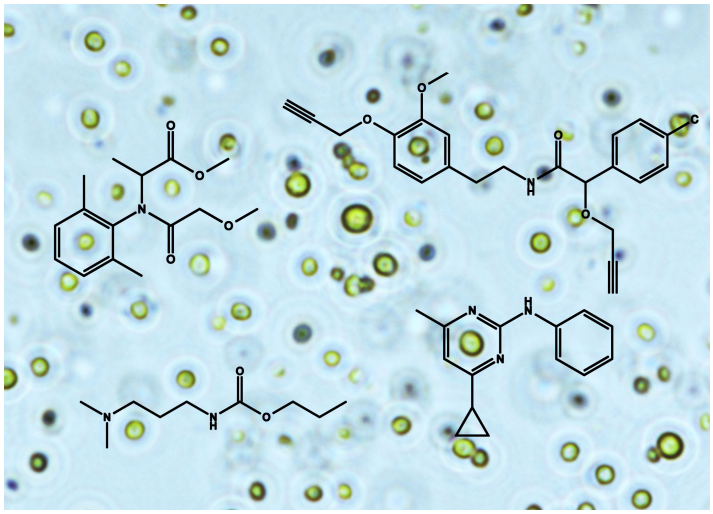
Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Architecture, Horticulture and Crop Production Science

Phycoremediation of pesticides using microalgae

Möjligheter med odling av mikroalger för rening av bekämpningsmedelsrester i vatten

Embla Ardal



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ABSTRACT

Every year, pesticides are found in surface and ground waters in Sweden. Fungicides are in common usage and applied in high amounts against potato late blight. The present thesis examined the possible removal of four fungicides (metalaxyl, cyprodinil, propamocarb and mandipropamid) from water using the microalgae *Chlorella vulgaris*. Microorganisms are capable of decomposing a range of organic pollutants and the main focus in previously published studies has been on bacteria and fungi. Microalgae are mostly studied due to their high capacity in biosorbing heavy metals. Removal of organic pollutants such as pesticides has been reported, however in fewer studies. The work was divided into two main experiments; one short-term experiment (60 min) using dead and living cells, and one long-term experiment (4 days) using growing cells. In the long-term experiment, the presence of growing algae resulted in around 50% lower propamocarb concentration in the medium compared with final concentration in the control without algae. In the short-term study, the presence of live algae cells led to a 30% reduction of propamocarb, compared to the concentration in the control. The main mechanism behind the reduction of propamocarb in the water phase is proposed to be biosorption onto the algal cells. This conclusion is based on the short duration required for removal to occur. For the other three studied fungicides no removal from the water phase by the algal treatments was observed.

SAMMANFATTNING

Varje år detekteras pesticidrester i yt- och grundvattnet i Sverige. Fungicider används i stor skala mot potatisbladmögel. Mikroorganismer är kapabla att bryta ner en rad olika organiska föroreningar och fokus på den här typen av studier har oftast legat på bakterier och svampar. När liknande studier gjorts på mikroalger har fokus legat på deras goda förmåga att biosorbera tungmetaller. Dock har rening av vatten från organiska föroreningar såsom pesticider rapporterats men dessa har varit få. Detta uppsatsarbete undersökte därför möjligheterna att minska mängden av fyra fungicider (metalaxyl, cyprodinil, propamocarb and mandipropamid) lösta i vatten med hjälp av mikroalgen *Chlorella vulgaris*. Uppsatsarbetet var uppdelat i två delar; ett korttids-experiment (60 min) med biomassa från levande och döda mikroalger, och ett långtids-experiment (4 dagar) med levande alger. I långtids-experimentet sjönk koncentrationen av propamocarb i lösningen med ca 50% i närvaro av levande alger jämfört med kontrollen där inga alger fanns närvarande. I korttids-experimentet minskade koncentrationen av propamocarb med ungefär 30% i närvaron av levande alger jämfört med kontrollen utan alger. Huvudorsaken till minskningen av propamocarbs beror troligtvis på biosorption som sker på algernas cellväggar. Denna slutsats har dragits baserat på den korta tidsperiod för under vilken minskningen av propamocarb sker. Ingen av de övriga tre fungiciderna minskade i koncentration på grund av mikroalgens närvaro.

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1. INTRODUCTION

A non-toxic environment is one of the ambitious environmental quality objectives stated in 1999 and again in 2005 by the Swedish government. The objective requires that presence of substances in the environment, created of or extracted by humans, shall not be a threat to human health or to the biodiversity and the levels of these substances should be close to zero (Naturvårdsverket, Miljömål 2014). One important step to reach this goal is to restrict use of substances detrimental to the wider environment (REACH, 2014). Parallel with this work remediation systems are also needed to reduce environmental contamination.

Residues of organic compounds, such as pesticides and drug residues represent a serious environmental problem. Heavy metals also pose a serious problem, though considerable effort has been made to develop methods to remove them from wastewaters. Chemical precipitation and adsorption methods for heavy metal wastewater treatment have several disadvantages, notably high costs and inefficiency when metals occur at low concentrations (Mehta & Gaur, 2005; Monteiro et al., 2012). It should also be pointed out that wastewater treatment plants are not designed for uptake of complex organic compounds (Daneshvar et al., 2010) instead they have been designed to reduce the amount of easily degraded organic carbon, nitrogen and phosphorus. Many complex organic pollutants leave wastewater treatment plants relatively untreated and may leak out into the surrounding environment. Methods available today and suggested for the removal of complex organic compounds in wastewaters include activated carbon and oxidation using ozone, UV-radiation and hydrogen peroxide, and membrane filtration (Naturvårdsverket, 2008; Zaini et al., 2010). The suggested methods are relatively energy demanding and expensive, and would result in an increase of the average local water tariff by 10-40% (MistraPharma, 2011).

There is a need for sustainable remediation methods with reasonable cost that can remove contaminants such as heavy metals and organic compounds effectively even when they are found in low concentrations. Biological cleaning methods, or so-called “bioremediation” may provide cost-efficient alternatives to conventional treatment processes (Monteiro et al., 2012). Bioremediation is the collective name for cleaning processes utilising biological material and commonly used are microorganisms such as bacteria, fungi and algae. The microorganisms can take up heavy metals or organic compounds and the uptake can either be an active or a passive process.

The active uptake process is termed “bioaccumulation” (Kaduková & Virčíková, 2005). It is driven by energy and therefore requires living organisms capable of taking up compounds across the cell membrane for accumulation and/or metabolization, dependent on the type of compound present (Velásquez & Dussan, 2009). Many microorganisms are able to metabolize organic compounds, using the carbon as an energy source (Grigg et al., 1997; Massoud et al., 2008).

Heavy metals are naturally not biodegradable, although they can be accumulated in living organisms (Priyadarshani et al., 2011).

The passive uptake process is termed "biosorption" which is a physico-chemical mechanism that happens immediately at chemical structures on the cell wall and is a property of both living and dead organisms (and their components). Most biosorption studies have been carried out on microbial systems such as fungi, bacteria and microalgae, however other biological material can be used for biosorption (Mehta & Gaur, 2005; Gadd, 2009). Algae are considered to be very effective organisms in biosorption of heavy metals (Priyadarshani, 2011; Monteiro et al., 2012: Mehta & Gaur, 2005). Studies also show that microalgae furthermore are able to biosorb and also metabolize organic compounds including pesticides (Warshawsky et al., 1990; Cerniglia, 1993; Tsang et al., 1999; Tam et al., 2002; Akzy & Tezer, 2005; Ghasemi et al., 2011; Priyadarshani et al., 2011).

The focus of the present thesis is water remediation of pesticides using microalgae. Results from tests made in Swedish agricultural areas show that residues of pesticides are common in surface water (Lindström et al., 2013; Regionala pesticiddatabasen, 2014). The situation for pesticides is that they often never pass through any wastewater treatment and instead leak directly out to the surrounding environment through agricultural run-offs and percolation through the soil profile. Additionally pesticides may also be introduced to the aquatic environment via industrial waste disposal (Tikoo et al., 1996) and domestic sewage (Priyadarshani et al., 2011). Pesticide residues often reach ground and surface waters where they can be a threat to living organisms. As fungicides are in extensive use in Sweden and since their environmental effects have generally received less attention than insecticides and herbicides (Bengtsson, 2011; Wightwick et al., 2012) this thesis focuses on four fungicides: metalaxyl, cyprodinil, propamocarb and mandipropamid all of which are often found in Swedish surface water (Lindström et al., 2013).

1.1 Objectives

This study examines the possibilities for utilisation of the microalgae *Chlorella vulgaris* to simultaneously remove a number of fungicides in concentrations representative of reported residue values in monitoring reports (Lindström et al., 2013). This report evaluates the levels of reduction of each one of the pesticides, using biomass of both living and dead cells in a "short-term" experiment and growing cells in a "long-term" experiment. The main questions for this study are:

- Does concentration of the selected fungicides in water decrease after 60 min exposure to biomass of *C. vulgaris* as compared to no microalgae exposure?

- Under these conditions, are there any differences in removal between dead and living algal cells?

- Will the concentration of the fungicides in water decrease after 4 days exposure to growing cells of *C. vulgaris* as compared to no microalgae exposure?

- Which one of the two processes, biosorption or biodegradation, is most efficient in removal of the selected fungicides and thereby best suited for remediation processes?

1.2 Scope

The practical work of this study is restricted to one microalgae species, *Chlorella vulgaris*. The experimental set-up of this study allowed simultaneous detection of 37 pesticides that were treated with *C. vulgaris*. This report focuses on four prevalent synthetic organic fungicides: metalaxyl, cyprodinil, propamocarb and mandipropamid.

2. BACKGROUND

2.1 Pesticides residues in water

In Sweden, environmental monitoring of pesticides started in 2002 with the Swedish Environmental Protection Agency (Naturvårdsverket) as financier. The studies are part of the national monitoring program and examine the impact of agriculture on the quality of surface water, groundwater and sediment as well the presence of pesticides in air and in precipitation. Samples are taken every year in streams in four watersheds (800-1700 ha) in the four major agricultural regions in Sweden; Scania, Halland, Östergötland and Västergötland. Furthermore, the monitoring also includes two of the larger streams in Scania. The purpose is to examine the levels of different pesticides leaking out into the surrounding water and monitor the long-term change over time. Analyses are carried out in the accredited laboratory of Organic Chemistry at the Department of Water and Environmental Sciences at the Swedish University of Agricultural Sciences (SLU) (CKB, 2014). In 2012, 87 pesticides were found in surface waters with the greatest number of substances being found in Scania and Halland with presence corresponding to areas with the highest use of pesticides in Sweden. Herbicides are the most used pesticide group in Sweden followed by fungicides and insecticides. In 2012, 2111 tonnes herbicides were used in Sweden. Fungicides are also in large usage with 216 tonnes used while the use of insecticides is much lower; 35.1 tonnes (KemI, 2014).

The high amount and high concentration can perhaps explain why herbicides also are the group of pesticides most frequently found in surface waters. The four most commonly encountered substances in surface water samples in Sweden 2012 from the report by Lindström et al., (2013), were benzatone (100%), glyfostat (90%), isoproturon (75%) and MCPA (75%). The percentage shown is approximate and based on how many samples with the specific substance were detected from all of the samples taken. Fungicides were the second most common group of pesticides found in surface water samples, which correlated with the large-scale usage of these compounds. Fungicides that were most often found in surface waters samples were azoxystrobin (50%), metalaxyl (40%) and pikoxystrobin (40%) (Lindström et al., 2013).

2.1.1 Environmental effects of fungicide residues

Fungicides are applied in agriculture, horticulture, forestry and industry to protect produce and property from parasitic fungi (Larsson, 1990). Potato late blight, caused by *Phytophthora infestans* from the class *oomycetes,* is a serious pest in large parts of the world, including Sweden. The disease affects the transport of water and nutrients within the plant, which results in wilting and lower yields. Today in Sweden, potato late blight is mostly controlled with fungicides and accounts for around half of the fungicide usage, even though only 1% of the agricultural areas are used for potato growing (Bengtsson, 2011; Statistiska meddelanden, 2013; Furenged & Gertsson, 2014).

Fungicide residues in the environment can harm non-target organisms such as beneficial fungi e.g. mycorrhizal fungi and can cause significant damage to soil fauna (Larsson, 1990). One common side effect of residues of fungicides in the soil is that parts of the fungal biomass are impaired which may affect the ecosystem and the species composition in the soil. For example, when the proportion of fungi decreases, the proportion of bacteria will increase and thus take the empty place of the excluded fungi. Some fungicides are mobile in the soil while others have limited mobility. Some may linger in the soil for more than a year whilst others may degrade within months (Larsson, 1990). The environmental effects and fate of fungicides in wetlands (Friesen-Pankratz et al., 2003) and in surface waters is today largely unknown.

Fungicides are not a homogenous chemical group and therefore they cannot be expected to behave in the environment in a similar way. Their adsorption and transport in the environment depends on their physico-chemical properties that are affected by their overall chemical properties and the processes occurring in relation with the environment. These processes are sorption (adsorption and absorption), desorption, chemical and biological degradation, volatilisation, leaching and uptake by plants (Komárek et al., 2010).

One of the most common reductions of fungicides in soil is a result of microbial degradation (Larsson, 1990). Biodegradation is affected by two groups of factors; the chemical properties and the microbial consortium. Chemical properties of the pesticide such as molecular weight, functional groups and toxicity affect the metabolic degradation of a fungicide (Priyadarshani et al., 2011). Algae appear to be more able to metabolize organic compounds with low molecular weights than larger molecules (Semple et al., 1999; Juhasz & Revendra 2000; Ghasemi et al., 2011).

Another very important process that influences the fate of pesticides is sorption to organic matter. Water solubility, lipophilicity, volatility, molecular size, molecular structure and ionisation ability influences the sorption capacity of a specific fungicide. The octanol-water coefficient (K_{ow}), the distribution coefficients (K_d) and soil sorption coefficient (K_{oc}) are parameters that describe the distribution of a specific substance between water and organic materials and thereby also the substance tendency to be sorbed. The K_{ow} describes the distribution of a compound between water and a lipophilic phase; the higher the K_{ow} the more likely the fungicide will bind to organic matter in soils, sediments and living tissues. The K_{ow} also correlates with the bioconcentration factor (BCF) in living organisms. This means that compounds with a high K_{ow} are more prone to bioaccumulate in fat tissues of living organisms. Log K_{ow} values that are higher than 7 - 8 indicate strong adsorption, which makes these chemicals almost immobile in the environment (Casserly et al., 1983; see review: Komárek et al., 2010).

The distribution coefficient K_d (l kg^{-1}) describes the distribution of a compound between the liquid and the solid phase. A fungicide with a high K_d indicates stronger adsorption to the soil matrix. The soil sorption coefficients K_{oc} describe the K_d but for a specific soil. Pesticides with higher K_d should have a lower risk for water contamination but there are evidence for pesticides present in water with a K_d higher than 1000 (Komárek et al., 2010).

The pH value in the environment has an important influence on the biosorption. One example are the correlations between the sorption of metal ions to soil (and algae) and low pH (2-3) (see section 2.3.1). However, all metals have a specific pH optimum for being biosorbed and some metals are favoured by high pH (Mehta & Gaur, 2005; Aksu & Dönmez, 2006). The pH value can also influence sorption of pesticides to soil. Gondara et al. (2013) have shown a correlation between a decreasing pH and the increase in sorption of certain hydrophilic pesticides on the soil organic matter. This response is related to the effect of pH on the ionisation of soil carboxyl groups that become more hydrophilic favouring binding of water molecules.

The vapour pressure describes how volatile a substance is and pesticides with a high vapour pressure have an increased risk for spray drift. Substances with high water solubility are easily transported via percolating water through the soil profile and can easily end up in water bodies

(Norberg, 2004). The persistence of a pesticide in the aqueous environment depends greatly on how easily it is degraded via hydrolysis and photolysis (PPDB, 2014).

2.2 Algae

Algae represent a large group of photoautotrophic organisms that include around 30 000 species, ranging from unicellular (microalgae) to more complex multicellular organisms (macroalgae). Previously, cyanobacteria were included under the title microalgae, and some authors are still including this photosynthesising group of prokaryotic organisms under this category (Priyadarshani et al., 2011). Here when describing microalgae, reference is made only to eukaryotic microorganisms.

Algae are photosynthetic organisms and are able to transform light energy into chemical energy in a similar way to higher plants. However, they grow comparatively faster, especially microalgae, which results from the fixation of CO_2 being 10-50 times faster than in plants (Subashchandrabose et al., 2013). When compared to plants microalgae have a more simple cell structure and they are also often surrounded by fluid allowing easier uptake of water and nutrients (Chacoón-Lee & González-Mariño, 2010).

Microalgae (and cyanobacteria) are highly adaptive and many species can furthermore grow heterotrophically and mixotrophically. Environmental conditions such as low light and/or nutrient deficiency have been reported to encourage mixotrophy (Stoecker et al., 2006; Subashchandrabose et al., 2013). Mixotrophy is defined as the ability to use light and organic carbon simultaneously as energy sources. This provides microalgae with substantial competitive advantages over fungi and bacteria in degrading organic pollutants (Subashchandrabose et al., 2013).

Algae are taxonomically divided based on their pigments, storage compounds and the main compounds present in their cell wall. The major classes are Clorophyta (green algae), Rhodophyta (red algae), Phaeophyta (brown algae), Euglenophyta, Pyrrophyta and Chrysophyta. *Chlorella vulgaris*, used in the present study, belongs to Clorophyta, which includes microalgae and multicellular algae. They are similar to terrestrial plants in that they possess chlorophyll a and b and some carotenoids, their cell wall is made of cellulose and their main storage products are starch (Harwood & Guschina, 2008).

In the late 1950s, the Western world become interested in microalgae cultivation due to its high protein content, which could provide a potential food source to support rapid population growth (Chacoón-Lee & González-Mariño, 2010). Other application areas for microalgae that have since been investigated are agricultural feed, fuel and pharmaceuticals (Olaizola, 2003). Also the use

of microalgae for remediation of nutrients (N and P) from wastewater is a well-established technology. The reduction of nutrients in wastewater may be due to the uptake and assimilation, which occur as the algae grow (Larsdotter, 2006). Additionally, nutrients may also be reduced by processes such as precipitation (phosphorus) and volatilisation (ammonia) due to high pH induced by the algae (Hammouda et al., 1994). In fact, the mentioned processes have been demonstrated when nutrient rich water sources such as residual water from greenhouse production were treated with microalgae (Ardal, 2012; Hultberg et al., 2013).

2.3 Use of microalgae for remediation

As mentioned earlier, the physiochemical methods for removing metals from wastewaters are expensive and also ineffective when metals occurs in low concentrations (Mehta & Gaur, 2005; Priyadarshani et al., 2011; Monteiro et al., 2012) and biological methods are therefore of interest. Microalgae have certain advantages for biological cleaning processes in water. They are efficient in the uptake of excessive nitrogen and phosphorous from wastewater (including farm and greenhouse run-offs) due to their fast growth rate and have good potential for removal of atmospheric deposition such as CO_2 from coal fired power plants (Zeiler et al., 1995; Priyadarshani, 2011).

Microalgae have been used in many studies on remediation of pollutants, both heavy metals and organic chemicals, and are primarily in focus due to their efficacy in scavenging metals from effluent wastewater by biosorption. However as mentioned previously, algae can take up and remove organic pollutants both via both biosorption and/or metabolization (Priyadarshani et al., 2011). Also interactions between algae and bacteria affect the biodegradation of pesticides (Ghasemi et al., 2011).

2.3.1 Algal biosorption of heavy metals and organic compounds including pesticides

Biosorption involves a number of physico-chemical metabolic-independent processes that include absorption, adsorption, surface complexation, ion exchange, and precipitation. Biosorption happens instantly and consists of passive processes that take place in the cell wall of both living and dead cells (and their excreted and derived products). Most biosorption studies have been carried out on microbial systems such as fungi, bacteria and microalgae, however other biological material may be used for biosorption such as plant biomass (bark, leaves, sawdust), animal biomass (crustaceans, hair), seaweeds (macroalgae), waste organic sludge, and several kinds of waste material and bio-products. Most biosorption research has focused on toxic metals, e.g. mercury, lead, cadmium, copper, arsenic, chromium as well as radionuclides such as uranium, cobalt, strontium, thorium, etc. However, the term “biosorption” is now additionally

applied on particulates and all kinds of organic substances (Gadd, 2009). Algae are considered to be very efficient for biosorption of heavy metals found in water (Monteiro et al., 2012).

Algal cell walls are surrounded by a three-dimensional network of macromolecules (polysaccharides and proteins). These carry negatively charged functional groups such as carboxyl, hydroxyl, phosphate or amine groups. Since metal ions in water solution generally are in cationic form, they are adsorbed to the algal cell walls. The algal metal biosorption is pH dependent and in general, acidic conditions of pH 3–5 are most favourable for the sorption of metal ions (Mehta & Gaur, 2005; Aksu & Dönmez, 2006).

Using dead biomass for biosorption has some notable advantages over using live cells and indeed some studies have shown that heavy metal biosorption was several times greater in dead cells as compared to live (Tsang et al., 1999; Tam et al., 2002; Mehta & Gaur, 2005). Additionally, there is obviously no risk for damaging the cells allowing them to be exposed to pollutants with high toxicity and they do not require addition of further nutrients. Dead biomass has also the ability to be recycled (Aksu & Dönmez, 2006). Metal ions bound to the algal cell wall may be removed by washing the biomass after each cycle with deionised water and desorption agents (HCL, NaOH, $CaCl_2$) (Chen et al., 2012). However, it must be noted that metabolic processes in living cells may contribute to the overall removal process (Gadd, 2009).

Some examples on studies on biosorption of heavy metals using green microscopic algae are: removal of Cu using *Tetraselmis chuii, Scenedesmus obliquus, Chlorella pyrenoidosa* and *Closterium lunula*; Ni using *Spirogyra* sp.; Se using *Spirogyra* sp.; Cd, Hg, Pb, As and Co using *Spirogyra hyaline*; Cu, Co, Pb and Zn using *Scenedesmus bijuga*; Cd, Zn and Cr using *Scenedesmus acutus Chlorella vulgaris*; Cd, Pb and Hg using *Chlorella vulgaris, Chlorococcum* sp., *Scenedesmus acutus*; Cr (VI) using *Chlorella minutissima*; Zn, Cu, As, Pb, Cd, Cr, Ni, Hg using *Chlorella pyrenoidosa* etc. (see review: Priyadarshani et al., 2011). The ability for microalgae to biosorb metal ions from aqueous solutions has resulted in much attention on wastewater treatments where algal biomass can be used to reduce the amount of toxic chemicals (Priyadarshani et al., 2011).

Algae are furthermore able to biosorb organic compounds and have an affinity for hydrophobic non-polar compounds (Casserly et al., 1983). Examples of biosorption studies on organic compounds include reactive dyes (Akzy & Tezer, 2005) and the anti-fouling biocide used in paint, tributylin (Tsang et al., 1999; Tam et al., 2002). Pesticides that have been removed by algae are visible in Table 1 and additional biosorption studies on pesticides include aldrin, dieldrin, endrin, lindane, carbaryl and chlordane (Klekner & Kosaric, 1992).

Table 1: Biosorption of pesticides and related compounds by microalgae (adapted from Priyadarshani et al., 2011).

Microalgae	**Pesticide**
Chlamydomonas sp.	Mirex
Chlorella sp.	Toxaphene, methoxychlor
Chlorococcum sp.	Mirex
Cylindrotheca sp	DDT
Dunaliella sp.	Mirex
Euglena gracilis	DDT, parathion
Scenedesmus obliquus	DDT, parathion
Selenastrum capricornutum	Benzene, toluene, chlorobenzene, 1,2-dichlorobenzene, nitrobenzene naphthalene, 2,6- dinitrotoluene, phenanthrene, di-n- butylphthalate, pyrene

Friesen-Pankratz et al., (2003) evaluated the removal of the herbicide atrazine and the insecticide lindane using the green algae *Selenastrum capricornutum* during 11 days of treatment. Their results showed that presence of the algae decreased the aqueous persistence of both pesticides and they therefore speculated whether the algae biosorb the pesticides to facilitate their degradation. There was less lindane left in the aqueous phase after 11 days compared to atrazine. This can possibly be explained with lindane having a greater sorption potential (log K_{ow} 3.72) compared to atrazine (log K_{ow} 2.34). However, lindane has a higher vapour pressure than atrazine and are therefore is more prone to volatilisation from the water phase.

2.3.2 Algal metabolization of pesticides

Microorganisms can use a range of organic pollutants, including pesticides, as an energy source for their growth and at the same time detoxify and mineralize the compounds (Grigg et al., 1997; Massoud et al., 2008). Another mode of degradation is when microorganisms produce enzymes that break the bonds in the pesticide molecules without the microorganisms deriving any benefit from it (Larsson, 1990). Disadvantages with the metabolization of pesticides are that some are extremely difficult to break down and some degradation products can themselves be hazardous (Gadd, 2009).

Many microorganisms can metabolize organic compounds in a way similar to mammals, birds, fish and some plants. This largely depends of the cytochrome P450 superfamily of monooxygenase enzymes (Schocken et al., 1997). Cytochrome P450s are universally found in animals and in higher plants but moreover are found in bacteria, fungi, viruses and protists and have been detected in the algal phylum Clorophyta (green algae), Rhodophyta (red algae) and Chromophyta. Using different pathways these enzymes oxidize endogenous substrates such as

lipids, steroidal hormones as well as xentibiotic substances such as pesticides, and in particular herbicides (Pflugmacher & Sandermann, 1998; Guengerich, 2008; Lamb et al., 2009). Involvement of P450s in biotransformation of foreign compounds has been recorded in the *Chlorella* sp. microalgae (Thies & Grimme, 1994, 1995) and P450 activity has been documented in *C. fusca and C. sorokiniana* in the presence of the pro-herbicide Metflurazon (Thies et al 1996).

Other studies on metabolization of pesticides and related compounds using green algae have been carried out on organophosphorus pesticides such as methyl parathion, parathion, malathion, phorate, quinalphos and monocrotophos (Megharaj et al., 1994). Furthermore, it has been known since 1980 that eukaryotic algae and cyanobacteria can metabolize PAHs. Green, red and brown algae and cyanobacteria could oxidize naphthalene (the main ingredient in moth balls) under photoautotrophic conditions (Cerniglia, 1993) and the green algae *Selenastrum capricornutum* could under photoautotrophic conditions metabolize benzo[a]pyrene (Warshawsky et al., 1990). Other reports of pesticides that have been metabolized by green microalgae are lindane, phenol, chlordimeform, DDT (Table 2.) (Priyadarshani et al., 2011).

Table 2. Microalgae in biotransformation of pesticides and related compounds (adapted from Priyadarshani et al., 2011).

Microalgae	**Pesticides**
Chlamydomonas sp.	Lindane, naphthalene, phenol
Chlorella sp.	Lindane, chlordimeform
Dunaliella sp.	DDT, naphthalene
Euglena gracilis	Phenol
Scenedesmus obliquus	Naphthalene sulfonic acid
Selenastrum capricornutum	Benzo[a]pyrene

So far, few studies have demonstrated degradation of organic compounds with high molecular weights, indeed it appears that algae are more able to metabolize compounds with low molecular weight such as smaller polycyclic aromatic hydrocarbons (PAHs). Hydroxylations of naphthalene (a two-ring PAH, 128 g mol-1) and phenanthrene (a three-ring PAH, 178 g mol−1) have been demonstrated (Semple et al., 1999; Juhasz & Revendra 2000; Ghasemi et al., 2011).

As previously mentioned microbial consortium affects the biodegradation of pesticides. This includes what microorganisms are used and how many are present, the contact between microorganisms and substrate and the environmental conditions around them (notably pH, temperature, salinity, nutrients, light quality and intensity, available water, oxygen tension and redox potential, surface binding and presence of alternative carbon substrates). One important aspect of the degradation of organic compounds using algae is the interaction with bacteria. They

can act as degradation partners where algae via photosynthesis provide O_2 for the bacteria and in turn get CO_2 released from the heterotrophic bacteria. This synergistic combination can enhance the degradation potential of the whole consortium (Ghasemi et al., 2011). Bacteria are more or less always present in algal cultures even if no bacteria were intentionally added. The influence of bacteria in degradation studies of organic compounds using algae should therefore be considered.

2.3.3 Approaches for remediation processes

For many years biological approaches have been used in the removal of organic and inorganic pollutants. Some examples are standard sewage treatments, wetland approaches, biofilm reactors for pollutants, water and soil bioremediation processes and phytoremediation processes (using plants) (Gadd, 2009). The disadvantages of using freely suspended microorganisms in bioremediation studies are primarily the small particle size that makes them difficult to separate from the effluent. However, using immobilized or dead biomass in packed or fluidized-bed reactors could facilitate the process. Other alternatives that have been used are biofilms made of live immobilized biomass. Methods for immobilization are encapsulation or cross-linking and the materials used are agar, alginates, cellulose, silica gel etc. (Gadd, 2009).

2.4 Chlorella vulgaris

Chlorella vulgaris, a microalgae only 2-10 μm in diameter, is a member of the class Chlorophyta and has been cultivated for several years and considered a possible food source (Chacoón-Lee & González-Mariño, 2010). It is a commonly cultivated microalgae that is inexpensive and easily cultivated (Spolaore et al., 2005). It grows rapidly and is able to double in cell number every 8 hours in favourable light and nutritional conditions at a temperature between 20-35 °C (Putt, 2008). *Chlorella vulgaris* can furthermore grow in autotrophic, mixotrophic and heterotrophic modes (Heredia-Arroyo et al., 2011). Today, *C. vulgaris* is a common model organism in scientific remediation studies of both heavy metals and organic compounds and has been well used in wastewaters treatments for removal of metals (see review: Priyadarshani et al., 2011) and nutrients (Larsdotter, 2006).

2.4.1 *Chlorella* sp. and remediation of pesticides

Several studies have investigated *Chlorella* sp. for its ability to biosorb and accumulate heavy metals (Aksu & Dönmez, 2006) and organic compounds (Tsang et al., 1999; Tam et al., 2002).

In a study by Kaduková & Virčíková (2005) on *C. vulgaris*, copper biosorption in dead cells and accumulation in live cells was compared. The binding capacity of living cells was significantly lower than that of dead cells. The copper seriously damaged the cell surface of live cells, which lowered the capacity and made it impossible to recycle the biomass. Moreover, the damaged cells released the accumulated copper back into solution.

Metabolization studies

Chlorella sp. was able to metabolize the organophosphorus insecticide fenamiphos. During 4 days treatment, *Chlorella* sp. transformed more than 99% of the insecticide and thus has great potential in detoxification of fenamiphos. (Cáceres et al., 2008). In another study on *C. vulgaris*, the algae were able to degrade the fungicides carboxin and its analogue oxycarboxin during 15 days of treatment. In this study *C. vulgaris* oxidized carboxin to the degradation product, sulphoxide and degraded oxycarboxin more extensively producing six degradation products (Balasubramanya & Patil, 1980).

Scragg et al., (2003) evaluated the effects of 2,4-dichlorophenol (used in the synthesis of the herbicide 2,4-dichlorophenoxyacetic acid) on *Chlorella* VT-1. HPLC analysis showed that a large proportion of the 2,4-dichlorophenol was removed from the culture medium during the first 6 days, however no degradation products were found. Compared to the start concentration 2.2% 2,4-dichlorophenol was remaining in the medium and 1.5% was extracted from the cells. Scragg et al., (2003) suggests that *Chlorella* VT-1 act like higher plants and transform and store the chlorophenol within cells, rather than degrade it. Furthermore, live *Chlorella* sp. was capable of degrading around 800 mg l^{-1} phenol (100% of the phenol added) in 7 days while the concentration was stable in the control without algae. *Chlorella* sp. did furthermore convert 2,4-dinitrophenom to an isomer of dimethylbenzenediol (Klekner & Kosaric, 1992).

Biosorption studies

Using *Chlorella* sp. for removal of the persistent contaminant, tributylin (TBT), a biocide used in wood preservations and in antifouling paint, has been demonstrated in several studies and biosorption has been shown to be the main mechanism for said removal (Tam et al., 2002; Luan et al., 2006). Luan et al., (2006) evaluated the removal and degradation of TBT on alginate-immobilized algal beads of *C. vulgaris* as compared to blank alginate beads. More than 90% of the TBT was removed within 1 day, both in algal and blank beads irrespective of the initial TBT concentration (24.2, 122 and 244 µg TBT l^{-1}). However, the main part of the removal was due to biosorption of the alginate and only some of the TBT was adsorbed into the actual cell walls and less than 10% was accumulated within the cells.

In a study by Tam et al., (2002) the removal of TBT with dead and live *C. miniata* and *C. sorokiniana* were evaluated. Results showed that dead cells removed 85% of the initial TBT after only 5 minutes and were much more effective than live cells in removal efficiency which

reached the same level of removal after 14 days. Tsang et al., carried out a similar study in 1999 on live *C. vulgaris* and 40% of the TBT was removed after 2 days of culture. Both studies claim that biosorption was the main mechanism for the TBT removal. However, all three species could biodegrade/metabolize TBT as was evident from the detection of breakdown products within the cells after 1 day in *C. sorokiniana, C. vulgaris* and 7 days for *C. miniata* (Tsang et al., 1999; Tam et al., 2002).

2.5 Selected fungicides

2.5.1 Metalaxyl

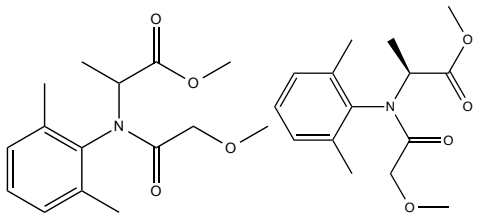

Figure 1. Left; metalaxyl, right: metalaxyl-M

Metalaxyl ($C_{15}H_{21}NO_4$), *N*-2,6-dimethylphenyl)-*N*-(methoxyacetyl)alaine methyl ester, is a phenylamine fungicide used worldwide against plant pathogenic oomycetes on a wide range of crops, in Sweden intended for use in potatoes (Norberg, 2004). Metalaxyl was first registered as a racemic formula (a blend of the two chiral enantiomers: rac-metalaxyl) in 1977 and thirty years later, in 1996 metalaxyl-M was launched which is enriched with the *R*-enantiomer (≥ 90%) of metalaxyl. Metalaxyl-M has systemic and residual effect and is a widely used fungicide, which can be explained by its stable properties and its broad-spectrum activity (Yao et al., 2009).

Metalaxyl is poorly sorbed by soil and highly soluble in water (8.4 g l^{-1} at 22ºC), which makes the substance mobile and increases the risk for the substance reaching ground and surface water by agricultural run-offs and leakage through the soil profile (Yao et al., 2009). The soil sorption coefficient (K_{oc}) is low (30-300 l kg^{-1}) which indicates that metalaxyl has a low tendency to be adsorbed of the soil matrix, and other organic matter (Komárek et al, 2010). Metalaxyl has a high to medium molecular weight (279.33 g mol^{-1}) and a low vapour pressure (PPDB, 2014), which reduces its volatility. Metalaxyl is not easily biodegradable and in water/sediment systems, the substances are distributed evenly between both water and sediment. Studies indicate that metalaxyl is stable to hydrolysis in normal pH conditions and it is also stable photolytically in water exposed to normal sun light conditions (Yao et al., 2009) and the half-life of in water is 106 days. Furthermore, metalaxyl is not expected to bioaccumulate and has a K_{ow} at 1.7 (Wightwick et al., 2012).

Metalaxyl is a liver toxicant and is moderately toxic to humans (PPDB, 2014) and the water quality standard for metalaxyl in Sweden are 60 μg l^{-1} (Lindström et al., 2013). Toxicity tests on the green microscopic algae *Scenedesmus subspicatus* and *Scenedesmus quadricauda*, show that metalaxyl is considerably non-toxic to green algae (Norberg, 2004; Massoud et al., 2008).

Earlier studies show that metalaxyl can be metabolized by microorganisms such as the bacteria *Pseudomonas* sp. and the fungi *Aspergillus niger* (Massoud et al., 2008). To my knowledge no biosorption quantification of metalaxyl by algae has been conducted. In a study on the sorption of soils it was shown that lowering the pH increased the metalaxyl adsorption. The fungicide gets positively charged in acid environments and gets more attracted to the negatively charged functional groups on the soil particles (Arias et al., 2006; Komárek et al., 2010; Gondara et al., 2013).

During 2012, one ton of metalaxyl-M was sold in Sweden (KemI, 2014) and the same year 40% of all surface water samples taken contained metalaxyl, which was the second, most common fungicide found in surface waters after azoxystrobin (Lindström et al., 2013). The frequency of metalaxyl detection has varied from year to year, however it has been present in Swedish surface waters since pesticide monitoring commenced in 2002 (Regionala pesticiddatabasen, 2014).

Metalaxyl are also sometimes found in the groundwater and was the most common substance found in groundwater samples in Halland 2012 (Lindström et al., 2013) and in Scania 2010 (together with propikonazol) (Regionala pesticiddatabasen, 2014). In 2010 metalaxyl also was the most common substance found in drinking water with two findings, found in Scania, out of 54 samples taken in whole of Sweden (Regionala pesticiddatabasen, 2014).

2.5.2 Cyprodinil

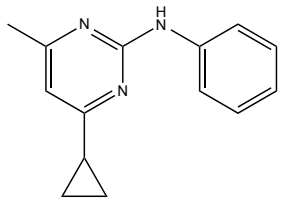

Figure 2. Cyprodinil

Cyprodinil ($C_{14}H_{15}N_3$), (4-cyclopropyl-6-methyl-N-phenylpyrimidine), is an anilinopyrimidine fungicide with a systemic effect for foliar applications on cereals and strawberries. It inhibits the penetration of the fungi into the plant and thus reduces mycelial growth inside and on the surface of the leaves.

It is a non-volatile (4.7-5.1 x 10^{-4} Pa in 25 ºC) substance that with the molecular weight 225.29 g mol^{-1} and is slightly soluble in water (0.013 g l^{-1} at 25 °C). Cyprodinil has a high log K_{ow} value (4.0 at 25 ºC) and is not very mobile in soil and therefore not presumed to leak and reach surface and ground water. The K_{oc} is 1706 l kg^{-1} suggesting a tendency to be adsorbed of the soil matrix, and other organic matter (Komárek et al., 2010).

Cyprodinil is stable for hydrolysis but degradable through photolysis in water. After 13.5 days in aqueous systems at pH 7, 50% of the fungicide dissipates through photolysis (PPDB, 2014). The major metabolite, CGA 249287, is also stable for hydrolysis but shows lower toxicity toward aquatic organisms than cyprodinil. Cyprodinil is expected to bioaccumulate in fish and is moreover toxic to the crustacean *Daphnia magna*. Toxicity tests on *Pseudokirchneriela subcapitata* show that it is considerably non-toxic to algae (Norberg, 2004). The proposed water quality standard for cyprodinil is 0.2 µg l^{-1} (Lindström et al, 2013).

In 2012, 10.9 tonnes of cyprodinil was sold in Sweden (KemI, 2014) and same year cyprodinil was present in 20% of all samples taken of surface waters (Lindström et al., 2013).

Earlier studies show that microorganisms can degrade cyprodinil. Ten out of the 12 cultures tested (9 filamentous fungi, 2 streptomycetes and 1 bacteria) could metabolize cyprodinil and produced a monohydroxylated metabolite. The microorganisms degraded cyprodinil with a range of 1.2 – 35.6% of the initial concentration (Schocken et al., 1997).

2.5.3 Propamocarb

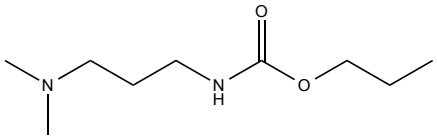

Figure 3. Propamocarb

Propamocarb ($C_9H_{20}N_2O_2$) propyl [3-(dimethylamino)propyl]carbamate, is a carbamate used on potatoes, lettuce, cucumbers, tomatoes and ornamentals (Kreuger et al., 2009) commonly applied as propamocarb hydrochloride. Propamocarb has a lower molecular weight (188.3 g mol^{-1}) compared to the other fungicides in this study. It is highly water soluble (900 g l^{-1}) and has a low log K_{ow} 0.84 which indicates that propamocarb more likely will leak through the soil and reach water bodies rather than sorb to the soil particles. Propamocarb has a high vapour pressure (730 mPa at 25°C) making it volatile (Bayer Crop Science, 2014; PPDB, 2014).

In 2012, 3.7 tonnes of propamocarb were sold in Sweden (KemI, 2014) and in the same year propamocarb was present in 20% of the samples taken from surface waters (Lindström et al.,

2013) and in 23% in 2010. In 2010 propamocarb had the highest maximum concentration (22.0 μg l^{-1}) and highest average concentration (2.133 μg l^{-1}) among all 33 fungicides tested. Geographically, all 20 findings out of 87 samples taken in the whole of Sweden was found in Scania (26%) (Regionala Pesticiddatabasen, 2014). The water quality standard for propamocarb is 90 μg l^{-1} (Lindström et al., 2013).

To my knowledge no information is available on the hydrolysis, photolysis or biodegradation of propamocarb. However, propamocarb hydrochloride is described as being very stable to hydrolysis and photolysis in aqueous media and is slightly mobile in soil. Aquatic microorganisms rapidly decompose propamocarb hydrochloride (up to 97% within 35 days) (PMEP, 2014) and in a study on four bacterial strains, it was shown that all bacterial populations increased after 35 days in the presence of propamocarb hydrochloride. The two strains, *B. amyloliquefaciens* IN937a and *B. pumilus* SE34, were most rapid in the degradation (Myresiotis et al., 2012). It is suggested that both propamocarb and propamocarb hydrochloride has low toxicity to aquatic organisms including green algae (PPDB, 2014).

2.5.4 Mandipropamid

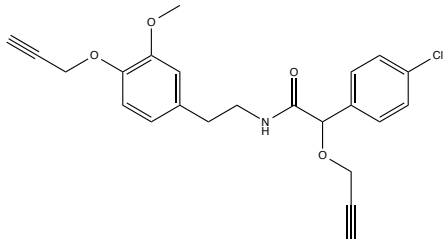

Figure 4. Mandipropamid

Mandipropamid ($C_{23}H_{22}ClNO_4$) ([4-chloro-N-[2-[3-methoxy-4-(2-propynyloxy)phenyl]ethyl]a-(2 propynyloxyl)-benzeneacetamide]) is used for the control of several foliar oomycete pathogens in a range of crops, belongs to the mandelamide/mandelic acid chemical group and was recently introduced to the market (Malhat & Mahmoud, 2012). Mandipropamid has an octanol/water coefficient (log K_{ow}) of 3.2 at 25ºC and the solubility in water and octanol is 4.2 mg l^{-1} and 4800 mg l^{-1} respectively. This indicates that mandipropamid has a clear tendency to partition into fat rather than water. The BCF in whole fish is 35-48 and makes it slightly prone to bioaccumulate. The mobility of mandipropamid in soil is low to medium and the K_{oc} (4 European and 3 American soils tested) range between 405-1294 ml g^{-1} and it is not expected to accumulate significantly in soil (Malhat & Mahmoud, 2012).

Mandipropamid is stable in water and is not degraded by hydrolysis. Though it is degradable through photolysis in water with a half-life that ranges from ”very rapidly degradable” to “fairly

degradable" depending on the light quality. The half-life corresponds to approximately 2 days in summer sunlight at 30-50ºN, in the top layer of an aqueous system (Malhat & Mahmoud, 2012).

Mandipropamid has a very low vapour pressure, 9.40×10^{-4} mPa (at 25°C), and it has a high molecular weight, 411.9 g mol^{-1}, which makes the risk for evaporation and spray drift low. Mandipropamid is slightly toxic to green microscopic algae *Selenastrum capricornutum* (APVMA, 2014).

In 2012, 8.2 tonnes of mandipropamid was sold in Sweden (KemI, 2014) and the substance was present in approximately 15% of all samples taken of surface waters same year. The water quality standard for mandipropamid is 8 µg l^{-1} (Lindström et al., 2013).

To my knowledge, no studies about the biodegradation capacity of mandipropamid have so far been reported, mainly due to the fact that this pesticide is relatively new on the market.

3. MATERIALS AND METHODS

3.1 Algae and growth conditions

Chlorella vulgaris strain 211/11B (http://www.ccap.ac.uk/strain_info.php?Strain_No=211/11B) was obtained from CCAP-SAMS (Culture Collection of Algae and Protozoa, The Scottish Association for Marine Science), Scotland.

The strain was pre-cultivated through inoculation with 10% (v/v) five-days-old algae culture every fifth day. The algae were grown in a standard growth medium for green algae, Z8 (NIVA, 1976), and kept under sterile conditions in a greenhouse at 20°C and with a photoperiod of 16:8 h (light:dark). An additional illumination of 100 µmol m^{-2} s^{-1} was added and the cultures were aerated with aquaria pumps to prevent the cells from settling. Algal growth was recorded through dry weight determination (g l^{-1}) and cell counts (density) in a Bürkner chamber (cells ml^{-1}) and the pH of the aqueous phase was measured in the end of the experiment.

3.2 Pesticides

The pesticide mix (Mix M2101/1B, Analytical Reference Material, Restek, USA), with the following 37 pesticides; acetamiprid, cyanazin, cyazofamid, cyprodinil, difenokonazol, etofumesat, fenmedifam, fenpropidin, fludioxonil, flurprimidol, flurtamon, flusilazol, flutriafol, fuberidazol, hexazinon, imidakloprid, karbofuran, karfentrazonetyl, klomazon, klorfenvinfos, kloridazon, kvinmerak, mandipropamid, metalaxyl, metamitron, metazaklor, metolaklor,

metrafenon, penkonazol, pirimikarb, propamokarb, propyzamid, protiokonazol-destio, pyroxsulam, spiroxamin,, terbutylazin, trinexapak-etyl samt triticonazol, was used. The standard was obtained from The Centre for Chemical Pesticides (CKB), SLU, Ultuna, Sweden. A theoretical starting concentration of 2.0 µg l^{-1} was obtained from an initial concentration of 20 µg ml^{-1}.

3.3 Experimental

Biosorption study/Short term

Dead biomass was prepared by cultivating *C. vulgaris* under the conditions described above for four days. The biomass was collected by centrifugation (3000 g, 15 min, JA-Avanti, Beckman Coulter), washed once with distilled water and the pellet was lyophilized in a freeze dryer for 24 h. The lyophilized biomass was stored under dark conditions at room temperature while the living biomass was produced under similar growth conditions. After four days, an equal amount of live biomass was centrifuged. Before the experiment, the dry biomass was ground by hand to a powder, using a glass rod. The pesticide mix was added to sterile distilled water to obtain an initial concentration of 2.0 µg l^{-1}. The treatments comprised of living algal biomass, dead algal biomass and a control without any biomass, with three replicates per treatment. The amount of biomass (dead or live) added to each replicate corresponded to 0.6 g dry weight per litre (6×10^7 cells ml^{-1}). There were three replicates per treatment and the total volume of each replicate was 150 ml. The treatments were stirred on a shaking table at a speed of 380 rpm for 1 h at room temperature. After one hour, the biomass was removed from the aqueous phase by centrifugation (4000 g, 20 min, JA-Avanti, Beckman Coulter) and the samples were stored in the freezer at -20°C until analysis. The pH values at the end of the experiment were measured and found to be 6.5 ± 0.06 in treatment with dead algae, 6.8 ± 0.0 in live algae and 6.4 ± 0.2 in the control.

Biodegradation study/Long-term

As described previously the pesticide mix was added to sterile Z8 to obtain a final concentration of 2.0 µg l^{-1}. The treatments included one treatment with growing *C. vulgaris* and a control without any biomass. There were three replicates per treatment and the total volume of each replicate was 150 ml. In the treatment with *C. vulgaris* an inoculum of 10% (v/v) of a five-day-old culture was added which resulted in a starting density of 3×10^6 cells ml^{-1}. The control treatment received an inoculum of 10% (v/v) of sterile Z8. The treatments were kept under the growing condition described above for 4 days. After the experiment the biomass was removed by centrifugation (4000 *g*, 20 min, JA-Avanti, Beckman Coulter) and samples of the aqueous phase were taken and stored in the freezer until analysis. After the experiment, the cell density was 3×10^7 cells ml^{-1} and the pH was measured to 9.3 ±0.06 in the treatment with algae and 7.43 ± 0.06 in the control.

3.4 Chromatographic analyses

Samples (70 ml) from the aqueous solution were sent to The Centre for Chemical Pesticides (CKB) SLU, Ultuna, for chromatographic analyses. They used the analytical method OMK 57 (CKB, 2014), which is based on a combination of liquid chromatography (LC) and mass spectroscopy (MS) specifically called LC-MS/MS (tandem-MS). This method can analyse large compounds simultaneously, which is advantageous in environmental monitoring. Tandem-MS provides low detection limits and very high security, which means that more substances can be tracked at lower level (Jansson & Kreuger, 2010).

3.5 Statistics

Mean and standard deviation was reported. The data were analysed by analysis of variance ANOVA followed by Tukey's multiple comparison test. Statistics were conducted with Minitab 16 (Minitab Inc, PA USA, 2013) and R 3.0.3 (R Core Team, 2014).

4. RESULTS

4.1. Biosorption/Short-term study

Propamocarb

In the short-term study, treatment with living cells resulted in significantly lower concentration of propamocarb compared to the final concentrations observed in treatments with dead cells and control treatments, respectively ($P<0.001$). There was no significant difference in final concentrations of propamocarb between the control and dead algae (Table 3, Figure 5).

Mandipropamid

There was a significant difference between the concentrations of mandipropamid between respective treatments ($P=0.0175$). A significantly lower amount of mandipropamid was observed in the control treatment as compared to the one with dead algae ($P=0.0175$). There was no significant difference between live and dead biomass or between live biomass and control (Table 3, Figure 5).

Cyprodinil

The concentration of cyprodinil in the treatment with the dead cells was significantly lower compared to the concentration in the treatment with live cells (P=0.0459). All other pairwise comparisons were not significantly different (Table 3, Figure 5).

Metalaxyl

There was no significant difference between biosorption of metalaxyl between respective treatments (P=0.148).

Table 3. Biosorption/short-term study; pesticide concentrations in the medium at the end of the experiment.

Pesticide	**Pesticide remaining in medium. Dead algae (µg l^{-1})**	**Pesticide remaining in medium. Live algae (µg l^{-1})**	**Pesticide remaining in medium. Control (µg l^{-1})**
Cyprodinil	0.39**a***± 0.02	0.47**b** ± 0.04	0.42**ab** ± 0.01
Mandipropamid	1.83**a** ± 0.15	1.53**ab** ± 0.06	1.17**b** ± 0.3
Metalaxyl	2.07**a** ± 0.06	1.93**a** ± 0.06	1.93**a** ± 0.12
Propamocarb	2.4**a** ± 0.0	1.67**b** ± 0.06	2.33**a** ± 0.06

*Values within rows followed by the same letter are not significantly different (P>0.05).

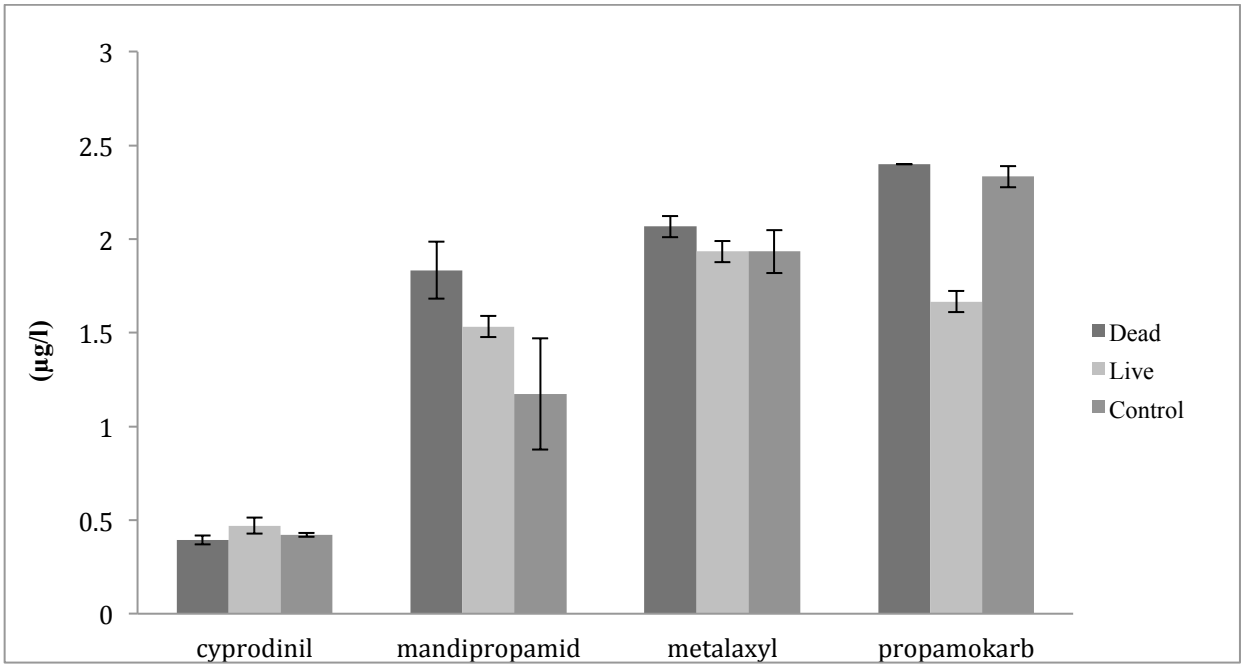

Figure 5. Biosorption/short-term study; pesticide concentrations in the medium at the end of the experiment.

4.2 Biodegradation/Long-term study

Propamocarb

The concentration of propamocarb was significantly lower in the treatment with growing algae compared to the control with no algae (P<0.001) (Table 4, Fig. 6).

Mandipropamid

There was no significant difference between the concentration of mandipropamid in the presence of algae and the control (P=0.0855).

Metalaxyl

No significant difference was observed between the concentration of metalaxyl in treatment with growing algae and the control (P=0.519).

Cyprodinil

There were no cyprodinil detected in the growing or the control after the long-term experiment.

Table 4. Biodegradation/long-term study; pesticide concentrations in the medium at the end of the experiment.

Pesticide	**Pesticide remaining in medium. Growing algae (µg l^{-1})**	**Pesticide remaining in medium. Control (µg l^{-1})**
Cyprodinil	-	-
Mandipropamid	0.87**a*** ± 0.04	0.70**a** ± 0.12
Metalaxyl	1.67**a** ± 0.06	1.63**a** ± 0.06
Propamocarb	1.17**a** ± 0.06	2.33**b** ± 0.06

*Values within rows followed by the same letter are not significantly different (P>0.05)

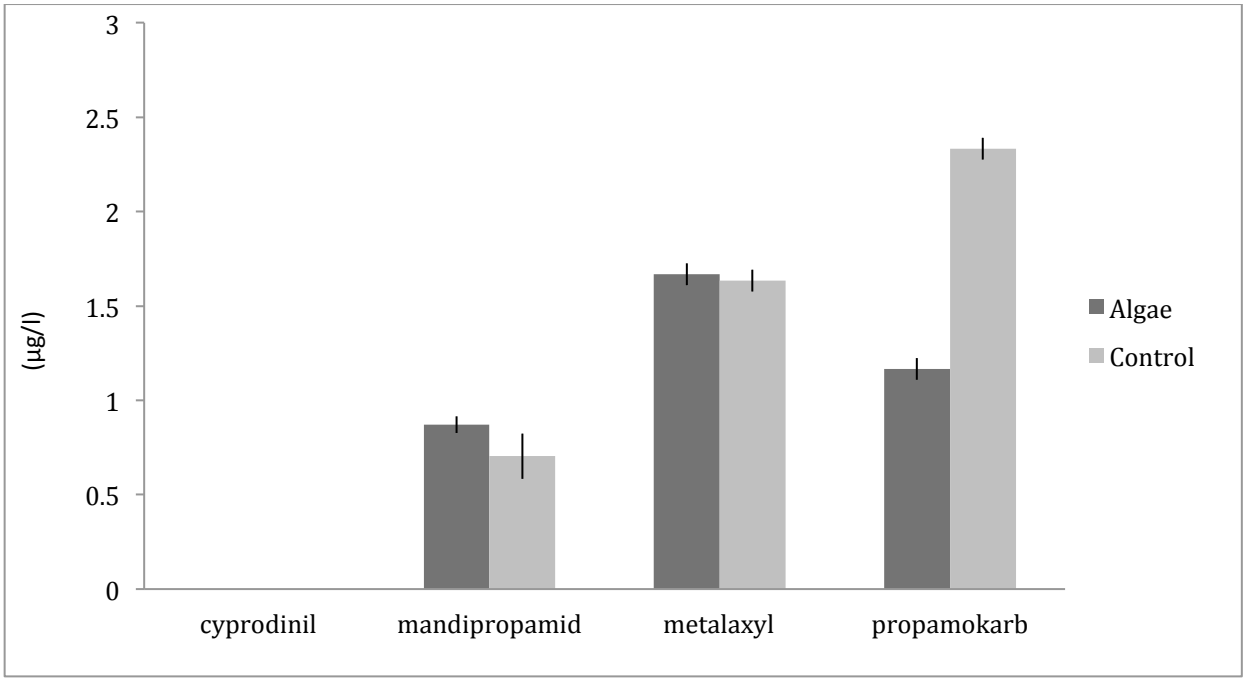

Figure 6. Biodegradation/long-term study; pesticide concentrations found in the medium at the end of the experiment.

5. DISCUSSION

Pesticide residues are commonly found in the aquatic environment as a result of agricultural run-offs and through industry and domestic sewage (Tikoo et al., 1996; Priyadarshani et al., 2011; Lindström et al., 2013; www.kemi.se) and these substances may be a hazard to both the ecosystem and human health (Larsson, 1990; REACH, 2014). There is thus a need for cost efficient remediation systems that could process these pesticides in contaminated water bodies or capture these substances in wastewater treatments before they can contaminate the wider environment. Bioremediation is a promising alternative, using living or inactivated organisms for purification processes that are less expensive than currently available methods (using activated carbon or ozonation) (Naturvårdsverket, 2008; Zaini et al., 2010). Microalgae have the ability to remove pesticides in water both through biosorption and through metabolism (Warshawsky et al., 1990; Cerniglia, 1993; Tsang et al., 1999; Tam et al., 2002; Akzy & Tezer, 2005; Ghasemi et al., 2011; Priyadarshani et al., 2011). In this study *Chlorella vulgaris* was exposed to a mix of 37 pesticides in two experiments: a short-term (60 min) and one long-term (4 days) experiment was conducted with subsequent focus on four prevalent fungicides in the environment; propamocarb, mandipropamid, cyprodinil and metalaxyl.

5.1 Biosorption/Short-term study

Propamocarb

The presence of living biomass of *C. vulgaris* decreased the concentration of propamocarb in the medium with around 30% compared to the control (with no algae) and also compared to the treatment with dead biomass (dead 2.4 ± 0.0 μg l^{-1}; live 1.67 ± 0.06 μg l^{-1}; control 2.33 ± 0.06 μg l^{-1}) (Table 3, Figure 5). The removal of propamocarb over such a short period (60 min) suggests biosorption as the probable mechanism. This is because the short duration of the experiment is unlikely to provide sufficient time for active uptake or metabolization processes. However, the low log K_{ow} value (0.84) (PPDB, 2014) and the not very high value for the soil sorption coefficient K_{oc} (719) (Bayer Crop Science, 2014) gives propamocarb a low tendency to be biosorbed and thus contradicts this theory. However, biosorption can be influenced by other physico-chemical factors including molecular size, molecular structure and ionzability (Komárek et al., 2010).

If biosorption was responsible for the removal of propamocarb, then it is unclear why it did not occur for treatment with dead biomass, as biosorption can both occur on dead and living cells (Gadd, 2009). Perhaps there are differences between the charged functional groups in the cell walls of dead and live cells. One possibility involves particle size; it was difficult to homogenize the dry dead biomass to equally small particles as found in live biomass. Larger algal particles reduce the total surface area available for the pesticides to be biosorbed to. One reason may be that the metabolic processes in living cells can affect the overall removal process (Gadd, 2009). Another explanation why live cells and not dead cells removed propamocarb could be that the uptake was due to active bioaccumulation and not passive biosorption (Kaduková & Virčíková, 2005). As expected, the concentration in the control did not decrease at all. This can be explained with propamocarb most likely being stable to both hydrolysis and photolysis, as is the case for the closely related propamocarb hydrochloride (PMEP, 2014).

Mandipropamid

Significantly lower mandipropamid concentrations were found in the control as compared to the treatment with dead biomass, in the short-term study (Table 3, Figure 5).

Mandipropamid is degradable by photolysis (half-life 2 days in summer light) and perhaps the suspended dead algal biomass shaded this process. The lack of biosorption of mandipropamid may be explained with its soil sorption coefficient not being very high (K_{oc} 405-1294 ml g^{-1}) (Malhat & Mahmoud, 2012). Common for pesticides that have shown ability for biosorption is that they have a high value for K_{oc} and are immobile in the soil, e.g. DDT; K_{oc} 151000 (PPDB, 2014).

Cyprodinil

The concentration of cyprodinil in the short-term experiment was very low in all treatments (dead 0.39 ± 0.02 μg l^{-1}; live 0.47 ± 0.04 μg l^{-1}; control 0.42 ± 0.01 μg l^{-1}) (Table 3, Figure 5). There was however a slight difference between the concentrations of cyprodinil in the presence of dead and live biomass with a lower concentration recorded in the presence of dead biomass. Cyprodinil has a tendency to be adsorbed and has the highest value for log K_{ow} (4.0) among the four fungicides in this study. It also has a high K_{oc} (1706 l kg^{-1}) (Komárek et al., 2010) and was possibly adsorbed to the dead cells in higher extent as compared to the live cells. As with propamocarb, the removals of cyprodinil differ significantly between dead and living cells (Table 3, Figure 5) and the reason for this in unknown.

The reason for the loss of cyprodinil in the short-term study was not likely to be due to hydrolysis, photolysis or evaporation (Komárek et al., 2010). Instead, one possibility could involve potential handling errors with the pesticide before or after the experiment. Some degradation may have occurred in some of the pesticides in the pesticide mix during initial shipment and also in the samples when transported back to the lab. The high adsorption capacity of cyprodinil could possibly make the pesticide bind onto some of the plastic material present, for example the pipette tip or more likely the plastic jars in which the samples were transported. Many plastic materials are able to adsorb organic compounds such as pesticides (Teuten et al., 2009).

Metalaxyl

There was no significant difference of the metalaxyl concentration between the treatments in the short-term experiment and the concentrations were all close the start concentration (dead 2.07 ± 0.06 μg l^{-1}; live 1.93 ± 0.06 μg l^{-1}; control 1.93 ± 0.12 μg l^{-1}) (Table 3, Figure 5). Biosorption processes have probably not occurred and thus can be explained with metalaxyl having low values for log K_{ow} (1.7) (Wightwick et al., 2012) and K_{oc} (30-300 l kg^{-1}) (Komárek et al, 2010). According to Komárek et al. (2010) there is a correlation between a decreasing pH value below 5 and an increased sorption of metalaxyl. In acidic environment, metalaxyl gets positively charged and can adsorb to the negatively charged functional groups on the algal cell walls (Mehta & Gaur, 2005; Arias et al., 2006; Komárek et al., 2010; Gondara et al., 2013). In the present short-term study, the pH value was above 5 (dead 6.5; live 6.8). Perhaps biosorption would have occurred at lower pH values. Other chemical properties for metalaxyl that can explain its strong persistence are that it is non-volatile (PPDB, 2014) and stable for hydrolysis and photolysis (Yao et al., 2009).

5.2 Biodegradation/Long-term study

Propamocarb

At the end of the four day experiment, there was a 50 % lower concentration of propamocarb in the presence of algae compared to the control in the long-term study (algae 1.17 ± 0.06 μg l^{-1}; control 2.33 ± 0.06 μg l^{-1}) (Table 4, Figure 6). In the short-term experiment 30 % of the propamocarb was lost after 60 min and thus there was only 20 % more removal compared to the long-term experiment. This suggests that the main mechanism for the propamocarb removal in the long-term experiment is the same as in the short-term experiment, namely biosorption. Still, it is unclear if the remaining 20 % loss was removed via biosorption or due to other mechanisms. However, in the long-term experiment there was enough time for some metabolization processes to occur by the algae.

Reports show that the propamocarb hydrochloride is able to become biodegraded by bacteria and aquatic microorganisms (Myresiotis et al., 2012; (PMEP, 2014) and since they are very similar compounds, propamocarb is probably also able to be biodegraded in a similar way. The relative low molecular weight of propamocarb (188.3 g mol^{-1}) could be a favourable factor for the metabolization process to occur in the algae, as previous reports points out a correlation between low molecular weight and successful metabolization in algae (e.g. naphthalene; 128 g mol^{-1} and phenanthrene; 178 g mol^{-1}) (Semple et al., 1999; Juhasz & Revendra 2000; Ghasemi et al., 2011). Larger pesticides can also be metabolised by algae; previous studies show that *Chlorella* sp. can metabolise pesticides such as fenamiphos (235.30 g mol^{-1}) (Cáceres et al., 2008) and carboxin (303.36 g mol^{-1}) (Balasubramanya & Patil, 1980).

The algae may either have biosorbed, metabolised or facilitated the degradation of propamocarb, or it can be due to a combination of those. The concentration in the control in the long-term experiment is the same as in the short-term experiment (Table 3 and 4). These results demonstrate that propamocarb is very stable to hydro- and photolysis in aqueous media, as has been shown for propamocarb hydrochloride (Myresiotis et al., 2012).

Mandipropamid

There was no removal of mandipropamid that could be explained with the presence of algae, as there was no significant difference between the concentrations in the control compared with the treatment (algae 0.87 ± 0.04 μg l^{-1}; control 0.70 ± 0.12 μg l^{-1}) (Table 4, Figure 6). The end concentration was approximately 65% lower than the initial concentration. This could be explained by mandipropamid being rapidly degradable via photolysis with a half-life of approximately 2 days in summer sunlight equivalent in the top layer in an aqueous system (Malhat & Mahmoud, 2012; APVMA, 2014).

Metalaxyl

There was no difference between the concentration of metalaxyl in the treatment with algae and in the control (algae 1.67 ± 0.06 μg l^{-1}; control 1.63 ± 0.06 μg l^{-1}) (Table 4, Figure 6), which indicates that there was no removal due to the presence of the algae. Even though metalaxyl is proven to be biodegradable by the bacteria *Pseudomonas* sp. and the fungi *Aspergillus niger* (Massoud et al., 2008) it was not biodegraded by *C. vulgaris* in this study. Of note, the initial concentration of metalaxyl used in the Massoud study was considerably higher (100 μg ml^{-1}) and the duration of the experiment was much longer (4 weeks) as compared with the present study (2 μg l^{-1}) and (4 days). Perhaps we would have different results in this study if concentration and duration were increased.

The observed concentrations were not much lower in this study than in the short-term study. It is possible that this may be explained with the stable properties of this compound as detailed above (see discussion on the short-term study).

Cyprodinil

There were no traces of cyprodinil in either the algae treatment or in the control, so this cannot be due to the presence of algae (Table 4, Figure 6). Similarly, the concentrations were low in all three treatments as were in the short-term experiment. Thus, it is probable that something unexpected happened with the pesticide before or after the experiment. In this experiment with duration of 4 days, an additional amount of cyprodinil had disappeared compared to the short-term study. This disappearance could perhaps partly be explained with cyprodinil being moderately fast degraded via photolysis in water (PPDB, 2014).

5.3 Using algae for remediation

Microalgae are well suited for remediation of heavy metal polluted waters and the easily produced species *Chlorella vulgaris* is a promising organism to work with. This raises the possibility of developing a filter made of dead algal biomass that could be used for uptake of heavy metals in the treatment of wastewaters. In the present work it was demonstrated that it was also possible to remove pesticides from water by short time treatment with algal cells. Our results also show that the effect varies with the chemical nature of the fungicide and it's interaction with the microalgal cell wall. An algal biomass filter could potentially be developed for the remediation of pesticides in agricultural water disposals. As many of the pesticides used in Sweden are applied against potato late blight these filters could be located in waters close to potato fields. As mentioned previously, using dead biomass instead of live has the advantages that the product will be stable and no risk for damaging the cells is expected. Dead biomass has also the ability to be recycled (Aksu & Dönmez, 2006). The biosorbed pollutants could be

washed away from the algal biomass (Chen et al., 2012) and processed in a safe way (Friesen-Pankratz et al., 2003) and thereafter the biomass filter itself could be reused.

One other approach is to use live *Chlorella vulgaris* in remediation systems, providing metabolization in addition to biosorption. As discussed earlier, metabolic factors taking place in living cells can affect the overall removal process. A side effect is that the pH increases in the presence of growing algae, which can have a further effect on the interaction with the pesticide. Other advantages of using live *C. vulgaris* is that it can grow in autotrophic, mixotrophic and heterotrophic modes (Heredia-Arroyo et al., 2011) which gives the algae competitive advantages over fungi and bacteria in degrading organic pollutants in certain environments (Subashchandrabose et al., 2013). Microalgae thus have the ability to grow in environments where other organisms cannot live, e.g. off-shore sites polluted with xentibiotics such as pesticides (Pflugmacher & Sandermann, 1998). Microalgae are efficient in building up biomass (Chacoón-Lee & González-Mariño, 2010; Subashchandrabose et al., 2013) and in addition to the ability to remove pesticides and heavy metals; *C. vulgaris* can take up nutrients from horticultural and agricultural fertilizer leakage (Ardal, 2012; Hultberg et al., 2013). All these abilities make live *C. vulgaris* ideally suited for remediation in agricultural water disposals. One previous approach is to use constructed wetlands dominated by microalgae as a treatment for pesticide contaminated water (Friesen-Pankratz et al., 2003). In further research, it would be interesting to develop a bioremediation system that resembles something in-between a biofilter and wetland approach.

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