesticides bioaccumulate in organisms, leading to elevated concentrations in the higher trophic levels. Pesticides are proven to reduce biodiversity, both directly by contamination and bioaccumulation, and indirectly by reducing the population of weeds and insect on which higher orders feed [3]. On the other hand, pesticides play an essential role in pest control and cannot yet be replaced. Agriculture wouldn't be able to feed the growing world population without crop protection, which is estimated to prevent more than 400 billion dollars losses each year [4]. Besides, pesticides help to reduce infectious diseases incidence, by killing the disease vector. Table 1 shows the losses of food production, due to diseases, insects, and weeds.

Attainable crop production (2002 prices)	**$ 1.5 trillion**
Actual crop production (≈ 36.5%)	$950 billion
Production without crop protection	$455 billion
Losses prevented by crop protection	$415 billion
Actual annual losses to world crop production	$550 billion
Losses caused by diseases only (14.1%)	$220 billion

Table 1: Examples of losses in the food production, with and without pesticides. Extracted from [4]

Pesticides have been used since ancient times by different cultures, using easy obtainable plants, animals, or mineral products. Pyrethrum, extracted from dried Chrysanthemum cinerariaefolium flowers (Fig. 1), from which modern pyrethroid pesticides derive, has been used as a pesticide for over 2000 years. While pyrethrins are extracted from dried Crysanthemum flowers, pyrethroids were sintetized for the first time in the 1950s [5].

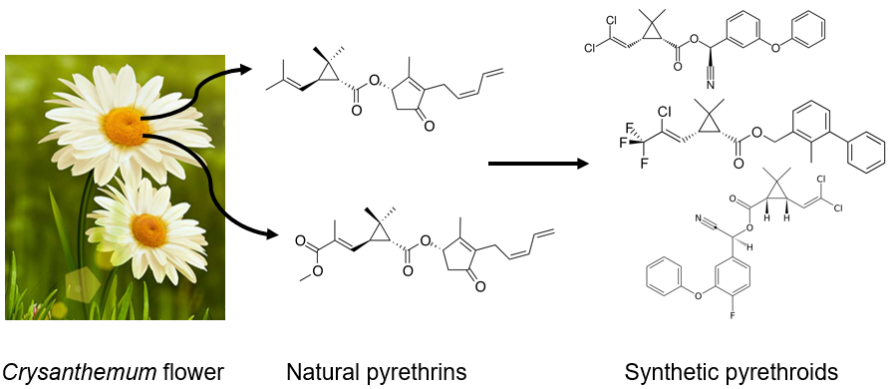

Figure 1: Chemical structure of some pyrethrins and pyrethroids.

With industrial revolution, synthetic chemicals such as nitrophenols and naphthalene started being used to control pests, but they usually caused damage the crops, due to low selectivity.

In the 1940s, the pesticide market showed a great development with the discovery of OCPs, which lowered food prices and insect-born diseases rates. It wasn't until the 1960's when the threat to human health and the environment was discovered and less harmful pesticides as OP and pyretroids replaced them. In 1974 the broad spectrum herbicide glyphosate was discovered, showing low off-target toxicity and persistence. The appearance of genetic modified crops, resistant to herbicides and insecticides, greatly increased the use of this chemicals, but recent studies have shown they also constitute a threat to some chronically exposed species [6], [7].

The threat that pesticide accumulation posed, pushed the development of more sustainable pest control strategies, such as Integrated Pest Management. This strategy aims to reduce pesticide usage, not by trying to eradicate pests, which would rapidly yield resistant strains, but instead looking for an equilibrium between the pesticide and the pests, thus keeping an economically tolerable percentage of them [8]. In 2001, the Stockholm Convention banned the use and production of some persistent organic pollutants, including several OCPs, but they are still being used in Central America, China, India, and some African countries [9]. Despite the improvements in the field, pesticides still hold an important threat to environment, because of the high persistence of the now banned pesticides, which still contaminate fields and water systems [10]; and the overuse of modern pesticides, which leads to bioaccumulation and thus increased persistence and toxicity [11]. Figure 2 shows pesticide contamination risk of surface waters around the globe.

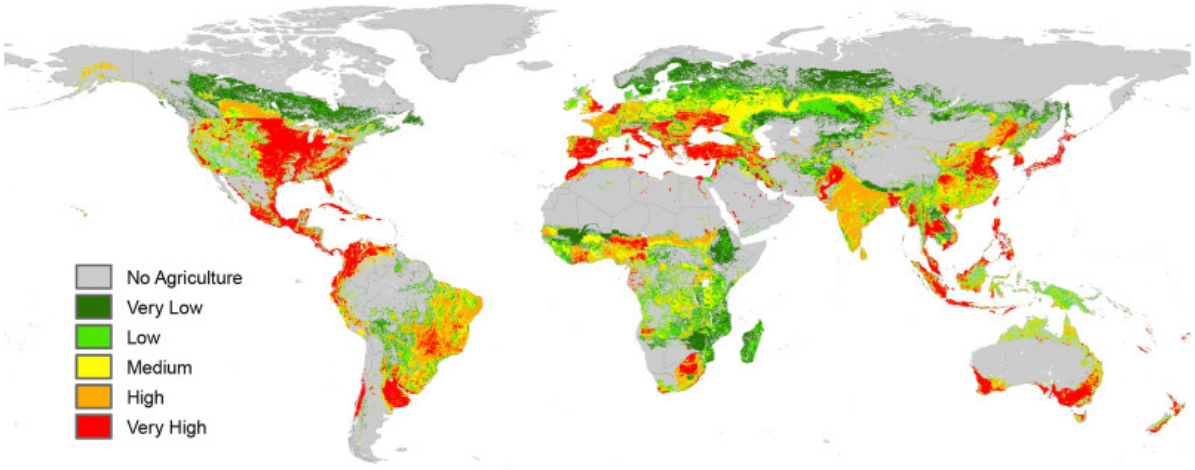

Figure 2: World map of the pesticide contamination risk in surface waters, Modelled based on environmental and anthropogenic factors of pesticide run-off. Extracted from [12]

1.2 Fate of pesticides in the environment

1.2.1 Factors affecting pesticide fate

Pesticide environmental fate is driven by three main factors: initial distribution, mobility and persistence, which, in turn, are determined by several different properties of both, the pesticide and the site of application (Fig. 3). Understanding how all these factors interact is crucial to predict their fate in the environment, and therefore, being able to wisely choose the right pesticide and the concentration for each case, in order to develop approaches to remediate their off-site pollution.

Figure 3: Main factors affecting the fate of the pesticide. Adapted from [13]

The first factor to consider when studying a pesticide's fate is its initial distribution, even though it does not exclusively depend on its properties. This, in turn, will depend on the formulation, method and rate of application, and on environmental and site characteristics such as soil composition, topography, amount and type of vegetation, and weather conditions [13].

After application, pesticides can adsorb to soil, dissolve in water, or become airborne by volatilization or erosion, before they are finally degraded. This further depends on their affinity to soil, water solubility, and vapor pressure, as well as on environmental and site characteristics, and affects their mobility (Fig. 4).

Pesticide degradability is measured by its persistence, which is expressed in half-life (time for 50% of the pesticide to be broken down). The degradation could be mediated by light, abiotic chemicals,

or microorganisms (or combination thereof); therefore, the rate depends on many factors, which will be addressed later on in the chapter.

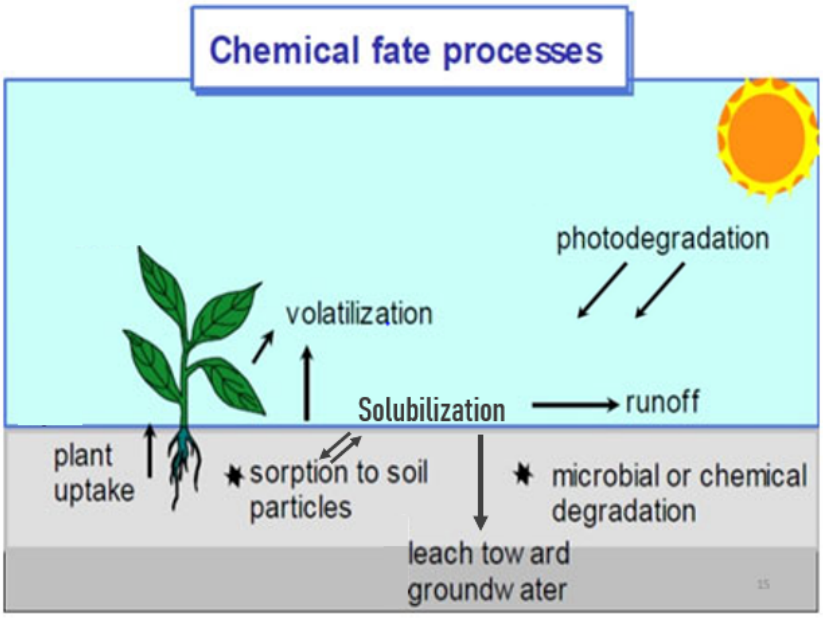

Figure 4: Different processes affecting mobility and persistence of pesticides. Adapted from [13]

Mobility, along with persistence, determines which portion of the pesticide will move off-site and stop providing its crop protection effect, thus representing an economic loss and an environmental threat. The potential to move and accumulate into aquifers is rated by the Groundwater Ubiquity Score (GUS), taking into account the sorption coefficient (Koc) and half-life [14] (Tab. 2).

GUS Value	Potential for movement toward groundwater
Below 0	Extremely Low
1-1.8	Low
1.8-2.8	Moderate
Above 2.8	High

Table 2: GUS values help to estimate the pesticide potential to reach groundwaters. GUS=log10(half-life)*[4-log10(Koc)]. Extracted from [14].

High sorption coefficients help pesticides to remain in the root zone, where they can be taken by plants and degraded by microorganisms, but, at the same time, a strong bond makes them less available for microbial degradation and plant uptake (Table 3). Weakly adsorbed pesticides, on the other hand, easily move off-site and pollute groundwater [15]. Sorption coefficient is influenced mostly by pH (which determines the pesticides charge;), the soil moisture, because water molecules could compete for soil binding sites with pesticides, as well as organic matter content and texture of the soil, which affect the amount of binding surface [16].

Water solubility is influenced by temperature, the presence of other chemicals, and pH. It increases

the mobility and plant uptake, which prevents runoff and leaching. Lastly, volatilization from soil (determined by vapor pressure, sorption coefficient, and water solubility) greatly increases mobility. Nevertheless, airborne pesticides are often degraded rapidly in the atmosphere [17].

SUBSTANCE	Soil Half-life (days)	Water solubility (mg/L)	Sorption coefficient (Soil Koc)	Pesticide Movement Rating
Organochlorines				
DDT	2000	0.0055	2000000	Extremely Low
Chlordane	350	0.06	20000	Extremely Low
Lindane	400	7	1100	Moderate
2.4 D	10	890	20	Moderate
Organophosphates				
Malathion	1	130	1800	Extremely Low
Parathion	14	24	5000	Very Low
Triazines				
Atrazine	60	33	100	High
Simazine	60	6.2	130	High
Pyretroids				
Bifenthrin	26	0.1	240000	Extremely Low
Cypermethrin	30	0.004	100000	Extremely Low
Carbammates				
Carbofuran	50	351	22	Very High

Table 3: Soil half-life, water solubility, and sorption coefficient of different pesticides, and the subsequent pesticide movement rating. Adapted from [18], [19].

1.2.2 Pesticides degradation

Abiotic degradation

Abiotic degradation of pesticides comprises chemical and photochemical reactions, not catalyzed by enzymes. They hold an important role in some specific environments, though generally, enzyme-mediated degradation is the most important process and some degradation pathways can only be performed by enzymes [20].

Photodegradation is a light-mediated, chemical degradation. It is divided in direct or indirect photodegradation, depending on whether the photon is absorbed directly by the pesticide or by other molecules, which then turn into reactive species that react with the pesticide (Fig. 5). The overlap of electronic absorption spectrums of pesticides and sunlight spectrum is not common, making indirect transformation the main photodegradation pathway [21]. This should be considered mainly in surface waters, where light turns dissolved organic matter into reactive species, which

can degrade pesticides [22].

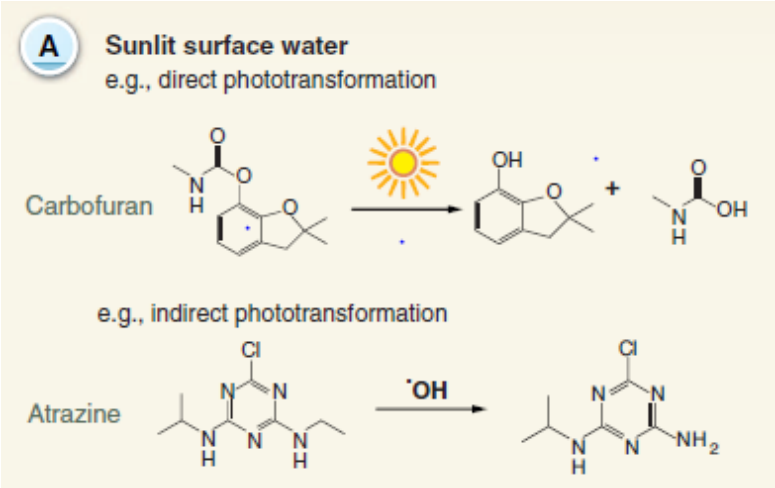

Figure 5: Examples of direct and indirect photodegradation in surface waters. Extracted from [20].

Non-light mediated chemical degradation is usually a slow process, but it prevails over biodegradation in specific conditions like high pH, low redox, or low nutrient environments like groundwater, where the retention time is in the order of years (Fig 6). It also requires the presence of abiotic catalysts, which many times come from microbial metabolism [23].

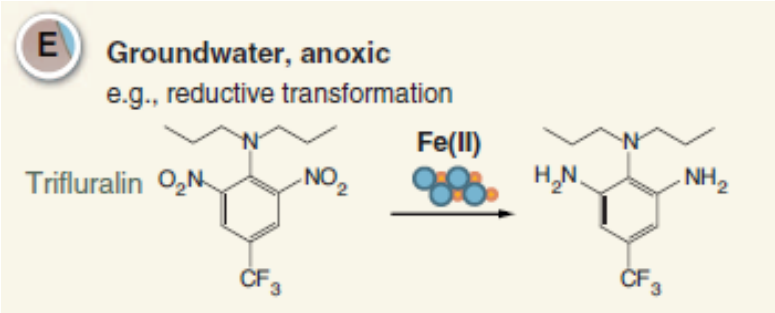

Figure 6: Non light-mediated chemical degradation of trifluralin in groundwater. Extracted from [20].

Biodegradation

Many organisms can degrade pesticides. Eukaryotic organisms, like many plants, animals, or fungi, use this ability to neutralize their harmful effects, while bacteria typically break down the pesticide to assimilate its nutrients. Bacteria are accounted for most of the pesticide biodegradation. Their ability to transfer genes horizontally, combined with a fast evolution rate, enables them to quickly adapt and extend the biodegradation potential of a new mutant to a whole bacterial community [24], while the focus of pesticides on eukaryotic organisms makes them often resistant to their toxicity.

This biodegradation rate varies greatly from site to site, and it does not correlate with the total

amount of culturable bacteria or the community diversity, since pesticide degraders often constitute a minor fraction of the bacterial population and are not among the dominant groups. For that case, the tools developed to predict the biodegradation of pesticides in the field based on the abundance of specific communities, did also not yield successful results [25].

Depth, pH, temperature, soil moisture, and clay or organic matter content, have proven to be the best predictors of microbial degradation. Biodegradation rates diminish with increasing soil depth [26], correlating generally with the decline of nutrient levels, while pH and temperature determine the microbial community, the enzyme efficiencies, and the adsorbed and dissolved pesticide ratio [27], [28]. This explains why each pesticide shows its own optimal degrading conditions, depending mostly on the combination of the above-mentioned parameters and the bacteria that degrade them. Last but not least, soil moisture is important for the microbial physiology and, along with clay and organic content, influences pesticide sorption [29].

Degrading a pesticide takes usually more than one enzymatic step and, along this process, transformation products can also be obtained, either due to random degradation, or because of enzymes catalyzing the first steps more efficiently than the last ones [30]. Even though the toxicity of such intermediates is, in most cases, lowered through degradation, if the active moiety remains intact, they can keep the same toxicity and show additive effects when combined with the parent compound, or might even increase their toxicity [31].

So to summarize, the degradation of a recalcitrant compound such as pesticides, depends on several factors, such as the induced synthesis of a specific degrading enzyme, the possibility to enter the cell due to suitable permease, availability of the compound due to insolubility or adsorption, excessive toxicity of the parent compound or its metabolic product, availability of the proper electron acceptor, favorable environmental conditions (temperature, light, pH, O2, moisture, etc.) and the availability of other nutrients and growth factors. Not surprisingly, their fate is difficult to predict, and in some cases their toxic effects have been found only 20-30 years after the approval of the parental compound [32], thus underlining the importance of a precautionary approach.

1.3 Pesticide classes

Pesticides can be divided into different categories, such as herbicides, insecticides, fungicides, rodenticides, nematicides and plant growth regulators, depending on their target [3].

Some of the most commonly applied insecticides in modern agriculture belong to organo-phosphate, organo-chlorinated pesticides and carbamate groups (Fig. 7). A typical example of organo-phospohate is O-dimethyl O-(4-nitro-phenyl) phosphorothioate, known as Methyl parathion, which is used to control insects such as aphids and mites in a wide variety of crops, including cereals, fruits, vegetables, ornamental plants, cotton and field crops [33], [34]. It is a broad-spectrum non-systemic pesticide that kills pests by stomach poisoning; an acaricide that has some fumigant action also [33], [35].

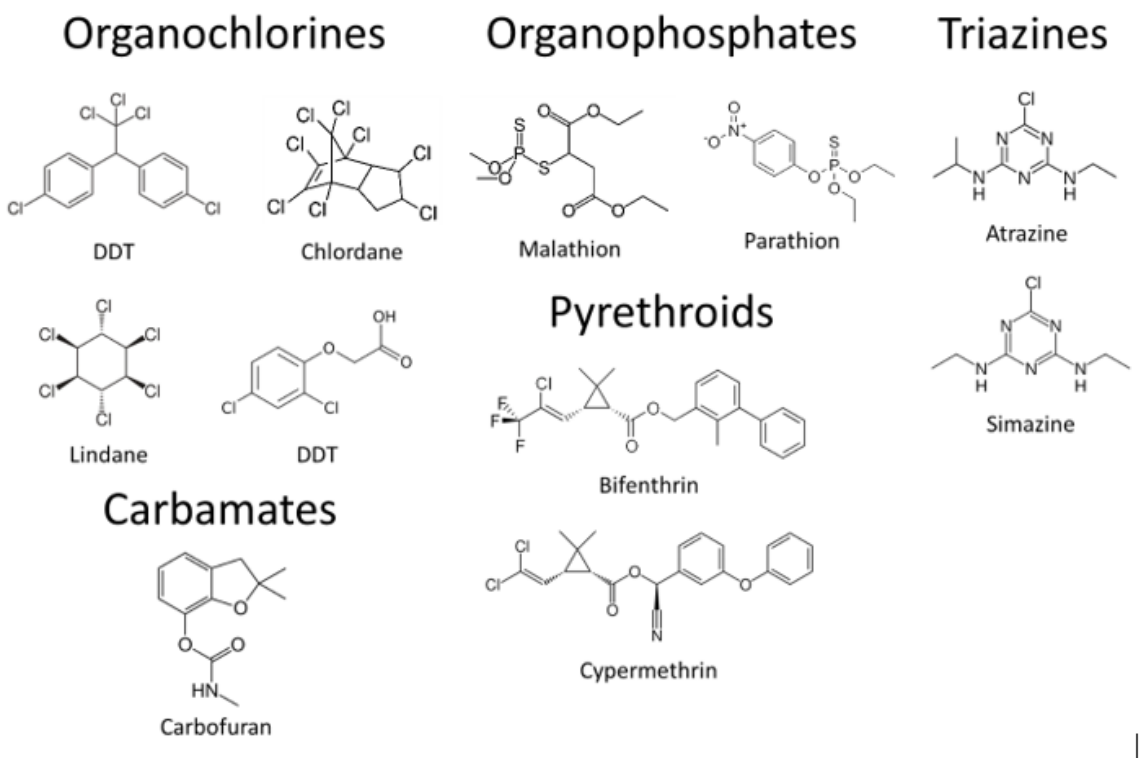

Figure 7: Chemical structures of typical organochlorinated and organophosphorus pesticides, triazines, carbamate and pyrethroids pesticides, recently investigated for their biodegradability.

A moderately persistent organophospohrous pesticide is represented by Chlorpyrifos (0,0-diethyl 0-(3,5,5-trichloro-2-pyridyl) phosphorothioate), widely used for insect control in cereal, cotton, fruit, nut and vegetable crops [35], [36]. Its half-life in soil ranges from less than 1 day to 240 days, depending on the soil type, moisture, pH and initial concentrations [37]. Despite the moderate persistency, its residues have been associated with decreased levels of the hormone thyroxine (T4) and an increase in estradiol levels in sheep. A comparison of the persistence of different pesticides is shown in Table 4.

A different example is represented by lindane (an isomer of hexachlorocyclohexane), a highly chlorinated, recalcitrant pesticide belonging to the organochlorinated pesticides. It has been extensively used as a general broad-spectrum insecticide for various purposes, including killing of plant-eating insects, pest control on domestic animals, as well as mosquito control [38].

Carbofuran (2,3-dihydro-2–2-dimethyl-7-benzofuranyl-methylcarbamate) is a good example for a widely-used carbamate. It is a very effective systemic and contact insecticide and nematicide, which is often employed against a wide range of agricultural pests [39].

SUBSTANCE	Time for 75-100% reduction
Chlorinated insecticides	
DDT	4 years
Aldrin	3 years
Chlordane	5 years
Heptachlor	2 years
Lindane	3 years
Organophosphate insecticides	
Dianinon	12 weeks
Malathion	1 week
Parathion	1 week
Herbicides	
2,4 D	4 weeks
2,4,5-T	20 weeks
Dalapon	8 weeks
Atrazine	40 weeks
Simazine	48 weeks
Propazine	1.5 years

Table 4: Persistence of pesticides/xenobiotics. Adapted from [40]

It is important to take into account, not only the persistence, which consequences were already underlined, but the toxicity of the chemical compounds. This two parameters are not necessarily correlated, and pesticides with high persistence might well have low toxicity or vice versa (see i.e. Table 8). Moreover, the toxicity can obviously vary, depending on the target organism taken into consideration.

Insecticide class	Examples	Persistence	Toxicity to mammals
Organochlorides	DDT, dieldrin, toxaphene, chlordane, lindane	High	Relatively Low
Organophosphates	Parathion, malathion, acephate, phorate, chlorpyrifos	Moderate	High
Carbamates	Carbaryl, methomyl, aldicarb, carbofuran	Low	High to Moderate
Pyrethroids	Permethrin, bifenthrin, esfenvalerate, decamethrin	Low	Low

Figure 8: Persistence and toxicity of pesticides/xenobiotics. Adapted from [40]

More and more studies are now investigating the possible degradation pathways of different pesticides, such as parathion and methyl-parathion, endosulfan, lindane, carbofuran, glyphosate, etc. A well-studied example is for instance endosulfan [41], [42]. There is a wide range of microorganisms, mostly bacteria, fungi and cyanobacteria, capable of utilizing this organochlorinated pesticide as

a sole source of carbon and/or sulfur [43]. An example of the pathways possibly involved in the biotransformation of endosulfan by microorganisms and associated enzymes is shown below (Fig. 9).

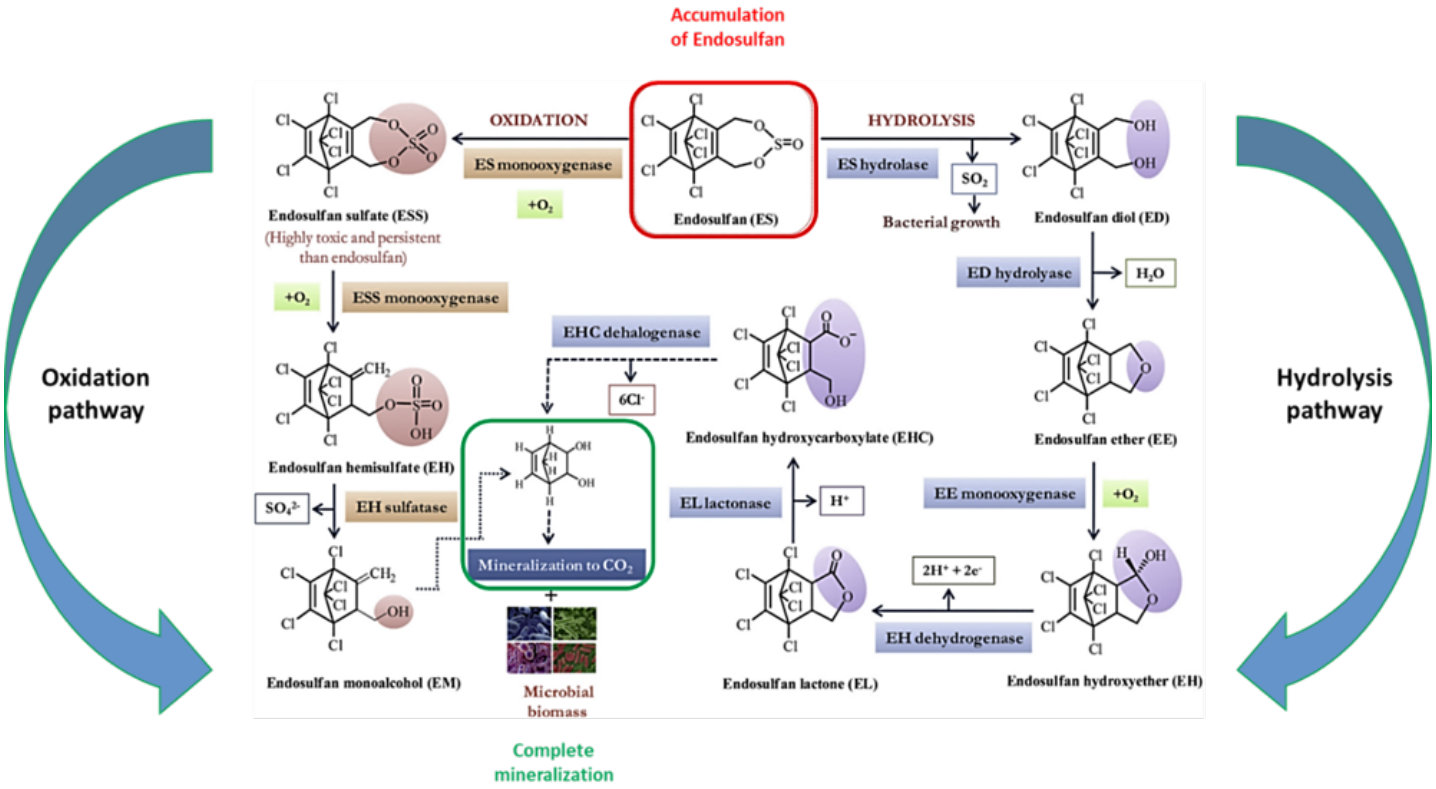

Figure 9: Suggested pathways involved in the bioconversion of endosulfan. Adapted from [43]

1.4 Pesticide remediation

To reduce or eliminate pesticides residues, physical, chemical, or biological processes can be applied. Physical methods, such as excavation and landfilling used to be the main solutions to remediate highly polluted soil containing banned pesticides in the 1970s and 1980s, but they should be seen as a provisional waste disposal solution, and not as a remediation technology, as these landfills still constitute a problem in developing countries [44]. Chemical treatments are usually costly because most of them have to be carried out ex-situ, and are not very environmentally friendly because of their aggressiveness to soil and soil microbiota.

Biological treatment, or bioremediation, is a more eco-friendly and low-cost process, as it can be applied in-situ and covers from traditional methods as composting (enriching the contaminated soil microbiota by addition of organic wastes as manure to promote microbial degradation), to the use of specific nutrients, microorganisms, plants, or even earthworms, to speed up the natural degradation of pesticides [45].

The two most tested approaches for the bioremediation of polluted environments in the last years have been biostimulation and bioaugmentation. Biostimulation is the addition of nutrients to

contaminated areas to boost the growth of microorganisms capable of degrading the toxic molecule. These are usually rate-limiting nutrients such as nitrogen, phosphorus, easily degradable carbon source, or surfactants to help the uptake of the organic pollutant [46], [47]. Bioaugmentation, on the other hand, implies the addition of single strains or consortia to the contaminated site, which can be indigenous (enriched and adapted) or exogenous [48]. The use of either or both techniques depends on the degrading potential of the indigenous communities, and on the type and extent of contamination.

It is worth noting that in several cases, the bioremediation of soil is most likely to occur in the presence of a mixture of pesticides, which can interact with each other, thus making the reconstruction of the exact degradation pathways more challenging. A good example of the complexity of this task can be found in the study by Krishna and Philip [33], who studied the biodegradation mechanisms of mixed pesticides. The authors reported that traces of many intermediate compounds were detected and that there was no significant accumulation of these compounds as immediate degradation of intermediates occurred after their formation. This means that, despite nowadays a significant amount of pesticide degradation studies as well as data from regulatory testing are available, it still remains a challenging to precisely anticipate the pathways of pesticide degradation (especially in field conditions). This poses a major drawback, since even though an array of abiotic and biotic transformations may effectively remove pesticides from the environment, on the other hand these processes may lead to the accumulation of potentially hazardous products in secondary compartments [20].

1.4.1 Screening for pesticide degrading microorganisms

To develop effective tools for pesticide bioremediation, efficient degraders for each of the pesticides used must be found. Taking samples from contaminated sites and screening them for pesticide degraders is a classical approach to obtain a wide diversity of microorganisms, which can, subsequently, be adapted, immobilized, combined with other bacteria, etc. to improve their degradation rate [49]. In recent years, new technologies such as high throughput screening with automated robots and microtiter plates have allowed to significantly speed-up the process of microbial discovery, thanks to the possibility to rapidly test and process large numbers of experimental conditions [20]. The identified microorganisms could then be introduced in the same site they were taken from (autochthonous bioaugmentation), or in other sites contaminated with similar pesticides (allochthonous bioaugmentation) used for the screening [50].

As mentioned above, inoculum samples are usually taken from contaminated sites, i.e. from intensive farming fields, pesticide manufacture wastewater treatment plants or storage sites, or polluted soil landfills [51], [52]. The enduring presence of pesticides in these places can lead to an increase of adapted/selected bacteria able to tolerate and metabolize these compounds, thus having an advantage over the other microorganisms. This can result in an increased tolerance to higher pesticide concentrations and a higher degradation rate [45]. It is worth reminding that many conditions, such as soil depth, pH, or clay content, influence the diversity of the microbial community (as well as the persistence and availability of pesticides), and thus taking numerous

samples from different areas is anticipated to increase the diversity of the community screened [37].

These inoculum samples are then typically subjected to a selective enrichment process, to increase the abundance of the pesticide degraders in the community. This is commonly done by inoculating the sample in a liquid minimal medium containing the desired pesticide/s as the sole carbon source. However, some studies have also identified bacteria that can use pesticides as the sole nitrogen [51] sulphur [53], or phosphorus [54] source. Besides, Jing-liang reported that Rhodococcus jialiangiae djl-6-2 degraded the fungicide carbendazim faster when it was used as the sole nitrogen source than when it was used as the sole carbon source, or even when used as both, carbon and nitrogen source [55].

Clearly, the duration of the enrichment depends on the biodegradability of the pesticide, the concentration, and its toxicity, so a different enrichment strategy should be designed for each pesticide (or mix of pesticides) investigated. Moreover, due to their high toxicity of many compounds, the concentration of carbon source used is often too low to monitor the bacterial growth with turbidity measurements, therefore other methods, such as colony forming units (CFU) count should be performed [40]. To measure the biodegradation of the substrate, chromatographic methods such as GC-MS or HPLC-UV are mostly used. The use of 14C labelled pesticides gives a more reliable biodegradation measurement but involves the added risks of working with radioactivity [20].

Finally, the obtained enriched culture can be either tested directly for biodegradation efficiency, or it can be used to obtain efficient pure strains, through classical isolation methods such as streak plates (using i.e. minimal medium agar supplemented with the pesticide), agar rolls or dilution-to-extinction techniques [40]. The pure strains can then be identified by methods such as biochemical tests or 16S rDNA sequencing. The next steps would be studying the optimal conditions of the bacterial degradation, both in liquid medium and in soil microcosm, to finally obtain an efficient bioremediation tool.

1.4.2 Designing pesticide degrading consortia

With the focus on finding efficient solutions to remediate pesticide-polluted soils, the use of microbial mixed consortia (MMC) shows several advantages when compared to pure cultures [56]. MMC usually exhibit higher growth rates on persistent/recalcitrant compounds, and metabolize a wider range of substrates, since they combine catabolic pathways of different strains [57]. Consequently, MMC often present increased survival and pollutant degradation rates than pure cultures. Moreover, co-evolved MMC have proven to have lower growth requirements (or stated differently, a more efficient use of the resources) thanks to cross-feeding mechanisms, and are thus more robust to external contamination from other microorganisms [58]. This allows operating more efficiently in non-sterile conditions [59], a necessary pre-requisite for in-situ bioremediation. In fact, the fate of the inoculum is a major challenge in the case of in-situ remediation, and becomes a critical factor in the case of pure strains.

On the other hand, even though enriched MMC capable of effectively degrading target pesticides can be achieved directly from contaminated sites, this method lacks reproducibility as the community

obtained is affected by the specific conditions (i.e. the time and place, the variable biodiversity) of the sampling site. In addition, the MMC would be subjected to changes over time, related to different culture, operating or storage conditions. Therefore, the community diversity of the enriched culture must be assessed, and methods to reproduce it developed, to guarantee the proper and long-term implementation of MMCs as a bioremediation tool [60].

Taking this into account, a simple approach to obtain an efficient and reproducible pesticide-degrading consortium is by mixing the isolated strains together, in different combinations, and choose the most efficient degrader for the target pesticide [61].

The diversity of pesticide-degrading strains will be lowered allowing for a better control, but, on the other hand, specific exogenous bacteria can be added to the defined mixed consortium [62] to provide the culture with desired specific abilities, such as biosurfactant production, or plant growth promotion. Biosurfactants are amphiphilic molecules produced by microorganisms, which increase the bioavailability of hydrophobic molecules, improving the degradation rates of many pesticides [63]. Plant growth promoting bacteria help the plant to acquire limiting nutrients such as nitrogen or phosphorus and decreases the growth of some pathogens, thus decreasing the amount of fertilizer and pesticide needed.

1.5 Aim and approaches

This project comes under a colaboration between Aalborg University and FMC corporation which aim is to develop bioaugmentation strategies which lower the persistance of different given pesticides. This framework constitutes two projects: the present master thesis, and a group bachelor thesis (Fig. 10).

To achieve this, the steps of enrichment, screening, and consortia design, laid out before in the introduction, will be followed. In total, three different environmental samples will be used as inoculum for the selective enrichment, namely, soil from contaminated farming fields, soil from the surroundings of a pesticide formulating facility, and activated sludge from the wastewater treatment plant of a pesticide formulating facility. These will be enriched using five different pesticides, separately, as the sole carbon source: malathion, dimethoate, dicamba, bifenthrin, and glyphosate, of different pesticide classes. Then the strains isolated will be screened for pesticide degradation, and the most efficient ones will be used to design efficient pesticide degrading consortias. In parallel, *P. putida* and *S. marcescens*, well studied for their organophosphate pesticides degradation potential, [64], [48],will be screened for malathion and dimethoate degradation, and used to study the influence of surfactants in degradation, to further optimize the bioremediation approaches.

This study is complemented by a parallel bachelor thesis, where a glycerol fermenting (GF) culture, will be used to obtain potential *Klebsiella* pesticide degrading species, deeply studied for pesticide biodegradation [42], [65], by enriching it, separately, in the pesticides malathion, dimethoate and dicamba, either as the sole carbon source, or with the supplement of citrate. It will also participate in the screening of isolates from the environmental samples enrichment, to increase the number of strains screened.

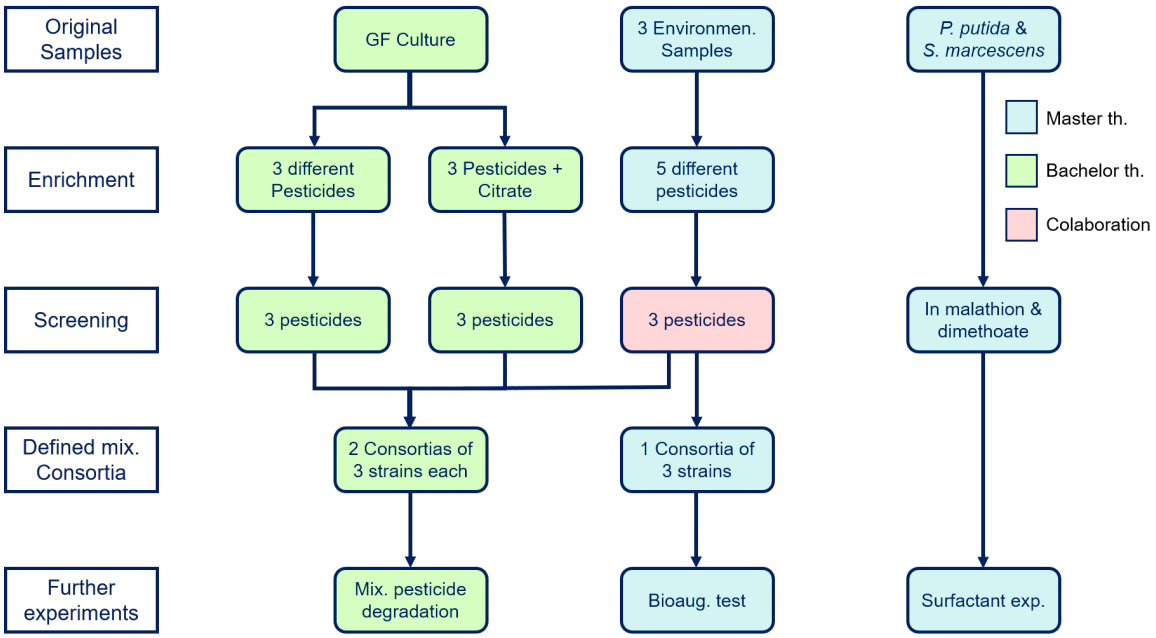

Figure 10: AAU-FMC collaboration overview. The green boxes sum up the steps followed in the bachelor thesis, while the blue boxes define the ones followed in the present master thesis. The red box refers to a joint work in the screening of the isolates from the environmental samples enrichment.

2 Materials and Methods

All experiments were performed in Aalborg University, in the Sustainable Biotechnology section of the Copenhagen Campus, except for the genomic analysis, which were performed in the Aalborg Campus, in the Chemistry and Bioscience department.

2.1 Chemicals and solutions

The pesticides malathion, dimethoate, dicamba, and bifenthrin were provided in standard form by FMC Corporation. The pesticide glyphosate was provided as the comercial product Envision by... They were stored in a ventilated cupboard at room temperature. Along the project, in the labeling of the different conditions, and the subsequent isolated strains, they are referred respectively as Mal, Dim, Dic, Bif, and Gly.

In order to prepare the M9 medium used, different solutions were prepared. 10X salt solution (pH=7.2) containing Na $_2HPO_4$, 75.2 g/L; KH_2PO_4, 30 g/L; NaCl, 5 g/L; and NH_4Cl, 5 g/L was prepared. $MgSO_4$, 1M; and $CaCl_2$, 1M; solutions were also made, and the three solutions were autoclaved separately, to avoid precipitation of salts. A 1000X trace elements solution containing $CuSO_4$, 0.04 g/L; KI, 0.1 g/L; $ZnSO_4$, 0.4 g/L; Na_2MoO_4, 0.2 g/L; $FeCl_3$, 0.2 g/L; and H_3BO_3, 0.5 g/L and filter sterilized was also used. To make the M9 medium, 100ml of 10X salts solution, 1ml of $MgSO_4$ solution, 0.3ml of $CaCl_2$ solution, and 1 ml of 1000X trace elements solution, were added to 897.7ml of destiled water.

The LB medium used contained tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 10 g/L. LB-agar was made adding 15 g/L agar to LB medium.

2.2 Sample collection and processing

The soil samples were provided by FMC Corporation, coming from five different locations in the surrounding aerea of the Cheminova pesticide formulating facility in Rønland; and by an Aalborg University professor, from two sites (with higher and lower pesticide contamination) of a pesticide contaminated agricultural field, in Hungary. The soil was taken from the first 10 cm depth to ensure an aerobic bacteria population, which have often been found to degrade pesticides [25]. The different samples were merged into two main samples: the soil coming from FMC and the soil coming from Hungary. Along the project, in the labeling of the different conditions, and the subsequent isolated strains, they are referred respectively as FS (FMC Soil) and HS (Hungary Soil). In this way we could analyze the whole biodiversity with fewer resources. The samples were then stored at 4°C, til they were used as inoculum in the following days.

Prior to the enrichment, the soil was dissolved at 100 g/L in M9 medium and agitated for 5 minutes. The samples were continuously stirred with magnets to obtain the inoculum for the enrichment.

The activated sludge sample was also provided from FMC corporation, and is referred in the project as Slu in the labeling of strains and conditions . It was obtained from the wastewater treatment plant of the Cheminova pesticide formulating facility, and stored at -20°C.

Prior to the start of the enrichment, the frozen sludge sample was thawn at room temperature and preactivated for two days in an M9 medium supplemented with glucose, 22 g/L; yeast extract, 5 g/L; and NaCl, 15 g/L; following FMC indications, using a 10% inoculum. The growth was monitored at 600nm with an spectrophotometre. The sample was transfered to the selective enrichment step with a 0.568 O.D., while being continuously stirred with a magnet.

2.3 Selective Enrichment

2.3.1 Enrichment overview

The three environmental samples were enriched separately in each of the 5 pesticides, in duplicates, to a total of 30 flasks, which underwent 6 enrichment steps with the same initial pesticide concentration. The enrichment strategy lasted for 11 weeks, following 3 initial 1 week steps, a 4^{th} 2 weeks step, and two last 3 weeks steps.

A 10% inoculum was used in M9 medium supplemented with the pesticide at the concentration listed in the Table 5, in a total working volume of 20 ml. 100 ml flasks were used for this purpose. The flasks were kept at 30°C, and shaking at 150rpm, along the enrichment strategy. The flasks were sealed with cotton stoppers to avoid contamination but prevent anaerobic conditions.

Pesticide	Concentration	Bibliography
Malathion	0.3 g/L	[66], [67], [68], [69]
Dimethoate	0.15 g/L	[64], [66], [70]
Dicamba	0.75 g/L	[71], [72]
Bifenthrin	0.25 g/L	[73], [74], [75]
Glyphosate	1 g/L	[76], [77]

Table 5: Concentration of the different pesticides in the selective enrichment. The concentration used was chosen as a mean of the concentrations used in some of the latest articles studying these pesticides biodegradation.

The pesticides were measured in a balance and dissolved directly in each of the flasks due to the low solubility of some of the pesticides, which prevented the making of concentrated stock solutions. The inocula and samples were taken while continuously stirring the culture with magnets. At the end of the enrichment, three sets of samples were taken: 1ml samples to monitor the growth; 2ml samples to be used in the next step inocula (4ml in the alternative enrichment); and 5ml to be analysed in a metagenomic study, frozen at -20°C until then. The rest of the culture was temporarily frozen at -20°C.

Alternative enrichment

From the third step of the enrichment, an alternative enrichment, was carried out in parallel, using a 20% instead of a 10% inoculum. The two best growing cultures for each pesticide, according to the O.D measurements, were chosen, and the conditions were exactly the same as the ones of the main enrichment, except from the inoculum .

2.3.2 Monitoring the growth

1 ml samples were taking at the begining and end of each step, to measure the increase in the absorbance and the extracelular proteins content. They were measured in triplicates in a microtiter plate reader.

O.D. measurement

200μl triplicates were taken from each sample, to measure the O.D. at 600nm. The microplate was shaked for ten seconds before measuring the O.D., and M9 medium was used as blank.

Extracelular protein measurement

The commercial kit: Pierce^{TM} BCA Protein Assay Kit by Thermo scientific, was used. 25μl triplicates were taken from each sample, and mixed with 200μl of the A:B reagent mix (*what is the proportion*) in the microtiter wells. M9 medium was used as blanks. The plate with the samples was shaked for 30 seconds, incubated for 30 minutes at 37°C, and then cooled down for 15 minutes at room temperature. Finally the O.D was read at 562nm.

To check if there are pesticide-BCA interactions causing false-positive results, 1mg of each pesticide were mixed separately with the AB reagent in 1 ml of the reagent. The change of colour caused by the reaction was be easily observed.

2.3.3 Plating cultures

From the end of the 4th step onwards, the cultures were plated at the end of the step in LB-Agar, to check if there is still growth. For this purpose, 100μl of each culture was used as inoculum, without any dilution, and spread in a plate with an L-shaped rod. They were incubated for 24 hours at 37°C before checking the presence of colonies.

Controls, using sterile M9 medium as inoculum and the same plates, LB-Agar, tips, and L-shaped rods, were used to ensure that all the material was sterile and none of the colonies are contamination.

2.4 Isolation of strains

At the end of the 6th step of the enrichment, the cultures were plated in LB-agar, taking 100μl sample as inoculum. The plates were incubated 24h at 37°C, after which different colonies were picked from each plate, based on morphology, and spread individually on LB plates. This step was repeated 4 times, to finally obtain isolated strains.

The isolated strains were frozen at -80°C in glycerol, using 4mm glassbeads. For this, 500ul of 15% glycerol was pipetted in each agar plate with the isolated bacteria. Then, around 15 glassbeads were placed in the plate and it was shaked so the glassbeads were covered by the bacteria. Then the glassbeads were transfered to criotubes with a sterilised spatula, and were frozen at a low rate by placing them in an isopropanol-containing box in a -80° freezer.

Controls were done in all the isolation steps to ensure that none of the material used was contaminated.

The strains were labeled after the sample they were coming from, the pesticide they were adapted to in the enrichment, and the duplicate number (1 or 2). The latter numbers refer to the different strains isolated from a single enrichment flask.

2.5 Degradation experiments

Before the experiments, strains were pre-activated for 24h in LB medium. Their final pre-activation O.D. was measured in a microplate reader at 600nm, so the inoculum used in the experiment leads a 0.1 O.D. at time 0. The surfactants were used at their critical micellar concenctration, 15mg/L for Tween80; and 1.5 mg/L for the biosurfactant, a mixture of mannosylerythritol lipids (MEL) produced by *Candida Antarctica* PYCC5048. The degradation experiments were carried out in M9 medium supplemented with the selected pesticide to a total of 20ml working volume, in a 30°C and 150 rpm incubator. The flasks were sealed with cotton stoppers to avoid contamination but prevent anaerobic conditions.Samples were taken periodically to measure the pesticide concentration in the HPLC-UV, and the controls were plated in LB-Agar, (37°C, 24h incubation) at the end of the experiments, to look for possible contamination.

2.5.1 Isolates screening

All the experiments were performed in triplicates, in 50 ml flasks, with the pesticide concentration listed in Table 6. Two strains were taken from each of the isolates libraries of three of the pesticide enrichments (malathion, dimethoate, and dicamba), along with *P. putida* and *S. marcescens*. Each of the isolates were cultured with the pesticide they are adapted to, while *P. putida* and *S. marcescens* were cultured with either malathion or dimethoate. Negative controls were made for each of the pesticides, without a bacterial inoculum.

Pesticide	Concentration
Malathion	0.125 g/L
Dimethoate	0.1 g/L
Dicamba	0.5 g/L

Table 6: Concentration of the different pesticides in the isolates screening.

The experiment lasted for 14 days, and samples were taken at day 0, day 7, and day 14. At the end, the total volume in each of the flasks was measured, to be used in the normalization of the pesticide concentration.

2.5.2 Influence of surfactants in biodegradation

Two strains known to degrade several organophosphorus pesticides were used, *Serratia marcescens* and *Pseudomonas putida* [64], [78]; two organophosphorus pesticides, malathion and dimethoate; and two surfactants, Tween80 and the biosuractant. Each strain was tested with both pesticides separately, and either or none of the surfactants. A negative control was made for each pesticide, disolving it in M9 medium without a bacterial inoculum, and with Tween80 as surfactant. Therefore, fourteen 100ml flasks were prepared, containing the selected bacteria, surfactant at their CMC concentration, and 0.1 g/L dimethoate or 0. 125 g/L malathion in 20ml of working volume. The volume was kept costant, in spite of evaporation, pouring the required M9 medium every week.

The experiment lasted for 14 days, and samples were taken at day 0, day 2, day 5, day 8, day 11, and day 14.

2.5.3 Consortia designing

All the experiments were done in triplicates with with dicamba as the carbon source, at the concentration used in the enrichment, 0.75 mg/L. 5 different inocula were used, and each of them was tried with and without surfactant (Tween80). The inocula were: the end stage of the enriched sludge sample; the strain HS Dic 22, isolated in this study; a combination of the strains HS Dic 22, HS Mal 22, and HS Dim 21, all isolated in this study; a combination of the sludge and the single strain inocula; and a combination of the sludge and the consortia inocula. The combinations were done so each of the individual cultures represented an even proportion of the O.D in the inoculum.

The experiment lasted for 14 days, and samples were taken at day 0 and day 14. At the end, the total volume in each of the flasks was measured, to be used in the normalization of the pesticide concentration.

2.6 Genomic analysis

2.6.1 DNA extraction

DNA was extracted from two sets of samples: the three isolated strains used in the consortia designing experiment, to be sequenced for identification; and the original AS sample and the six enrichment steps, to perform a metagenomic study by amplicon sequencing. The isolates were pre-activated for 48 hours in LB medium before the DNA extraction, and 0.5ml were taken to extract the DNA, centrifugated and resuspended in 0.5 sodium phosphate buffer (PBS). The sludge and enrichment samples were not preactivated, and 4.5ml were centrifugated (except from the original sludge sample, were only 0.5ml was used for DNA extraction) and resuspended in 0.5ml PBS to extract their DNA . The FastDNA® Spin Kit for Soil was used to extract the DNA of both sets of samples.

Cells were broken by bead beating in a FastPrep-24 instrument, at 6 m/s, during four series of 40s, cooling down the samples on ice for 2min in between series. The samples were then centrifugated at 10.000g and 4°C for 10min to obtain a supernatant without the beads and cell debris. Proteins were precipitated with a Protein Precipitation Solution at 14.000g for 5min, and the supernatant was mixed with a DNA binding matrix. After letting it settle down, the supernatant was discarded and the matrix was transfered to a SPIN™ Filter. The filter was centrifuged at 14.000g for 1min and washed with ethanol-SEWS-M solution and a 14.000g, 1min centrifugation. Then it was centifuged again for 2min, and the filter was allowed to dry for 5min, and finally the DNA was eluted in 60μl of DES solution. The DNA was then stored at -20°C to process it in the following days.

2.6.2 Evaluating DNA concentration and quality

DNA concentration was measured using a Qubit fluorescence assay kit. Initially, a Qubit dsDNA HS assay kit was used, and the samples which concentration exceeded the measurable range were analyzed using a Qubit dsDNA BR assay kit. The first one was used with 0 and 10ng/μl standards, while the second one was used with 0 and 100ng/μl standards. 2μl of the samples were mixed with 198μl of the reagent, and the 2 standards were mixed at a 10:190 μl ratio with the reagent. The standards and samples were then measured in a Qubit® 2.0 Fluorometer, and the concentration of the different samples was obtained through a regression line made with the standards.

DNA quality, after PCRs, was evaluated in a tapestation gel electrophoresis, with a Tapestation 2200 instrument and D1000 (for amplicon sequencing) or D5000 (for sanger sequencing) ScreenTapes. Two PCR products are randomly chosen, along with a negative (nuclease free water) and a positive control. 1μl of PCR product (or control) was mixed with 3μl of D1000 sample buffer and loaded in the Tapestation 2200 instrument along with the ScreenTape. The results were then analyzed with the TapeStation Analysis Software.

2.6.3 Nextera amplicon sequencing

The nextera amplicon sequencing was performed in the sludge and the 6 enrichment step samples.

Library preparation

The protocol used was based on the Illumina Nextera library preparation approach, were two PCRs are performed, the first one to amplify the target amplicon (amplicon PCR), and the second one to supply it with the Illumina adaptors and barcodes for sequencing (library PCR).

Before the first PCR, the extracted DNA concentration was measured with Qubit, and the samples were diluted to 5ng/μl in 60μl of nuclease free water.

The amplicon PCR was performed using a Platinum® Taq DNA Polymerase High Fidelity, and primers targeting the V4 region of the 16S rRNA (Table 7). All samples were run in duplicates to avoid PCR drift, and for each sample, 25.8μl of amplicon buffermix (buffer, dNTP and $MgSO_4$), 20μl of tailed primer mix (1 μM), 0.2μl of polymerase (5 U/μl), and 4μl of DNA, were taken. The reaction comprised the following steps: initial denaturalization (95°C, 2min); 35 cycles of denaturalization (95°C, 20s), annealing (50°C, 30s), and elongation (72°C, 60s); and final elongation (72°C, 5min). The duplicates were then pooled together and a cleanup was performed using Agencourt AMPure XP system, which uses magnetic beads that bind DNA, and two ethanol washes were carried out in a magnetic rack, til the DNA was disolved in nuclease free water.

Region	Primer name	Primer sequence
V4	515F	5'-GTGCCAGCMGCCGCGGTAA
	806R	5'-GGACTACHVHHHTWTCTAAT

Table 7: Primers used in the amplicon PCR.

Before the second PCR, the DNA concentration was measured and the samples were diluted to 5ng/μl, and the DNA quality was evaluated by a TapeStation gel electrophoresis.

The library PCR was performed using a PCRBIO Hifi Polymerase, and the provided adaptor primers, a specific pair for each sample. For each sample, 7.75μl of nuclease water, 5μl of X5 PCRBIO Reaction buffer (including $Mg_1{}^+$ and dNTPs), 0.25μl of polymerase (2 U/μl), 10μl of nexamp adaptor mixes (1 μM), and 2μl of PCR product. The reaction comprised the following steps: initial denaturalization (95°C, 2min); 8 cycles of denaturalization (95°C, 20s), annealing (55°C, 30s), and elongation (72°C, 60s); and final elongation (72°C, 5min). A cleanup was then performed in the same way as the previous one and the DNA was dissolved in 20μl of nuclease free water.

The DNA concentration was measured and the quality of the DNA products evaluated. The PCR products were then pooled together, taking the same amount of each of them, and reaching a total of 100ng of DNA. Finally the concentration and quality of the DNA is measured again before the Illumina sequencing.

Amplicon sequencing

The raw sequencing reads quality was checked using trimmomatic (version 0.32) [79] and merged using FLASH (version 1.2.7) [80]. The reads were then formatted for use with the UPARSE pipeline [81], screened for chimeric sequences and clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using USEARCH7. Taxonomy was assigned using the RDP algorithm implemented in QIIME [82] using SILVA database release S132 [83]. The microbial community data was analyzed using R (version 3.5.2) (http://www.R-project.org/) and RStudio (version 1.1.463) (http://www.rstudio.com) using the package ampvis2 (version 2.4.2) [84].

2.6.4 Sanger sequencing

PCR was performed using using a Taq PCR Core Kit (Qiagen); primers 27F: 5'AGAGTTTGATCMTGGCTCAG, and 1492R: 5'GGYTACCTTGTTACGACTT [85]; and 10ng of DNA, in 50μl working volume. The reaction comprised the following steps: initial denaturalization (94°C, 2min); 30 cycles of denaturalization (94°C, 30s), annealing (50°C, 30s), and elongation (72°C, 90s); and a final elongation (72°C, 10min). The PCR products were then purified using Qiaquick Gel Extraction Kit (Qiagen) and quantified with a qubit assay. The PCR fragment size was confirmed by a Tapestation gel electrophoresis, and sent for Sanger sequencing at Eurofins genomics, Germany.

2.7 HPLC-UV

Chromeleon™ Data System software was used on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific™), with a P680 pump. An LC18 with 5μm packing in a 250x4.6mm Luna column (Phenomenex®) was also used.

For the HPLC measures, 1ml samples were taken from the different experiments, and frozen at -20°C. Prior to HPLC analysis the samples were mixed with the chosen organic solvents, and filtered through a 0.45μm filter. The injection volume was 10μl.

Each sample was measured in triplicates for the first time each protocol was tested, and without replicates the following times.

Preparation of the standards

Three stock solutions were made, dissolving each pesticide in the respective HPLC organic solvent, malathion, 1 g/L in acetonitrile; dimethoate, 1 g/L in methanol; and dicamba, 1 g/L in methanol. The sets of standards in pure organic solvents were prepared mixing the pure organic solvent with the respective stock solution to achieve the concentrations listed in Table 8. The sets of standard in a water:organic solvent mixture had an extra step of adding water to the respective water:organic solvent ratio. These were 50:50 water:acetonitrile (v/v), for malathion; 50:50 water:methanol (v/v), for dimethoate; and 60:40 water:methanol (v/v), for dicamba. Tables 8 and 9 show the

concentration in the organic solvent, before adding the water. All the standards were filtered before injecting them in the HPLC column

Malathion	Acetonitrile	Water-Acetonitrile
1	9.59 mg/L	12.5 mg/L
2	19.19 mg/L	25 mg/L
3	38.38 mg/L	50 mg/L
4	76.75 mg/L	100 mg/L
5	153.5 mg/L	200 mg/L
6	307 mg/L	400 mg/L

Dimethoate	Methanol	Water-Methanol
1	12.5 mg/L	12.5 mg/L
2	25 mg/L	25 mg/L
3	50 mg/L	50 mg/L
4	100 mg/L	100 mg/L
5	200 mg/L	200 mg/L
6	400 mg/L	400 mg/L

Table 8: Concentration of malathion and dimethoate in the organic fraction of the standards.

Dicamba	Water-Methanol
1	31.25 mg/L
2	62.5 mg/L
3	125 mg/L
4	250 mg/L
5	500 mg/L
6	1000 mg/L

Table 9: Concentration of dicamba in the organic fraction of the standards.

Solubility test

For the solubility test, three 1/4 serial dilutions were made for each pesticide. Starting from 0.3 g/L malathion, 0.3 g/L dimethoate, and 800 mg/L, dicamba, in 20ml of total working volume. Five 500μl samples from each malathion and dimethoate solution flask, and 600μl from each dicamba solution flask, were taken and mixed with the correspondent organic solvent at the ratios mentioned in the preparation of the standards paragraph, filtered, and injected into the HPLC.

Recovery factor

To calculate the recovery factor, a 200 mg/L malathion, a 200 mg/L dimethoate, and a 666 mg/L dicamba solutions in 20ml water were made. Six samples were taken from each flask (500μl malathion and dimethoate, and 600μl dicamba), of which three of them were mixed with the correspondent pure organic solvent, and three with the correspondent 1 g/L pesticide stock solution. The recovery factor was calculated with the following formula:

$$R_{\mathrm{f}} = \frac{C_{\mathrm{w}} + C_{\mathrm{s}}}{C_{\mathrm{w+s}}}$$

Where C_{w} accounts for the pesticide concentration in the water sample, C_{s} accounts for the pesticide concentration in the stock solution, and $C_{\mathrm{w+s}}$ for the pesticide concentration of the

water sample spiked with the stock solution.

2.7.1 Malathion determination

Samples were mixed with acetonitrile at a 50:50(v/v) ratio, filtered, and injected in the HPLC column. The mobile phase used was a water (adjusted to pH=3.50 using 1N acetic acid): acetonitrile gradient, starting from 45% acetonitrile until 9 min, increased to 90% acetonitrile by 10 min, then returned to 45% acetonitrile at 13 min, and kept under this condition for 2 min to re-equilibrate. The flow rate was programmed at 0.5 ml/min from zero-9 min, increased to 2 ml/min by 10 min, and then returned to 0.5 ml/min at 13 min. The UV detector was set at 230nm [86].

2.7.2 Dimethoate determination

Samples were mixed with methanol at a 50:50(v/v) ratio, filtered, and injected in the HPLC column. The mobile phase used was acetonitrile:water in the ratio of 60:40(v/v). The flow rate was 1ml/min, and the detector wavelength used was 205nm. The temperature of the column was set to 30°C [87].

2.7.3 Dicamba

The samples were mixed with methanol at a 60:40(v/v) ratio, filtered, and injected in the HPLC column. The mobile phase used was a gradient of A: methanol–acetonitrile (82:18, v/v) and B: water acidified with TFA (0.17%, v/v). The gradient started from A:B (50:50) and linearly increased to 88% A in 35 min, held for 5 min, decreased to 50% A in 5min, and held for 12 min [88].

3 Results and Discussion

3.1 Enrichment

3.1.1 Growth monitoring

Two measurements were performed to monitor the growth along the enrichment steps: O.D., and extracellular proteins concentration. Due to the high soil and sludge concentrations in the first two steps of the enrichment, the measurements started to be taken from the third step of the enrichment.

Absorbance

Not much growth was detected during the third step of the enrichment (Fig 11), which lasted one week. The maximum values reached just 0.1 units of difference between the final and initial O.D. and there was a high variability within the duplicates. Therefore, it was decided to extend the fourth step to a 2 weeks period.

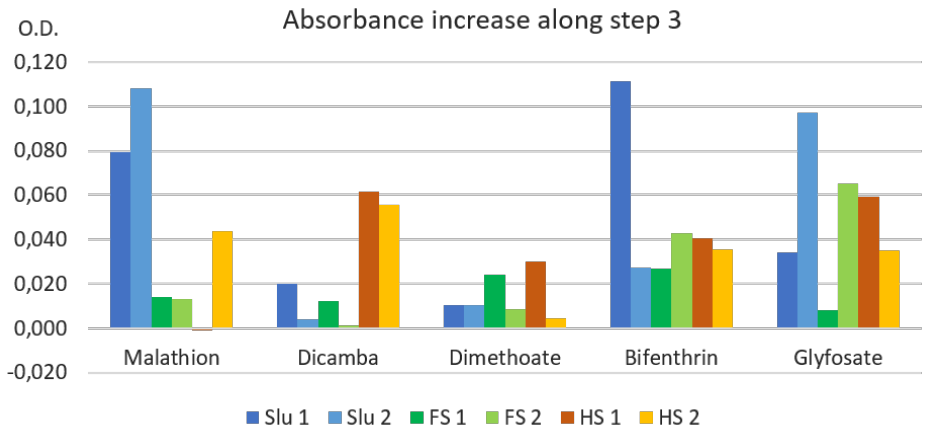

Figure 11: Increase in absorbance along the third step of the enrichment.

The fourth step of the enrichment, despite being longer than the third one, yielded a lower growth, with some of the flasks having even decreasing O.D (Fig 12). There was still a lot of variability within the duplicates, but also the most growing cultures were different ones in the third and in the fourth step. In response to this, the two best growers (in the third step) for each pesticide were unfrozen from the end of the second step of the enrichment, and the third step was repeated with a double inoculum concentration (20%), while the normal enrichment carried on.

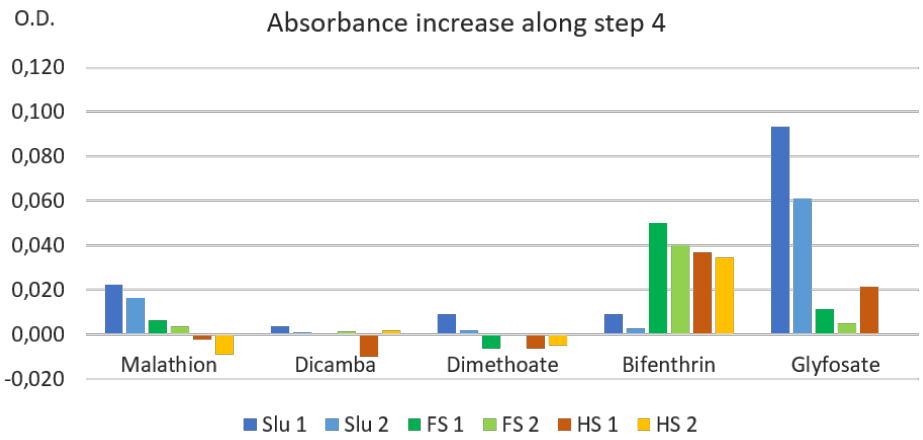

Figure 12: Increase in absorbance along the fourth step of the enrichment.

The alternative enrichment showed the lowest growth rates of the steps measured so far (13, so it was decided to stop performing O.D measurements, and check at the end of the step 5 of the main enrichment whether there were still bacterial communities growing in the enrichment, inoculating the different cultures in LB-plates.

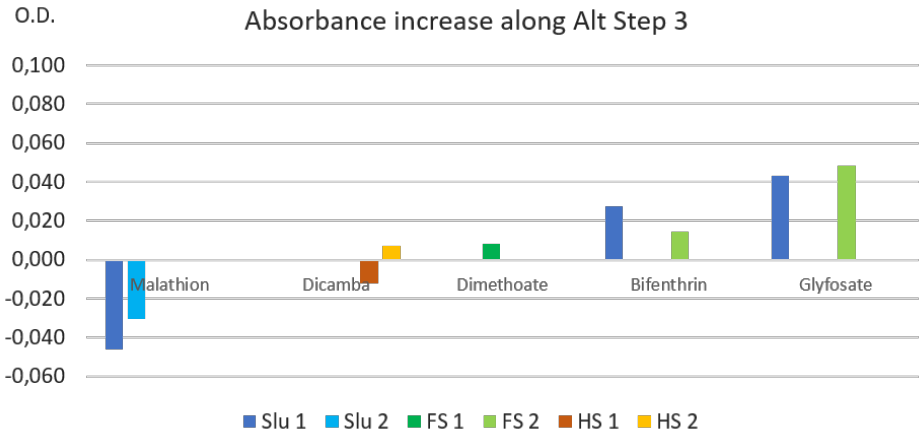

Figure 13: Increase in absorbance along the third step of the alternative enrichment (Alt).

Extracelular proteins

In parallel to the O.D. measurements, the BCA Protein Assay Kit was tested to monitor the bacterial growth in the pesticide enrichments, but due to the big difference in the signal between the different pesticides (Fig. 14), a qualitative test was made to see if any of the pesticides were interacting with reagent. The pesticides were mixed with the reagent at 1 g/L, and all but glyphosate showed a positive reaction. Not being the extractelular protein measurement reliable, it was not taken as a growth monitoring approach.

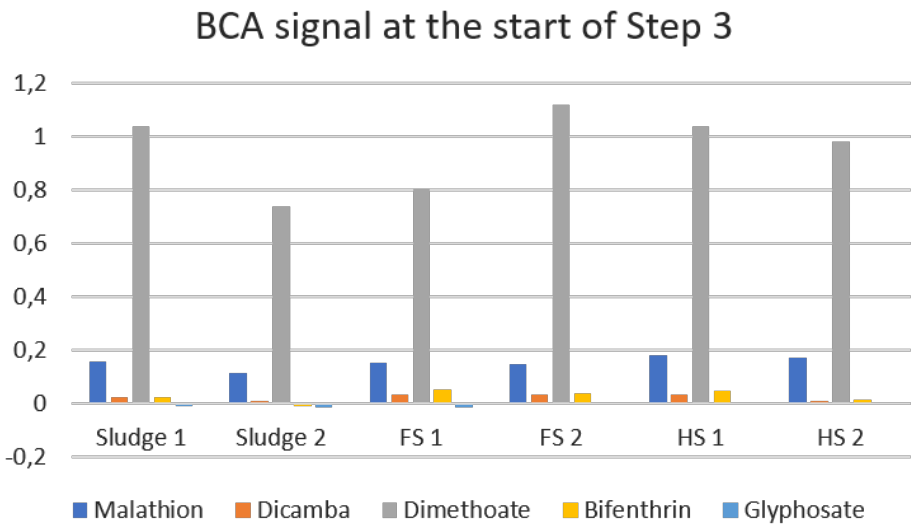

Figure 14: BCA signal at the start of Step 3 of the enrichment. The blank signal is substracted to all values before plotting.

Presence of colonies

After 5 steps of enrichment, all the cultures from the main and the alternative enrichment were checked for colonies (Fig. 15), which were found in all the cultures.

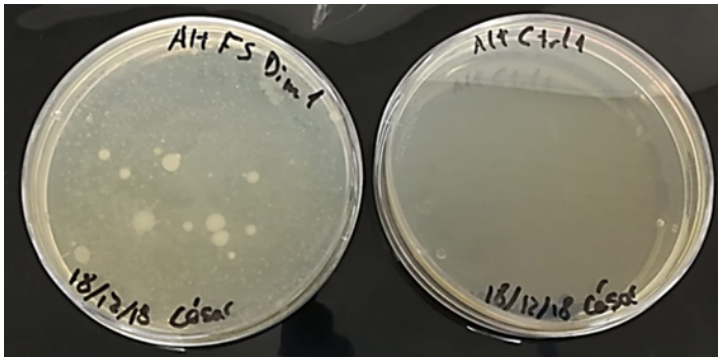

Figure 15: LB-plates inoculated at the end of the fifth step of the enrichment. The left plate was inoculated with one of the cultures of the alternative enrichment, while the right plate was inoculated with sterile M9 medium. The inculum used was 100μl, without dilutions.

3.1.2 Biodiversity assessment

It is important to remark that, though listed here in the enrichment subsection, the metagenomic data was obtained at the end of the project, and therefore, the conclusions extracted from it could be only applied in future projects.

Taking into account the enrichment and degradation assays, Slu Dic 1 culture was chosen to be used for the metagenomic study. This is because dicamba was the most promising pesticide in the

degradation studies, and therefore used in the last consortia desgining experiment. From dicamba enrichments, Slu Dic 1 was the culture showing a wider biodiversity in the isolation process.

Library preparation

DNA was extracted from 4.5ml of the samples taken at the end of each step, and from 0.5ml of the initial activated sludge. The DNA concentration, measured with Qubit after DNA extraction, and after the differet PCRs, can be seen in the appendix section. After each of the PCRs, a tapestation electrophoresis was also performed to ensure the quality of the DNA. For the first electrophoresis, a sample with an originally (before dilution) high concentration of DNA (first step of the enrichment), and a sample with an originally low concentration of DNA (third step) were randomly chosen.

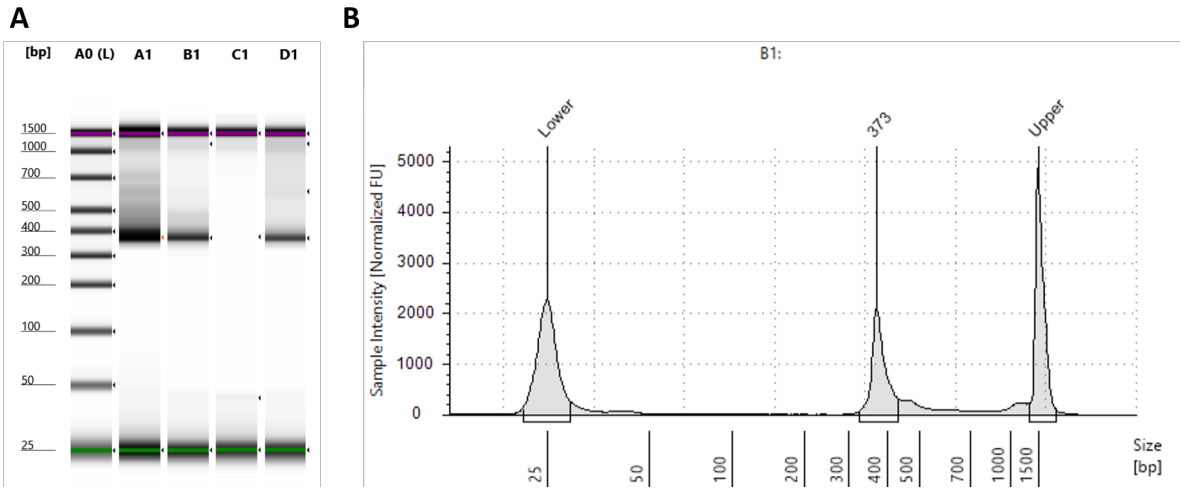

Figure 16: Tapestation electrophoresis of the first PCR products. **A**: Tapestation electrophoresis gel. The marker is shown on the left (A0), and the negative (C1) and positive (D1) controls on the right. The first and third step of the enrichment samples are respectively A1 and B1 lanes. **B**: Electropherogram of the B1 lane of the electrophoresis gel, with labeled lower and upper marker, and the 373 base pairs amplicon.

Figure 16 confirms that the first PCR amplified the 515F/806R part of the V4 region (around 300-350bp) properly. After the second PCR, the tapestation electrophoresis showed a slightly higher band, result of adding the illumina barcodes and adaptors to the amplicon (Fig. 17). In this second electrophoresis, the positive control has a different band size because it was amplified with different primers in the first electrophoresis (the same as D1 lane sample), but the rest of the conditions were the same and they were run in the same PCR plate in both electrophoresis. Having ensured that both PCR were succesful, the DNA was pooled, according to the Qubit results and sent to sequencing.

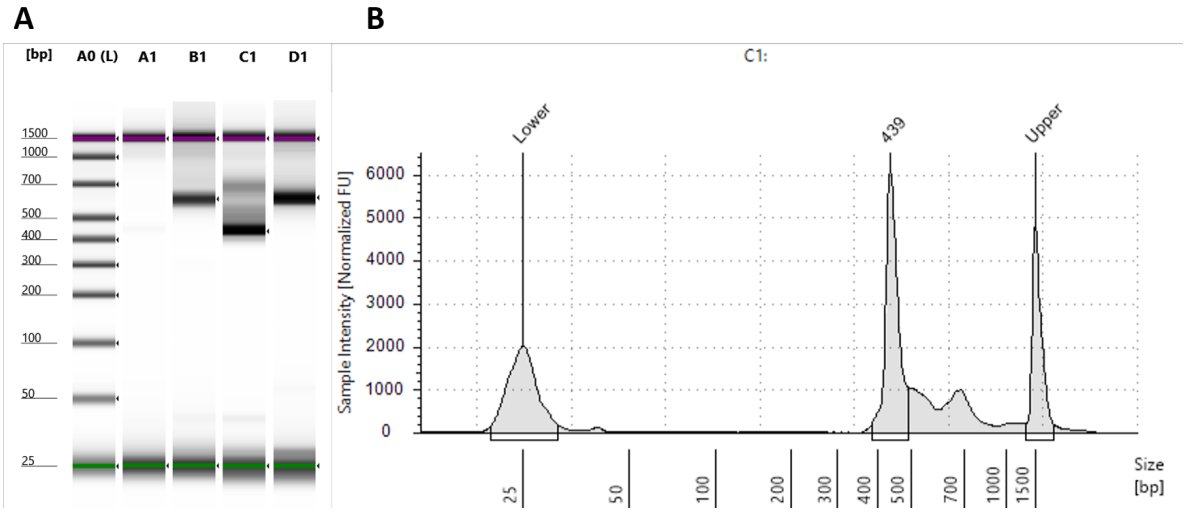

Figure 17: Tapestation electrophoresis of the second PCR products. **A**: Tapestation electrophoresis gel. The marker is shown on the left (A0), followed by the negative (A1) and positive (B1) controls. C1 lane represent our step 2 sample, while D1 represents a sample from a parallel project of the laboratory. **B**: Electropherogram of the C1 lane of the electrophoresis gel, with labeled lower and upper marker, and the 439 base pairs amplicon with the adaptors.

Sequencing

After the external sequencing and processing of the raw reads, 63,195 sequences, from the seven samples sent, were analyzed, and identified as 229 different OTUs. They represented 16 different bacterium phyla, but only 6 had an overall abundance greater than 1% of the sequences in any of the samples (Fig. 18, A). Proteobacteria alone accounted for a 82.79% of the reads, including gammaproteobacteria (44.88%) and alphaproteobacteria (37.81%). The next most abundant phyla were firmicutes (9.159%), actinobacteria (3.17%), and chloroflexi (1.146%).

The abundance of alphaproteobacteria is higher in the unenriched sludge (UnS) and in the last steps of the enrichment, while gammaproteobacteria is more abundant in Steps 1-5 of the enrichment, and sharply decreases in the last step (Fig. 18, B). Firmicutes phylum, represented mainly by the Lactobacillales family, was enriched only in the first half (steps 1-3); and actinobacteria, represented mainly by Nocardiaceae and Nocardioidaceae, appeared in the step 5, and mainly step 6 of the enrichment.

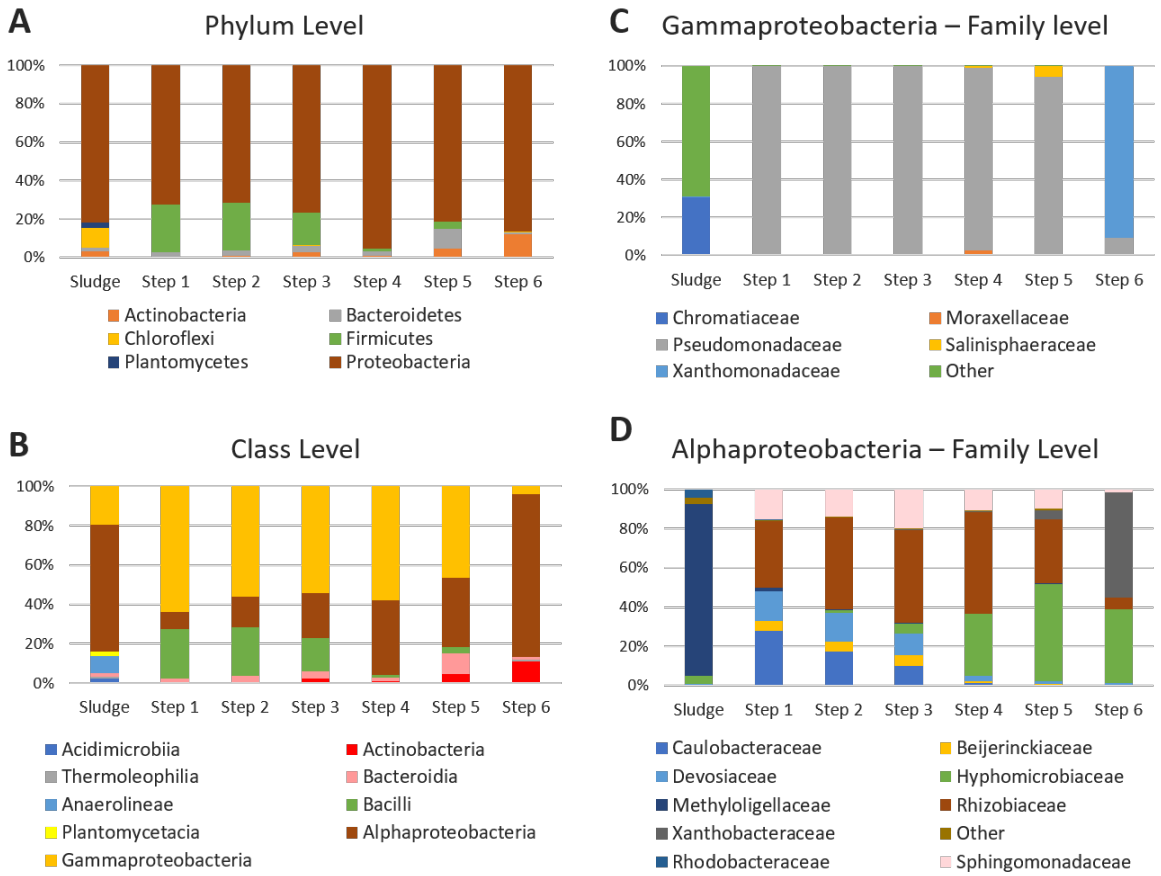

Figure 18: Composition of the bacterial communitiy at Phylum **(A)** and Class **(B)** level, and the family diversity within the gamma **(C)** and alphaproteobacteria **(D)** classes.

The composition of alpha and gammaproteobacteria also varies along the different stages (Fig. 18, C and D). Methyloligellaceae accounts for 51.41% of the Unenriched Sludge reads, but is almost extincted in the enrichment steps. Families like Devosiaceae, Beijerinkiaceae, and Caulobacteraceae, are present only in the first half of the enrichment, while Hyphomicrobiaceae appears in the second half, and xanthobacteraceae only in the last step of the enrichment. There is also, in the last step, a decrease of Rhizobiaceae and Sphingomonadaceae, which were relatively constant along the enrichment. Within gammaproteobacteria the community is also changed with the start of the enrichment, which is mainly represented by pseudomonadaceae family until the sixth step, when is suddenly colonized by Xanthomonadaceae.

In every sample, more than 50% of the reads come from just one or two genus (Fig. 10). In the sludge sample, Methyloceanibacter represent half of the bacterial community, and another 8% stands for an unidentified gammaproteobacteria. Pseudomonas is the most abundant genus in the steps 1-5, where tends to a slight decrease, but it suddenly disappears in the sixth step, where there is a drastic change in the community. Xanthobacteraceae raises from a 1.33% in the previous step to a 31.61% abundance in the sixth, Hyphomicrobiaceae reaches a 31.06%, and Rhodopseudomonas increases from a 0.27% to a 6.31%.

15 Most Abundant Genus	Sludge	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
Pseudomonas	0.00	63.98	55.97	53.96	54.97	42.57	0.33
Hyphomicrobiaceae	0.00	0.01	0.20	1.14	11.93	17.51	31.06
Methyloceanibacter	50.84	0.15	0.10	0.14	0.01	0.06	0.00
Streptococcus	0.00	9.83	13.67	9.61	0.78	0.52	0.00
Xanthobacteraceae	0.00	0.00	0.01	0.06	0.23	1.33	31.61
Rhizobiaceae	0.00	0.53	1.30	3.06	10.65	4.66	4.72
Shinella	0.00	1.90	4.93	6.61	7.37	1.77	0.10
Parapedobacter	0.00	2.17	3.02	3.59	1.39	9.73	0.66
Vagococcus	0.00	8.06	5.30	2.92	0.14	0.85	0.01
Sphingomonadaceae	0.00	1.33	2.15	4.57	4.00	1.49	0.93
Lactococcus	0.00	3.37	2.81	1.75	0.24	1.25	0.00
Gammaproteobacteria	8.30	0.00	0.01	0.02	0.00	0.00	0.00
Brevundimonas	0.00	2.42	2.65	2.24	0.47	0.22	0.03
Devosiaceae	0.00	1.13	2.02	2.19	0.91	0.31	0.96
Rhodopseudomonas	0.00	0.00	0.00	0.00	0.01	0.27	6.31

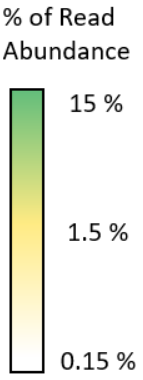

Table 10: List of the 15 most abundant genus, and their abundance (%), in each of the samples.

3.1.3 Discussion

Two parameters were analyzed to monitor the growth, cell density, and extracellular proteins concentration, but none of them showed to be effective. In the end, the cultures were plated at the end of the last two steps of the enrichment, as a qualitative check, to ensure that there is still some growth in the cultures.

Two approaches were tried out to increase the bacterial growth: increasing the duration of the enrichment step (Fig. 12), and repeating the steps with a double amount of inoculum (Fig. 13). The aim of these measures, were, respectively, to allow the pesticide degraders to consume the pollutant along the step, if the problem was a slow degradation; and increase the inoculum size, if the problem was that the pesticide degraders were being washed out in the transfers. However, both approaches yielded a lower growth than the one in the third step of the main enrichment, and the difference in the growths in different pesticides do not match with the expected longer half-life of bifenthrin [89], in respect to the rest of the pesticides [67], [90], [91], [92].

Taking into account that the initial inoculum (sludge and soil samples) is higher in nutrients than the M9 medium supplemented with the pesticide, it would make sense that the growth is greater in the first steps of the enrichments, until this organic matter is totally washed out. Moreover, the inoculum for the rerun of the third step in the alternative enrichment went through a freezing and unfreezing process, withouth pre-activation (which would have counteracted the ongoing enrichment) that could have affected the viability of the community.

Nevertheless, the concentration of pesticide used add much less carbon source to the medium than what is usual in nutrient mediums such as LB. Therefore, the growth cannot be expected to be as high as those with high carbon concentration. Another method, reliable for low density cultures, should have been applied before changing the conditions of the enrichment. One of this methods could have been colony count in the plated cultures (Fig. 15), with serial dilutions, at the begining

and at the end of the experiment, to change from a qualitative to a quantitative assessment [93]. However, the size of the enrichment (number of samples) should have been reduced to a large extent to implement this approach, due to the time consumption this method entails.

The extracellular protein concentration was not a good parameter either, for the evaluation of the growth in the cultures, due to the unspecific interaction between the reagent and four of the pesticides (Fig. 14. Even if the reagent was tested with different concentrations of the pesticide to elaborate a calibration of the interference, we would not be able to differ, from the signal, between the pesticides and proteins concentration in the culture, as both concentrations should be changing along the enrichment step.

The significance of monitoring the growth lies in the fact that, if the pesticide degrading bacteria does not grow enough before transferring it to the next step, even the enriched species, which can grow on the pollutant, will be eventually washed out. However, what growth monitoring shows is the overall biomass present, and therefore, a stable O.D. along the enrichment step could also be due to a change in the community, where the pesticide degraders grow and the rest of the organisms perish (As showed in Fig. 18 and Tab. 10).

The metagenomic study shows a great change in biodiversity, at the family and genus level, at two points of the enrichment, the first step (because of the shift from the sludge to the enrichment conditions), and the last step of the enrichment. The first change could be smoothed, to avoid a possible loss of biodiversity, by a previous adaptation to the pesticide before the enrichment, in the original medium [94]; and by a step-wise change from the sludge to the enrichment conditions. The second big change in biodiversity could be caused by a change in the conditions along the last step, like an oxygen deprivation, which killed the strict aerobes pseudomonas and enriched the anaerobic, like some *Hyphomicrobiaceae* [95] and *Rhodopseudomonas*; and microaerophilic, like some *Xanthobacteraceae* [96], bacteria. This second big change in biodiversity suggests that, in order to obtain the maximum biodiversity for the screening process, the isolation should be performed not only at the end, but at different steps of the enrichment.

It is important to note that the samples analyzed came all from the same environmental sample, activated sludge from the wastewater treatment plant of a pesticide formulating facility. This is also a very restrictive environment, and that is seen in the fact that, in the same way as in the enrichmennt steps, in the sludge more than 50% of the reads come from a single genus, *Methyloceanibacter*, a family composed of methylotrophs and methanotrophs. The biodiversity dynamics of the enrichments coming from the contaminated, and not contaminated soils, could be different from the ones showed in this study, and it would be interesting to evaluate and compare them in following studies.

In spite of this restrictive environment, just after one week of enrichment, the diversity is replaced by bacteria known to be resistant to and/or degrade several pesticides, like Pseudomonas [97], [98], and several *Lactobacillales* genus such as *Streptococcus*, *Vagococcus*, and *Lactococcus* [99], [100]. The fact that these genus are replaced by others along the enrichment does not necessarily mean that the latter are more efficient pesticide degraders, though it is true that in the first steps several genus could be resistant to the pesticide, but feeding on other residual carbon sources

from the original sample. Many *Hyphomicrobiaceae* strains, which appears from the fourth step of the enrichment, are known to be oligocarbophilic, unable to live in rich carbon source media [95]. This raises two issues: another kind of pesticide degraders could be isolated when supplying an additional carbon source to the media, and low organic carbon medium should be used to isolate this oligotrophs.

Therefore, by enriching in low carbon source medium, and isolating in rich carbon source agar plates, we are only selecting, not only bacteria which can grow on the pesticide, but that also can live in a broad range of carbon concentrations, needlessly narrowing down the isolated bacteria diversity.

3.2 Isolation

At the end of the sixth step of the enrichment, all the cultures were plated in LB-Agar to isolate the bacteria present (Fig. 19). After four isolation steps, picking and spreading different individual colonies, 42 isolates were frozen in glycerol at -80°C, using glassbeads.

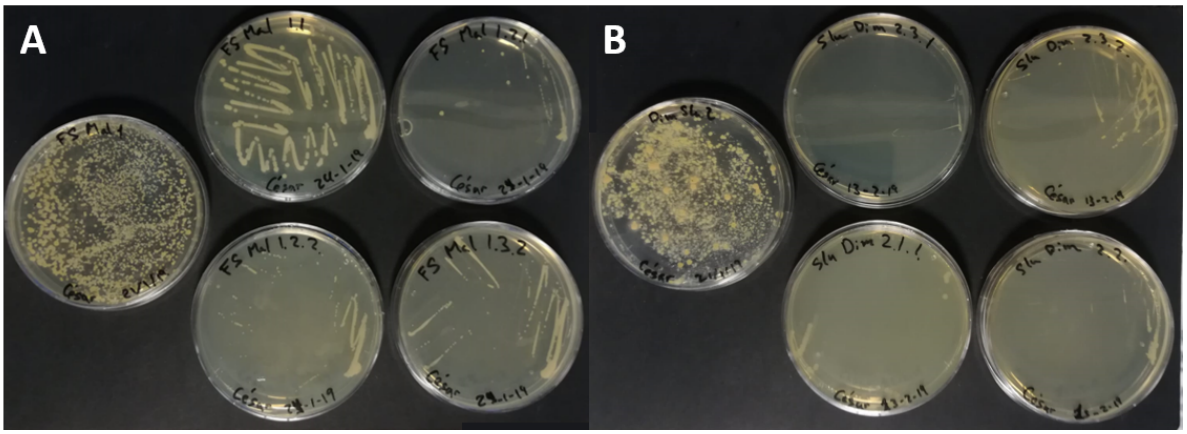

Figure 19: Isolation of bacteria from two malathion **(A)** and dimethoate **(B)** cultures. The plated mixed culture is placed on the left-hand side of each picture, and the bacteria isolated from them on the right-hand side.

3.3 HPLC tests

Before quantifying the pesticide degradation in the different degradation experiments, different tests were performed to validate the protocols and elaborate standard regression lines.

Is important to remark, that due to budget shortening, not all the tests performed were in the end measured in the HPLC, and thus no results are showed here in those cases.

3.3.1 Malathion

Standards

Two standards sets were elaborated, and measured in the HPLC (Fig. 20). The first one, as reported by Abu-Qare et. al. [86], in acetonitrile, and the second one in a water:acetonitrile 50:50 (v/v) mixture. As both types of standards showed a measurable linear regression, with a good R-squared, it was decided to use water:acetonitrile as the HPLC sample solvent for malathion determination and do not perform any extraction procedure.

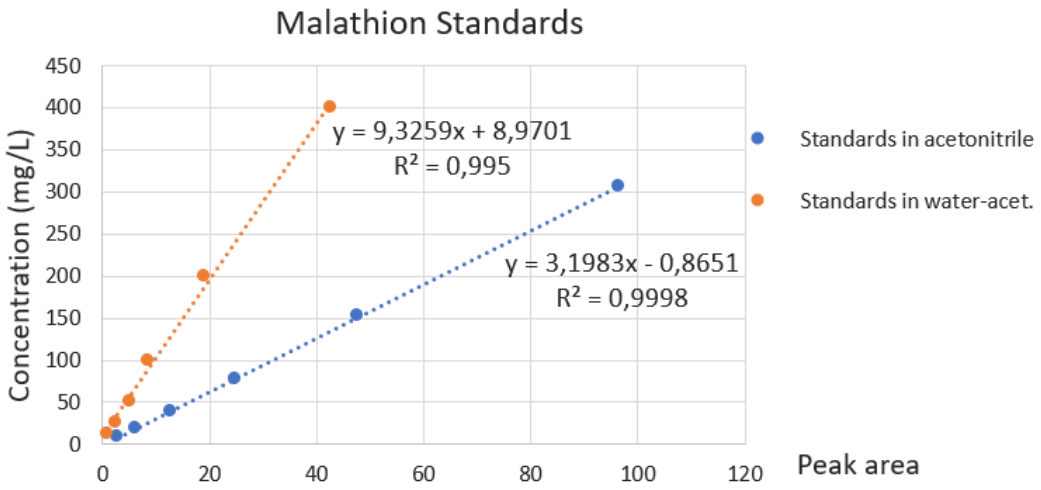

Figure 20: Regression lines of malathion standards in acetonitrile, and water:acetonitrile 50:50 (v/v), with their regression lines equation and R-square.

3.3.2 Dimethoate

Standards

In the same way it was done for malathion, two sets of dimethoate standards were prepared, in methanol, as reported by Kumar et.al [87]; and in water:methanol 50:50 (v/v). Both standards presented a measurable regression line with a good R-square (Fig. 21), so it was decided to use water:methanol as the HPLC sample solvent to avoid performing an extra extraction procedure.

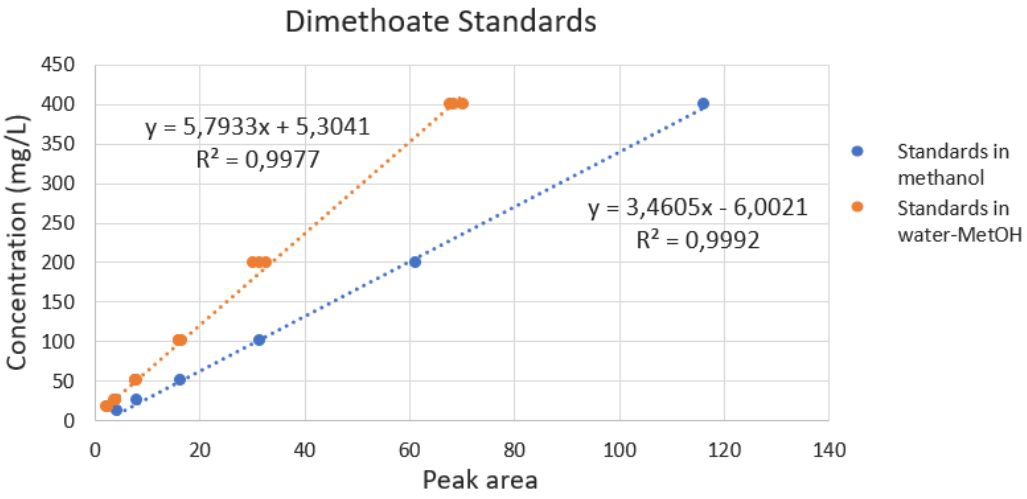

Figure 21: Regression lines of dimethoate standards in methanol, and water:methanol 50:50 (v/v), with their regression lines equation and R-square.

Solubility test

A test was performed to check if a single sample is representative of the pesticide concentration in the culture, at different concentrations. Three serial dilutions of dimethoate were made in 20ml working volume, and five samples were taken from each of them, from which two were measured in the HPLC. Figure 11 shows the expected concentration, which accounts for the theoretical concentration we would achieve with a perfect weighing of the pesticide and the following calculated dilutions; and the measured concentration by the HPLC. The coefficient of variation (CV) shows the standard deviation of the duplicates divided by their average, while the last calculation ilustrates how much of the expected concentration was achieved in the dilutions.

Expected Concentration (EC) (mg/L)	Measured Concentration (MC) (mg/L)	Coefficient of variation	MC/EC (%)
300.00	258.45	0.860653053	85.62765894
300.00	255.32		
75.00	71.20	1.464251511	93.96416182
75.00	69.74		
18.75	22.64	1.490439825	119.4683625
18.75	22.16		

Table 11: Dimethoate solubility test.

3.3.3 Dicamba

Standards

Only one set of standards was prepared in water:methanol 60:40 (v/v) [88]. The plotted graph showed a reliable regression line (Fig. 22.

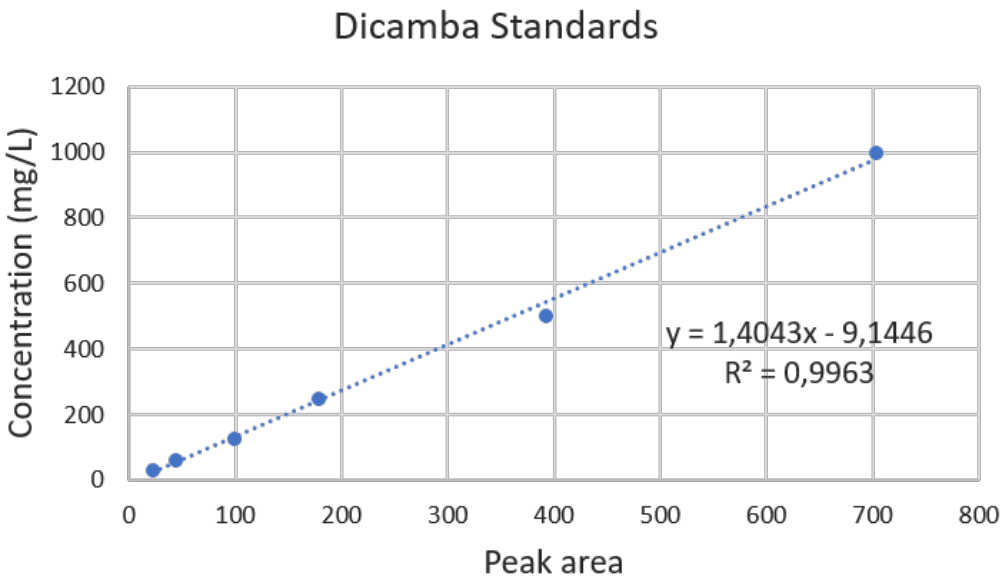

Figure 22: Regression line of dicamba standards in water:methanol 60:40 (v/v), with its regression line equation and R-square.

Solubility test and recovery factor

Three serial dilutions of dicamba in water were made, and five samples were taken from each of the dilutions, to check if a single sample is representative of the culture concentration, at different concentrations. Figure 12 lists the expected and measured concentration obtained, and summarize the CV of the replicates and the percentage of the expected concentration that the measured one represents.

Expected Concentration (EC) (mg/L)	Measured Concentration (MC) (mg/L)	CV (%)	MC/EC (%)
800	805.43	1.61036103	97.9191572
800	779.88		
800	779.75		
800	778.26		
800	773.44		
200	211.52	0.80137913	105.016968
200	212.05		
200	209.63		
200	208.72		
200	208.25		
50	49.75	0.40406599	99.1517129
50	49.83		
50	49.40		
50	49.47		
50	49.43		

Table 12: Dicamba solubility test.

To obtain the recovery factor, dicamba was dissolved in water at 666 mg/L, and each of the samples taken were mixed either with dicamba stock solution (1 g/L) in methanol or with pure methanol.

$$R_f = \frac{C_w + C_s}{C_{w+s}} = \frac{726.02mg/L + 666.66mg/L}{1198.52mg/L} = 1.16$$

3.3.4 Discussion

Only three of the five initial pesticides were chosen to be analysed in the HPLC. This was because of the complications derived from the low water solubility of bifenthrin, which make it difficult to take representative samples of the whole culture; and the lack of a pure standard of glyphosate, only the comercial product, with low purity, was obtained. The different tests performed aimed to ensure that the HPLC data given was representative of the pesticide concentration in the culture.

For that matter, the chosen protocol from the literature was first proven with standards, to check if there was a linear regression between the signal and the known concentration of the standards (Fig. 20, 21, and 22). Once that was achieved, with an R-square above 0.99 for the three pesticides, a change in the HPLC sample solvent was tested, for malathion and dimethoate, to a water:organic solvent mixture (Fig. 20, and 21), having checked that the mixture is compatible with the column, of weaker elution strength than the mobile phase, and compatible with the solubility requirements of the pesticide [101]. This was also achieved with R-squares above 0.99. Having water as part of the HPLC sample solvent would allow to mix directly the samples taken from the culture with the

organic solvent, and inject them into the column just after filtering them.

In pesticide degradation studies, the samples undergo generally an extraction protocol before being injected in the HPLC column, so the target compound is up-concentrated (in the cases where just trace concentrations are expected) [102]; and we get rid of compounds that could get stucked in the column and shorten its lifespan, or overlap with the target compound elution time, and thus interfere with its signal. In this study, however, the way above the detection limit pesticide concentration, the little bacterial growth seen, and the absence of similar compounds present (no pesticide mixture was used), made it possible to save both money and time in an additional extraction protocol.

The solubility tests solved two uncertainties: first, that a single measurement can be taken to find out the pesticide concentration in the culture, as the CV was below 2% in all cases measured; and second, that no solubility restraints are found within the concentrations tested, and thus the concentration tested is similar to the expected concentration. This last part was more accurate for dicamba, for which the highest deviation from the expected concentration was a 5% deviation, but for dimethoate it reached up to a 19% of deviation. For dimethoate, the starting sample (the most concentrated) concentration, is 15% below the expected one, and as the concentration decreases, it reaches a 19% concentration above the expected. This could be due to a wrong linear regression slope, which was only calculated once at the beginning of HPLC the experiments, to minimize solvents used, instead of every time a new HPLC set of samples was run.

In addition, the recovery factor was calculated to obtain a more reliable data of the real concentration in the culture. It did not affect the degradation percentages, because the same factor is applied to all the concentrations measured, but it confirmed our methodology to achieve the desired initial pesticide concentrations.

3.4 Degradation experiments

3.4.1 Isolates screening

Six isolates were selected from the malathion, dimethoate, and dicamba enrichments (two from each), and incubated for two weeks with the pesticide they were adapted to. *S. marcescens* and *P. putida* were also incubated with malathion and dimethoate.

From each triplicate made, two replicates were measure in the HPLC.

Malathion

The chromatograms, shown in Figure 23, showed a really low concentration in all the conditions from the experiment, and a different chromatogram than the ones seen before in the standards. Time 0 measurements were below the expected concentration (125 mg/L), and the duplicates measured were not similar to each other, so no degradation rates could be measured.

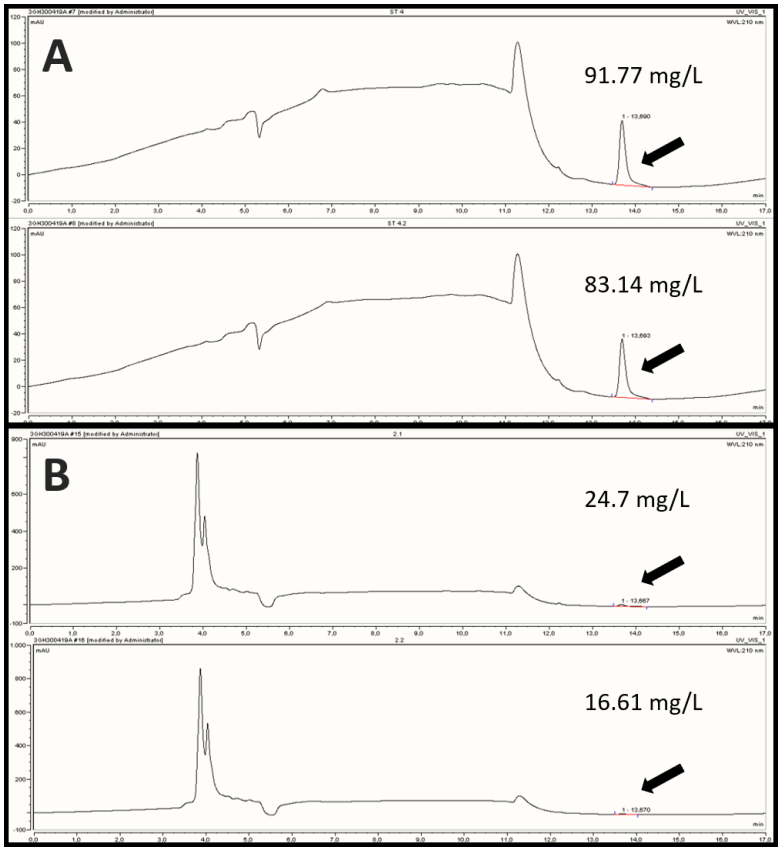

Figure 23: Malathion determination chromatograms, and the subsequent malathion concentration. **A:** Chromatogram and malathion concentration of two standard duplicates. **B:** Chromatogram and malathion concentration of the time 0 duplicates of one of the screened strains.

Dimethoate

As shown in Table 13, dimethoate is degraded faster in the controls than in the rest of the conditions. All the conditions, except from *S. marcescens* and the control, exhibit similar results in both replicates.

Dimethoate degradation (%)		1 week	2 weeks	Mean±SD
P. putida	Rep 1	21.11	25.90	26.59 ±0.98
	Rep 2	18.00	27.29	
S. marcescens	Rep 1	25.75	33.40	29.09 ±6.1
	Rep 2	16.25	24.78	
Slu Dim 22	Rep 1	18.54	27.85	27.96 ±0.15
	Rep 2	19.36	28.07	
HS Dim 21	Rep 1	19.32	28.69	28.59 ±0.14
	Rep 2	18.61	28.49	
Ctrl	Rep 1	38.51	46.31	39.42 ±9.74
	Rep 2	24.49	32.54	

Table 13: Dimethoate degradation along the isolate screening, with an initial pesticide concentration of 100 mg/L.

Dicamba

Table 14 shows an strain, HS Dic 22, which degrades 25% of dicamba in two weeks, while the degradation present in the control is of 16%. The duplicates present similar degradations both in the control and in the degrading strain, and the degradation present in the strain Slu Dic 11 is similar to the one in the control.

Dicamba degradation (%)		1 week	2 weeks	Mean±SD
Slu Dic 11	Rep 1	14.27	15.98	16.42 ±0.63
	Rep 2	9.37	16.87	
HS Dic 22	Rep 1	9.09	25.84	25.1 ±1.05
	Rep 2	11.69	24.36	
Ctrl	Rep 1	11.35	17.26	16.08 ±1.67
	Rep 2	6.31	14.90	

Table 14: Dicamba degradation along the isolate screening, with an initial pesticide concentration of 500 mg/L.

3.4.2 Influence of surfactants in biodegradation

From the malathion and dimethoate experiments, only dimethoate was measured. Fig. 24 show the degradation percentage at day two, five, and eight, of the experiment, substracting the degradation in the negative control. The data of the following days is not shown because the control starts to get degraded by day 11, and there are no positive degradations percentages after substracting the control degradation. At day 5 and day 8, dimethoate is degraded more efficiently in the flasks inoculated with *S. marcescens* and a surfactant, specially with the surfactant Tween80.

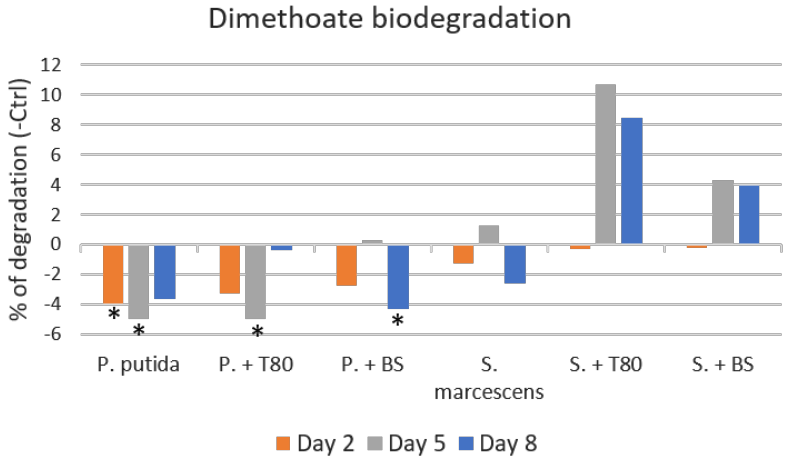

Figure 24: Dimethoate biodegradation along the experiment, with an initial concentration of 150 mg/L. BS accounts for the biosurfactant, while T80 for Tween 80. The columns marked with an asterisk do not represent the real value, which is probably an experimental or analytical error. The real data is listed in the appendix, table 16

3.4.3 Consortia design

A final experiment is performed to try to maximize the degradation of dicamba (Table 15), with a higher concentration of dicamba (750 (mg/L). The maximum dicamba degradation is found in the negative controls triplicates. The use of Tween 80 does not make a significant different at the concentration it was used (CMC= 15mg/L).

Dicamba degradation (%)		Triplicates			Mean	SD
Enrichment (1)	+ T80	7.86	8.99	7.29	8.05	0.87
	- T80	7.27	12.82	8.03	9.37	3.01
HS Dic 22 (2)	+ T80	7.01	8.25	6.32	7.19	0.98
	- T80	7.62	11.18	10.88	9.89	1.97
Consortia (3)	+ T80	9.87	8.28	10.64	9.60	1.20
	- T80	3.55	10.97	4.52	6.35	4.03
1 + 2	+ T80	7.54	2.90	2.34	4.26	2.85
	- T80	7.26	5.54	8.71	7.17	1.59
1 + 3	+ T80	7.30	6.49	9.91	7.90	1.78
	- T80	6.72	8.67	6.83	7.40	1.09
Ctrl	+ T80	13.70	16.20	12.35	14.08	1.95
	- T80	17.26	14.90		16.08	1.67

Table 15: Dicamba degradation along the experiment, with an initial concentration of 750 mg/L. Each of the conditions is tested with and without the surfactant Tween80

3.4.4 Discussion

The aim of the degradation experiments was to first obtain efficient pesticide degraders, and then optimize the degradation performed, building consortia with the most efficient degraders or supplying inducers such as surfactants. However, the screening yielded only one pesticide degrader, HS Dic 22, which was not able to degrade dicamba at higher concentrations; and another one, *S. marcescens* which could only degrade dimethoate with the aid of surfactants.

The screening of malathion degraders could not be satisfactory achieved, due an experimental error, which spoilt the malathion determination. More experiments should be performed to assess if this was an isolated error or an error in the methodology.

It should be taken into account that only 8 strains were screened in this study. Blaszak et. al., after a selective enrichment with the pesticide simazine, of simazine-resistant rich biotopes, isolated 12 bacteria, of which only one, identified as *Arthrobacter*, was able to degrade the pesticide [93]; and Shinde et. al. identified four efficient dimethoate and parathion degraders, out of the 96 bacterial and fungal strains isolated from soil in a mineral medium supplemented with the pestide [103]. As laid out in the introduction, the screening was complemented by a parallel project, which screened 12 more isolates from this project, and 16 *Klesbsiella* strains from a GF culture. No Klebsiella strain strain or consortia was found to perform a significant degradation, but, even though the study did not use replicates, and no time 0 was measured, two strains (HS Mal 22, and HS Dim 21) could be identified as potential efficient degraders, as they were able to grow (CFU counts) and degrade the three mentioned pesticides. These two strains, and the degrader found in this study, were sent to sequencing to be identified. If the strains are confirmed to be efficient pesticide degraders, by the sequencing data and subsequent degradation studies, the enrichment strategy performed would be proven to be an effective protocol for obtaining novel pesticide degraders for pesticide bioremediation studies.

P. putida and *S. marcescens* have been found to degrade several organophosphate pesticides. Nazarian et. al. used a *P. putida* strain isolated from contaminated soil to efficiently degrade dimethoate, 0.8 g/L, in a mineral solution [64]; and Cycon et. al. studied the ability of *S. marcescens* to degrade three organophosphorus pesticides, chlorpyrifos, fenitrothion, and parathion, 50 mg/L, in mineral media, and 58.9%, 70.5%, and 82.5% of the initial concentration was degraded after 14 days of incubation [78]. However in this study, the *P. putida* strain used was not able to degrade dimethoate, and *S. marcescens* was able to degrade it only in the presence of surfactants. The lack of replicates in the 'Influence of surfactants' experiment, and the several wrong data extracted make this result only semi-quantitative, an indication that more experiments should be performed in this direction, to assess the importance of surfactants in the pesticides degradation. In this experiment, the biosurfactant was less efficient than Tween 80 as a degradation enhancer, but it was extracted by colleagues from the yeast *Candida antarctica*, and a consortia featuring the tested *S. marcescens* strain, and *C. antarctica* or another biosurfactant producer, could be studied to efficiently degrade organophosporus pesticides.

Two different concentrations of dicamba were used along the enrichments, 750 mg/L, in the enrichment and in the consortia designing assay; and 500 mg/L in the screening, to be comparable

with the data obtained in a parallel ongoing screening. Both were within the range of concentrations used in degradation studies [71], [104], but in this study, 750 mg/L of dicamba seemed to be an inhibiting concentration for the strains used, which were already proven to degrade dicamba at 500 mg/L concentration. It could be discussed that a pre-activation in LB medium, prior to the incubation with the pesticide in a mineral medium, could be counterproductive for the bacterial medium, and that again we would only be selecting bacteria with a broad range of tolerance to carbon source concentration. For that matter, the pre-activated cultures were transfered to the pesticide-mineral medium without washing out the LB, so that we minimize the difference in the conditions, in the same way it was done in the screening.

Along the degradation experiments, a need to further optimize and validate the protocols used is evidenced in the fact that the negative controls show, in the dimethoate screening, a 25% CV; and in the dicamba consortia designing experiment, a two times higher degradation than the rest of the conditions. There is, therefore, a significant difference in the mean and standard deviation, between the negative controls and the conditions, and more tests should be done to understand its cause.

4 Conclusion and Future perspectives

After eleven weeks of enrichment, with 5 different pesticides as the sole carbon source, 42 potential pesticide degrading strains were obtained. O.D. measurements were proven not to be an effective method to monitor the growth in low carbon source mediums, and neither are BSA assays. The metagenomic study showed, that to optimize the enrichment protocol, to find the most efficient pesticide degraders, isolation and screening from different steps of the enrichment, or from higher carbon source enrichments, should be performed. All things considered, however, the enrichment strategy will be proven to be effective if the three potential degraders, sent to sequencing, are confirmed to be interesting for pesticide remediation.

HPLC protocols were developed and tested, for the determination of malathion, dimethoate, and dicamba. However, they should be further validated, until similar degradation results are obtained in the negative controls and in the non pesticide degrading strains.

The HPLC results suggest that biosurfactants could play a role in the degradation of dimethoate, and the screening should be repeated with the addition of biosurfactants and biosurfactant producing strains, to study the development of efficient dimethoate degrading consortia. Higher concentrations of dicamba were inhibiting the dicamba degrading strains found, and the experiments should be repeated at lower concentrations to confirm the degrading ability of the strains and study the development of the different consortia designing approaches.

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References

[1] Robin M. Whyatt, David E. Camann, Patrick L. Kinney, Andria Reyes, Judy Ramirez, Jessica Dietrich, Diurka Diaz, Darrell Holmes, and Frederica P. Perera. Residential pesticide use during pregnancy among a cohort of urban minority women. *Environmental Health Perspectives*, 2002.

[2] Ravindran Jayaraj, Pankajshan Megha, and Puthur Sreedev. Review Article. Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment, 2016.

[3] Isra Mahmood, Sameen Ruqia Imadi, Kanwal Shazadi, Alvina Gul, and Khalid Rehman Hakeem. Effects of pesticides on environment. In *Plant, Soil and Microbes: Volume 1: Implications in Crop Science*. 2016.

[4] George N. Agrios. *Plant Pathology*. Academic Press, 5th edition edition, 2005.

[5] J E Casida. Pyrethrum flowers and pyrethroid insecticides. *Environmental health perspectives*, 34:189–202, 2 1980.

[6] Ayoola SO. Toxicity of glyphosate herbicide on Nile tilapia (Oreochromis niloticus) juvenile. Technical Report 12, 2008.

[7] Christina M. Howe, Michael Berrill, Bruce D. Pauli, Caren C. Helbing, Kate Werry, and Nik Veldhoen. Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry*, 2004.

[8] Luis C. Rodriguez and Hermann M. Niemeyer. Integrated pest management, semiochemicals and microbial pest-control agents in Latin American agriculture, 2005.

[9] Lesa A. THOMPSON, Wageh Sobhy DARWISH, Yoshinori IKENAKA, Shouta M. M. NAKAYAMA, Hazuki MIZUKAWA, and Mayumi ISHIZUKA. Organochlorine pesticide contamination of foods in Africa: incidence and public health significance. *Journal of Veterinary Medical Science*, 2017.

[10] R. Lal, G. Pandey, P. Sharma, K. Kumari, S. Malhotra, R. Pandey, V. Raina, H.-P. E. Kohler, C. Holliger, C. Jackson, and J. G. Oakeshott. Biochemistry of Microbial Degradation of Hexachlorocyclohexane and Prospects for Bioremediation. *Microbiology and Molecular Biology Reviews*, 2010.

[11] Nathaniel L. Scholz, Erica Fleishman, Larry Brown, Inge Werner, Michael L. Johnson, Marjorie L. Brooks, Carys L. Mitchelmore, and Daniel Schlenk. A Perspective on Modern Pesticides, Pelagic Fish Declines, and Unknown Ecological Resilience in Highly Managed Ecosystems. *BioScience*, 2012.

[12] Alessio Ippolito, Mira Kattwinkel, Jes J. Rasmussen, Ralf B. Schäfer, Riccardo Fornaroli, and Matthias Liess. Modeling global distribution of agricultural insecticides in surface waters. *Environmental pollution (Barking, Essex : 1987)*, 2015.

[13] E. A. Kerle, J. J. Jenkins, and P.A. Vogue. Understanding pesticide persistence and mobility for groundwater and surface water protection. *Oregon State University*, 1994.

[14] D. I. Gustafson. Groundwater ubiquity score: A simple method for assessing pesticide leachability. *Environmental Toxicology and Chemistry*, 1989.

[15] C. Becerra-Castro, Á Prieto-Fernández, P. S. Kidd, N. Weyens, B. Rodríguez-Garrido, M. Touceda-González, M. J. Acea, and J. Vangronsveld. Improving performance of Cytisus striatus on substrates contaminated with hexachlorocyclohexane (HCH) isomers using bacterial inoculants: Developing a phytoremediation strategy. *Plant and Soil*, 2013.

[16] Kathleen E. Hall, Chittaranjan Ray, Seo Jin Ki, Kurt A. Spokas, and William C. Koskinen. Pesticide sorption and leaching potential on three Hawaiian soils. *Journal of Environmental Management*, 2015.

[17] Roger Atkinson, Rob Guicherit, Ronald A. Hites, Wolf-Ulrich Palm, James N. Seiber, and Pim De Voogt. Transformations of pesticides in the atmosphere. *Water, air, and soil pollution*, 115:219–243, 1999.

[18] R D Wauchope, T M Buttler, A G Hornsby, P W M Augustijn-Beckers, and J P Burt. The SCS/ARS/CES pesticide properties database for environmental decision-making. In *Reviews of environmental contamination and toxicology*, pages 1–155. Springer, 1992.

[19] P W M Augustijn-Beckers, A G Hornsby, and R D Wauchope. The SCS/ARS/CES pesticide properties database for environmental decision-making. II. Additional compounds. In *Reviews of environmental contamination and toxicology*, pages 1–82. Springer, 1994.

[20] Kathrin Fenner, Silvio Canonica, Lawrence P Wackett, and Martin Elsner. Evaluating Pesticide Degradation in the Environment: Blind Spots and Emerging Opportunities. Technical report.

[21] H.D. Burrows, M Canle L, J.A. Santaballa, and S Steenken. Reaction pathways and mechanisms of photodegradation of pesticides. *Journal of Photochemistry and Photobiology*, 67:71–108, 2002.

[22] Sebastian Huntscha, Heinz Singer, Silvio Canonica, René P. Schwarzenbach, and Kathrin Fenner. Input dynamics and fate in surface water of the herbicide metolachlor and of its highly mobile transformation product metolachlor ESA. *Environmental Science and Technology*, 2008.

[23] K A Barrett and MBs McBride. Oxidative degradation of glyphosate and aminomethylphosphonate by manganese oxide. *Environmental science & technology*, 39(23):9223–9228, 2005.

[24] Mervyn L De Souza, Jennifer Seffernick, Betsy Martinez, Michael J Sadowsky, and Lawrence P Wackett. The atrazine catabolism genes atzABC are widespread and highly conserved. *Journal of Bacteriology*, 180(7):1951–1954, 1998.

[25] Arnaud Dechesne, Nora Badawi, Jens Aamand, and Barth F. Smets. Fine scale spatial variability of microbial pesticide degradation in soil: Scales, controlling factors, and implications, 2014.

[26] Chittaranjan Ray, Tomas Vogel, and Jaromir Dusek. Modeling depth-variant and domain-specific sorption and biodegradation in dual-permeability media. *Journal of Contaminant Hydrology*, 2004.

[27] Christian L. Lauber, Micah Hamady, Rob Knight, and Noah Fierer. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology*, 2009.

[28] LM Reedich, MD Millican, and PL Koch. Temperature Impacts on Soil Microbial Communities and Potential Implications for the Biodegradation of Turfgrass Pesticides. *J Environ Qual*, 46(3):490–497, 2017.

[29] D. Or, B. F. Smets, J. M. Wraith, A. Dechesne, and S. P. Friedman. Physical constraints affecting bacterial habitats and activity in unsaturated porous media - a review. *Advances in Water Resources*, 2007.

[30] Marion Devers, Nadine Rouard, and Fabrice Martin-Laurent. Fitness drift of an atrazine-degrading population under atrazine selection pressure. *Environmental Microbiology*, 2008.

[31] Alistair B.A. Boxall, Chris J. Sinclair, Kathrin Fenner, Dana Kolpin, and Steve J Maund. When synthethic chemicals Degrade in the environment. *Environmental Science & Technology*, 38:368A–375A, 2004.

[32] Gianluigi Buttiglieri, Manuela Peschka, Tobias Frömel, Jutta Müller, Francesca Malpei, Peter Seel, and Thomas P. Knepper. Environmental occurrence and degradation of the herbicide n-chloridazon. *Water Research*, 2009.

[33] K. Rama Krishna and Ligy Philip. Biodegradation of mixed pesticides by mixed pesticide enriched cultures. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*, 2009.

[34] Nancy Pino, Maria Dominguez, and Gustavo Penuela. Isolation of a selected microbial consortium capable of degrading methyl parathion and p-nitrophenol from a contaminated soil site. *Journal of Environmental Science and Health, Part B*, 2011.

[35] Nancy Pino and Gustavo Peñuela. Simultaneous degradation of the pesticides methyl parathion and chlorpyrifos by an isolated bacterial consortium from a contaminated site. *International Biodeterioration and Biodegradation*, 2011.

[36] Hua FANG, Yunlong YU, Xiaoqiang CHU, Xiuguo WANG, Xiaoe YANG, and Jingquan YU. Degradation of chlorpyrifos in laboratory soil and its impact on soil microbial functional diversity. *Journal of Environmental Sciences*, 2009.

[37] Brajesh K. Singh, Allan Walker, J. Alun W. Morgan, and Denis J. Wright. Effects of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. *Applied and Environmental Microbiology*, 2003.

[38] T. A. Albanis, D. G. Hela, T. M. Sakellarides, and I. K. Konstantinou. Monitoring of pesticide residues and their metabolites in surface and underground waters of Imathia (N. Greece) by means of solid-phase extraction disks and gas chromatography. In *Journal of Chromatography A*, 1998.

[39] Ramesh C Gupta. Carbofuran toxicity. *Journal of Toxicology and Environmental Health*, 43(4):383–418, 12 1994.

[40] Michael T Madigan, John M Martinko, and Thomas D Brock. *Brock biology of microorganisms*. Pearson Prentice Hall, Upper Saddle River, NJ, 2006.

[41] Ryota Kataoka, Kazuhiro Takagi, and Futa Sakakibara. Biodegradation of endosulfan by Mortieralla sp. strain W8 in soil: Influence of different substrates on biodegradation. *Chemosphere*, 2011.

[42] Gi Seok Kwon, Ho Yong Sohn, Kee Sun Shin, Eungbin Kim, and Bu Il Seo. Biodegradation of the organochlorine insecticide, endosulfan, and the toxic metabolite, endosulfan sulfate, by Klebsiella oxytoca KE-8. *Applied Microbiology and Biotechnology*, 2005.

[43] Prabhu Thangadurai and Sumathi Suresh. Biodegradation of endosulfan by soil bacterial cultures. *International Biodeterioration and Biodegradation*, 2014.

[44] E. Morillo and J. Villaverde. Advanced technologies for the remediation of pesticide-contaminated soils, 2017.

[45] Chawla Niti, Suneja Sunita, Kukreja Kamlesh, and Kumar Rakesh. Bioremediation: An emerging technology for remediation of pesticides. Technical Report 4, 2013.

[46] Maria Nikolopoulou and Nikolas Kalogerakis. Biostimulation strategies for fresh and chronically polluted marine environments with petroleum hydrocarbons, 2009.

[47] Patcharaporn Pimmata, Alissara Reungsang, and Pensri Plangklang. Comparative bioremediation of carbofuran contaminated soil bynatural attenuation, bioaugmentation and biostimulation. *International Biodeterioration and Biodegradation*, 2013.

[48] M. Cycoń, A. Zmijowska, and Z. Piotrowska-Seget. Enhancement of deltamethrin degradation by soil bioaugmentation with two different strains of Serratia marcescens. *International Journal of Environmental Science and Technology*, 2014.

[49] Juliana M. Saez, Analía Álvarez, Claudia S. Benimeli, and María J. Amoroso. Enhanced lindane removal from soil slurry by immobilized Streptomyces consortium. *International Biodeterioration and Biodegradation*, 2014.

[50] Mariusz Cycoń, Agnieszka Mrozik, and Zofia Piotrowska-Seget. Bioaugmentation as a strategy for the remediation of pesticide-polluted soil: A review, 2017.

[51] Xiaoyan Yang, Huanyu Wei, Changxiong Zhu, and Bing Geng. Biodegradation of atrazine by the novel Citricoccus sp. strain TT3. *Ecotoxicology and Environmental Safety*, 2018.

[52] Pérez Mónica, Rueda O Darwin, Bangeppagari Manjunatha, Johana J Zúñiga, Ríos Diego, Rueda B Bryan, Sikandar I Mulla, and Naga R Maddela. Evaluation of various pesticides-degrading pure bacterial cultures isolated from pesticide-contaminated soils in Ecuador. *African Journal of Biotechnology*, 15(40):2224–2233, 2016.

[53] Murat Ozdal, Ozlem Gur Ozdal, Omer Faruk Algur, and Esabi Basaran Kurbanoglu. Biodegradation of α-endosulfan via hydrolysis pathway by Stenotrophomonas maltophilia OG2. *3 Biotech*, 2017.

[54] A. M. Cook, C. G. Daughton, and M. Alexander. Phosphorus-containing pesticide breakdown products: Quantitative utilization as phosphorus sources by bacteria. *Applied and Environmental Microbiology*, 1978.

[55] Xu Jing-Liang, Gu Xiang-Yang, Shen Biao, Wang Zhi-Chun, Wang Kun, and Li Shun-Peng. Isolation and characterization of a carbendazim-degrading Rhodococcus sp. djl-6. *Current Microbiology*, 2006.

[56] Cristiano Varrone, Georgis Floriotis, Tonje M B Heggeset, Simone B Le, Sidsel Markussen, Ioannis V Skiadas, and Hariklia N Gavala. Continuous fermentation and kinetic experiments for the conversion of crude glycerol derived from second-generation biodiesel into 1, 3 propanediol and butyric acid. *Biochemical engineering journal*, 128:149–161, 2017.

[57] Daniel Smith, Sam Alvey, and David E. Crowley. Cooperative catabolic pathways within an atrazine-degrading enrichment culture isolated from soil. *FEMS Microbiology Ecology*, 2005.

[58] Aleksej Zelezniak, Sergej Andrejev, Olga Ponomarova, Daniel R Mende, Peer Bork, and Kiran Raosaheb Patil. Metabolic dependencies drive species co-occurrence in diverse microbial communities. *Proceedings of the National Academy of Sciences*, 112(20):6449–6454, 2015.

[59] Cristiano Varrone, Ioannis V Skiadas, and Hariklia N Gavala. Effect of hydraulic retention time on the modelling and optimization of joint 1, 3 PDO and BuA production from 2G glycerol in a chemostat process. *Chemical Engineering Journal*, 347:525–534, 2018.

[60] Analia Alvarez, Juliana Maria Saez, José Sebastian Davila Costa, Veronica Leticia Colin, María Soledad Fuentes, Sergio Antonio Cuozzo, Claudia Susana Benimeli, Marta Alejandra Polti, and María Julia Amoroso. Actinobacteria: Current research and perspectives for bioremediation of pesticides and heavy metals, 2017.

[61] María S. Fuentes, Verónica L. Colin, María J. Amoroso, and Claudia S. Benimeli. Selection of an actinobacteria mixed culture for chlordane remediation. Pesticide effects on microbial morphology and bioemulsifier production. *Journal of Basic Microbiology*, 2016.

[62] K Bailey, S K KON, F DICKENS, J R P O'BRIEN, E J KING, C RIMINGTON, and BCJG KNIGHT A R TODD. Biochemical. *Engineering Fundamentals, McGraw-Hill, New York*, page 984, 1986.

[63] Greeshma Odukkathil and Namasivayam Vasudevan. Residues of endosulfan in surface and subsurface agricultural soil and its bioremediation. *Journal of Environmental Management*, 2016.

[64] Aboalfazl Nazarian. Utilization of dimethoate by wild type Pseudomonas putida from polluted sites in Iran. *Pakistan journal of biological sciences: PJBS*, 10(4):664–667, 2007.

[65] Yichen Huang, Lijuan Xiao, Feiyu Li, Mengshi Xiao, Derong Lin, Xiaomei Long, and Zhijun Wu. Microbial Degradation of Pesticide Residues and an Emphasis on the Degradation of Cypermethrin and 3-phenoxy Benzoic Acid: A Review. *Molecules (Basel, Switzerland)*, 23(9):2313, 9 2018.

[66] Abd Elaziz S A Ishag, Azhari O Abdelbagi, Ahmed M A Hammad, Elsiddig A E Elsheikh, Osama E Elsaid, J-H Hur, and Mark D Laing. Biodegradation of chlorpyrifos, malathion, and dimethoate by three strains of bacteria isolated from pesticide-polluted soils in Sudan. *Journal of agricultural and food chemistry*, 64(45):8491–8498, 2016.

[67] S R Geed, M K Kureel, A K Shukla, R S Singh, and B N Rai. Biodegradation of malathion and evaluation of kinetic parameters using three bacterial species. *Resource-Efficient Technologies*, 2:S3–S11, 2016.

[68] Baljinder Singh, Jagdeep Kaur, and Kashmir Singh. Biodegradation of malathion by Brevibacillus sp. strain KB2 and Bacillus cereus strain PU. *World Journal of Microbiology and Biotechnology*, 28(3):1133–1141, 2012.

[69] Gehan I Kh Marei, Youssef M M Mohammed, Entsar I Rabea, and Mohammed E I Badawy. Isolation, characterisation and efficacy of the bacterial strain Lysinibacillus sphaericus YMM in biodegradation of malathion insecticide in liquid media. *International Journal of Environmental Studies*, pages 1–18, 2018.

[70] Manisha Deb Mandal, Shyamapada Mandal, and Nishith Kumar Pal. Plasmid-mediated dimethoate degradation by Bacillus licheniformis isolated from a fresh water fish Labeo rohita. *BioMed Research International*, 2005(3):280–286, 2005.

[71] Li Yao, Xingjun Jia, Jiadong Zhao, Qin Cao, Xiangting Xie, Linlu Yu, Jian He, and Qing Tao. Degradation of the herbicide dicamba by two sphingomonads via different O-demethylation mechanisms. *International Biodeterioration & Biodegradation*, 104:324–332, 2015.

[72] James P Krueger, Robert G Butz, and Douglas J Cork. Use of dicamba-degrading microorganisms to protect dicamba susceptible plant species. *Journal of agricultural and food chemistry*, 39(5):1000–1003, 1991.

[73] Qun Zhang, Shuhuai Li, Chen Ma, Nancun Wu, Chunli Li, and Xinfeng Yang. Simultaneous biodegradation of bifenthrin and chlorpyrifos by Pseudomonas sp. CB2. *Journal of Environmental Science and Health, Part B*, 53(5):304–312, 2018.

[74] Mukhtar Ahmed, Farkhanda Jabeen, Muhammad Ali, Zarnab Ahmad, Fayyaz Ahmed, Muhammad Bilal Sarwar, Salah ud Din, Mubbashir Hassan, and Shah Jahan. Analysis of

Bifenthrin Degrading Bacteria from Rhizosphere of Plants Growing at Tannery Solid Waste. *American Journal of Plant Sciences*, 6(13):2042, 2015.

[75] Preeti Pandey, Geetika Pant, and G Sibi. Isolation and characterization of bifenthrin catabolizing bacterial strain Bacillus cibi from soil for pyrethroids biodegradation. *Online J. Biol. Sci*, 14:188, 2014.

[76] B J Malviya, V J Jadeja, H M Sherathiya, M V Parakhia, R S Tomar, M B Vaja, and D N Sherathia. Bioremediation of glyphosate by bacteria isolated from glyphosate contaminated soil. *Journal of Pure and Applied Microbiology*, 9(4):3315–3320, 2015.

[77] Ouided Benslama and Abderrahmane Boulahrouf. Isolation and characterization of glyphosate-degrading bacteria from different soils of Algeria. *African Journal of Microbiology Research*, 7(49):5587–5595, 2013.

[78] Mariusz Cycoń, Agnieszka Zmijowska, Marcin Wójcik, and Zofia Piotrowska-Seget. Biodegradation and bioremediation potential of diazinon-degrading Serratia marcescens to remove other organophosphorus pesticides from soils. *Journal of Environmental Management*, 2013.

[79] Anthony M Bolger, Marc Lohse, and Bjoern Usadel. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15):2114–2120, 4 2014.

[80] Tanja Magoč and Steven L Salzberg. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27(21):2957–2963, 2011.

[81] Robert C Edgar. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods*, 10(10):996, 2013.

[82] J Gregory Caporaso, Justin Kuczynski, Jesse Stombaugh, Kyle Bittinger, Frederic D Bushman, Elizabeth K Costello, Noah Fierer, Antonio Gonzalez Pena, Julia K Goodrich, and Jeffrey I Gordon. QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, 7(5):335, 2010.

[83] Christian Quast, Elmar Pruesse, Pelin Yilmaz, Jan Gerken, Timmy Schweer, Pablo Yarza, Jörg Peplies, and Frank Oliver Glöckner. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1):D590–D596, 2012.

[84] Kasper S Skytte Andersen, Rasmus H Kirkegaard, Soeren M Karst, and Mads Albertsen. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. *BioRxiv*, page 299537, 2018.

[85] D J Lane. 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics*, pages 115–175, 1991.

[86] Aqel W Abu-Qare and Mohamed B Abou-Donia. Simultaneous determination of malathion, permethrin, DEET (N, N-diethyl-m-toluamide), and their metabolites in rat plasma and urine

using high performance liquid chromatography. *Journal of pharmaceutical and biomedical analysis*, 26(2):291–299, 2001.

[87] P C Kumar and B M Gurupadayya. Analytical method development and validation of dimethoate pesticide using HPLC method. *Biochemistry & Analytical Biochemistry Journal*, 2:1009–2161, 2013.

[88] Thomas W Moy and William C Brumley. Multiresidue determination of acidic pesticides in water by HPLC-DAD with confirmation by GC-MS using conversion to the methyl ester with trimethylsilyldiazomethane. *Journal of chromatographic science*, 41(7):343–349, 2003.

[89] Brian N Meyer, Chung Lam, Sean Moore, and Russell L Jones. Laboratory degradation rates of 11 pyrethroids under aerobic and anaerobic conditions. *Journal of agricultural and food chemistry*, 61(20):4702–4708, 2013.

[90] Mark F Zaranyika and Justin Mlilo. Speciation and persistence of dimethoate in the aquatic environment: characterization in terms of a rate model that takes into account hydrolysis, photolysis, microbial degradation and adsorption of the pesticide by colloidal and sediment particles. *South African Journal of Chemistry*, 67(1):233–240, 2014.

[91] Joanna Nishimura, Kate Gazzo, and Robert Budd. Environmental Fate and Toxicology of Dicamba.

[92] Changpeng Zhang, Xiuqing Hu, Jinyan Luo, Zhiyi Wu, Li Wang, Bin Li, Yanli Wang, and Guochang Sun. Degradation dynamics of glyphosate in different types of citrus orchard soils in China. *Molecules*, 20(1):1161–1175, 2015.

[93] Magdalena Blaszak, Robert Pełech, and Paulina Graczyk. Screening of Microorganisms for Biodegradation of Simazine Pollution (Obsolete Pesticide Azotop 50 WP). *Water, air, and soil pollution*, 220(1-4):373–385, 9 2011.

[94] Tara D. Sutherland, Irene Horne, Michael J. Lacey, Rebecca L. Harcourt, Robyn J. Russell, and John G. Oakeshott. Enrichment of an endosulfan-degrading mixed bacterial culture. *Applied and Environmental Microbiology*, 66(7):2822–2828, 7 2000.

[95] Aharon Oren and Xue-Wei Xu. The Family Hyphomicrobiaceae. In Eugene Rosenberg, Edward F DeLong, Stephen Lory, Erko Stackebrandt, and Fabiano Thompson, editors, *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*, pages 247–281. Springer Berlin Heidelberg, Berlin, Heidelberg, 2014.

[96] Sophie-Marie Martirani-Von Abercron, Patricia Marín, Marta Solsona-Ferraz, Mayra-Alejandra Castañeda-Cataña, and Silvia Marqués. Naphthalene biodegradation under oxygen-limiting conditions: community dynamics and the relevance of biofilm-forming capacity. *Microbial biotechnology*, 10(6):1781–1796, 8 2017.

[97] Razia Alam Gilani, Mazhar Rafique, Abdul Rehman, Muhammad Farooq Hussain Munis, Shafiq ur Rehman, and Hassan Javed Chaudhary. Biodegradation of chlorpyrifos by bacterial genus Pseudomonas. *Journal of Basic Microbiology*, 56(2):105–119, 2 2016.

[98] K Jesitha, K M Nimisha, C M Manjusha, and P S Harikumar. Biodegradation of Endosulfan by Pseudomonas fluorescens. *Environmental Processes*, 2(1):225–240, 2015.

[99] Anastasiah Ngigi, Zachary Getenga, Hamadi Boga, and Paul Ndalut. Biodegradation of phenylurea herbicide diuron by microorganisms from long-term-treated sugarcane-cultivated soils in Kenya. *Toxicological & Environmental Chemistry*, 93(8):1623–1635, 2011.

[100] A. Azizi and A. Homayouni. Bacterial-degradation of pesticides residue in vegetables during fermentation. *Asian Journal of Chemistry*, 21(8):6255–6264, 10 2009.

[101] FDA. Pesticide Analitical Manual Vol. 1. In *Pesticide Analitical Manual Vol. 1*, chapter 6, pages 601–15. 1994.

[102] T Koal, A Asperger, J Efer, and W Engewald. Simultaneous determination of a wide spectrum of pesticides in water by means of fast on-line SPE-HPLC-MS-MS—a novel approach. *Chromatographia*, 57(1):S93–S101, 2003.

[103] S R Shinde, M V Bhailume, N B Patil, N N Patil, and V S Hamde. Screening, characterization and identification of soil isolates for degradation of organophosphorus group of pesticides (Dimethoate and Parathion). *Int. J. Curr. Microbiol. App. Sci*, 2:240–244, 2015.

[104] Andrew M Fogarty and Olli H Tuovinen. Microbiological degradation of the herbicide dicamba. *Journal of industrial microbiology*, 14(5):365–370, 1995.

6 Appendix

Dimethoate Deg. (%)	Day 2	Day 5	Day 8	Day 11	Day 14
S. marcescens	2.69	6.23	1.68	16.99	36.40
S. marcescens+ BS	3.71	9.27	8.24	28.53	41.33
S.marcescens + T80	3.65	15.70	12.77	41.38	47.29
P. putida	-40.04	-31.10	0.64	0.64	12.42
P. putida+ T80	1.18	5.30	-0.25	22.49	42.19
P. putida + Sur	0.65	75.86	3.91	28.73	45.94
Ctrl	3.93	4.97	4.30	27.67	49.21

Table 16: Complete values of the surfactant experiment. The values edited in the figure 24 are marked in red