Bioavailability of Pesticides to Benthic Invertebrates

The Role of Aquatic Biofilms and Humic Substances

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Abstract

In agricultural landscapes, pesticides are frequently contaminating nearby aquatic ecosystems. Unlike most other contaminants, pesticides are targeted against specific organism groups, but will also have negative effects on non-target organisms. When pesticides enter aquatic environments they tend to sorb to fine organic matter. Thus, benthic organisms inhabiting sediment and biofilms are constantly exposed to elevated concentrations. Depending on the bioavailability of these contaminants, benthic organisms play a key-role in the uptake and transfer to higher trophic levels. The bioavailability is partly deter-mined by the presence of organic matter, where their quantity and quality reflects the aquatic ecosystem's trophic status and catchment area features.

The broader aim of this thesis was to study the effects of different organic matter in artificial bottom substrate, and aquatic biofilms on pesticide bioavailability to benthic invertebrates with different feeding behaviour. We used organic matter of different sources, and short-term exposures to mimic peak exposures of pesticides in agricultural aquatic ecosystems.

The results show that sediments with microbial biofilms strongly increase chlorpyrifos bioavailability to selective feeding larvae. However, chlorpyrifos bioavailability showed a negative correlation to extracellular polymeric substances (EPS) and humic substances (HS) quantities in an artificial bottom substrate. Furthermore, uptake of carbofuran, lindane and chlorpyrifos in non-selective feeding snails were affected by dissolved organic matter (DOM) quantity and quality, and related to pesticide hydrophobicity. Also, pesticide fate in microcosm compartments was affected by DOM-quantity and quality depending on pesticide physicochemical characteristics. These results lead on to a study where we wanted to quantify pesticide sorption to molecular size-fractions of different DOM, however, unfortunately without success.

This thesis highlights the importance of quantity and quality of organic matter, based on our high resolution of different organic matter, and their impact on pesticide fate and bioavailability to lower trophic levels in aquatic ecosystems. Consequently, pesticide fate and bioavailability, and thus their effects on biota, changes between aquatic environments, and this should be taken into consideration in tests protocols for pesticide regulations.

Keywords: Bioavailability, Pesticides, Hydrophobicity, Dissolved Organic Matter (DOM), Humic Substances (HS), Extracellular Polymeric Substances (EPS), Aquatic Biofilms, Sediment, Benthic Invertebrates

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Widenfalk, A., Lundqvist, A. & Goedkoop, W. (2008) Sediment microbes and biofilms increase the bioavailability of chlorpyrifos in *Chironomus riparius* (Chironomidae, Diptera). *Ecotoxicology and Environmental Safety* 71, 490-497

II Lundqvist, A., Bertilsson, S. & Goedkoop, W. (2010) Effects of extracellular polymeric and humic substances on chlorpyrifos bioavailability to *Chironomus riparius*. *Ecotoxicology* 19, 614-622

III Lundqvist A., Bertilsson, S. & Goedkoop, W. Fate and bioavailability of insecticides across DOM-gradients in model ecosystems with biofilm and primary consumers (Submitted manuscript)

IV Lundqvist, A. Tattersdill, K., Jansson, C., Bertilsson, S. & Kylin, H. Model development, endeavouring to study the sorption of insecticides to different size fractions of aquatic humic substances (Manuscript)

Paper I and II are reproduced with permission of the publishers.

The contribution of Anna Lundqvist to the papers included in this thesis was as follows:

I The respondent was involved in the designing and planning of the study, as well as the lab work. In addition, the respondent was involved in the interpretation, writing and publishing.

II The respondent was responsible for the designing, planning, and the lab work of this study, as well as the interpretation, writing and publishing.

III The respondent was responsible for the designing, planning, and the lab work of this study, as well as the interpretation, writing and publishing.

IV The respondent was responsible for the designing, and the planning of this study. The respondent also participated in the lab work, and was responsible for the interpretation and writing.

Abbreviations

BCF	Bioconcentration Factor, L/kg
^{14}C	Radioactive Isotope of Carbon (6 Protons and 8 Neutrons)
CPM	Counts Per Minute
DDT	Dichlorodiphenyltrichloroethane
DEAE	Diethylaminoethyl
DOM	Dissolved Organic Matter
EPS	Extracellular Polymeric Substances
HOC	Hydrophobic Organic Contaminants
HPLC/MS/MS	High-Performance Liquid Chromatography Coupled with Tandem Mass Spectroscopy
HPSEC	High-Performance Size Exclusion Chromatography
HS	Humic Substances
K_{OW}	Octanol–Water Partitioning Coefficient
LV DOA	Lake Vikasjön Dissolved Organic Acid
LV HAFA	Lake Vikasjön Fulvic and Humic Acid
MilliQ	Highly Purified and Desalted Water (a Millipore™-system)
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
POP	Persistent Organic Pollutants
PSS	Polystyrene Sulphonates
SPMD	Semipermeable Membrane Device
SR FA	Suwannee River Fulvic Acid
SR HA	Suwannee River Humic Acid
TOC	Total Organic Carbon

1 Introduction

Organic contaminants, like pesticides, released in our environment often ends up in aquatic ecosystems, possessing high potential to negatively affect organisms living there.

1.1 Organic Contaminants in Aquatic Environments

The fate of organic contaminants in aquatic environments is determined by the partitioning into different environmental compartments (e.g., surface water, sediment and pore water), and the transformation processes of the specific compound in each of these compartments (Koelmans et al. 2001; Katagi 2006). Hydrophobic organic contaminants (HOC) rapidly partition to dissolved or particulate organic matter once they enter aquatic ecosystems. In sediments, they tend to sorb to fine-particulate organic fractions (Kukkonen & Landrum 1996), often resulting in higher concentrations in sediments than in the overlying water. Consequently, organisms living in sediment are frequently exposed to elevated levels of HOC. The bioavailability of these contaminants to benthic invertebrates is determined by their sorptive behaviour (Power & Chapman 1992), the physicochemical characteristics of organic matter both in sediment (Gunnarsson et al. 1999; Granberg & Forbes 2006) as well as in the overlying and pore water (Kukkonen & Oikari 1991), and the feeding behaviour of the organisms (Kaag et al. 1997). The introduction and further trophic transfer of sediment-associated contaminants in aquatic food webs is thus governed by their bioavailability at lower trophic levels (Reynoldson 1987; Power & Chapman 1992). There are many studies reporting on the role of organic matter for the fate and behaviour of persistent organic pollutants (POP) when released to aquatic environments, however few studies have addressed the fate of modern pesticides and how they affect the bioavailability of these contaminants to aquatic organisms.

To feed a growing global human population and to compensate for our continuously increasing demand on the biosphere, the so-called "Global Footprint" (World Wide Fund for Nature [WWF] 2010), pesticides are generally thought to be indispensible. Indeed, pesticide usage is increasing, both in the number of chemicals and their quantities (Carvalho 2006). Pesticides contaminate aquatic environments in agricultural landscapes as a consequence of pesticide application, mainly through spray-drift, runoff or field drainage (Kreuger et al. 1999). In contrast to most HOC found in the environment, pesticides are produced to be toxic to target organisms and highly biologically active. Among pesticides, insecticides have the largest impact on benthic invertebrates, since aquatic insects constitute a large part of this faunal group. Consequently, pesticides have negative impact on non-target aquatic organisms, populations, and communities (Liess & von der Ohe 2005; van den Brink et al. 2009), and on important ecosystem services both aquatic and terrestrial, for example water supply, pollination, and soil erosion prevention (Björklund et al. 1999; Sandhu et al. 2010). When pesticides enter aquatic environments they sorb to organic matter in sediments, dissolved organic matter (DOM) and aquatic biofilms (Gao et al. 1998; Katagi 2006), and depending on their persistence they can accumulate in sediments. For example, DDT was banned in Sweden 1975, but DDT residues were detected in sediment cores 15 years later of concentrations as high as nearly 100 µg/kg (dw) (Kreuger et al. 1999.) Even though the majority of modern pesticides are less persistent than pesticides used until the mid 1970's they still have the potential to accumulate in the environment (e.g., Kreuger et al. 1999), thus being bioavailable for uptake and further interactions with organisms at different trophic levels of aquatic food webs.

1.2 Contaminant Uptake in Benthic Invertebrates

Benthic invertebrates live in close relation to sediment and aquatic biofilms and are therefore constantly exposed to contaminants associated with these matrices. Thus, they play a key-role in the transfer of aquatic contaminants to higher trophic levels (Reynoldson 1987; DiPinto 1996).

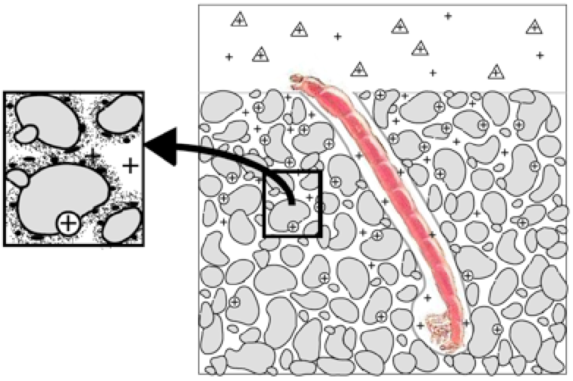


Figure 1 The benthic larval life stage of *Chironomus riparius* and its exposure routes to contaminants dissolved in overlying water and interstitial water (+), sorbed to dissolved organic matter (DOM, + in triangles), or sorbed onto sediment particles (+ in circles). The enlarged square to the left shows microbes (black spots) within the sediment and their surrounding extracellular polymeric substances (EPS), which also can sorb contaminants.

Benthic organisms are exposed to aquatic contaminants both through passive and dietary uptake routes (Fig. 1). Passive uptake is more important in aquatic insects that take up dissolved oxygen via gills or epithelium than those that breathe air (Buchwalter et al. 2002), and for the former it has been argued be the most important uptake mechanism (e.g., DiToro et al. 1991; Kaag 1997). However, benthic organisms feed more or less selectively on fine, organic-rich particles (Taghon, 1981), which constitute efficient sorption sites for hydrophobic contaminants (Kukkonen & Landrum 1996). Therefore, ingestion of sediment-associated contaminants have been emphasised and suggested to be the major route of uptake (e.g., Kaag 1997; Forbes et al. 1998; Weston et al., 2000). However, the major route of uptake can vary among different contaminants to the same organisms. For example, Selck and co-workers, found passive uptake of cadmium to be the major route of uptake (1998) whereas the opposite was found for a PAH (2003) for the sediment-dwelling *Capitella* sp I.

The bioavailability of organic contaminants in aquatic environments is affected by the presence of organic matter (Kukkonen & Oikari 1991; Gunnarsson et al. 1999; Haitzer et al. 1999; Granberg & Forbes 2006). DOM-quantity and sorption properties affects dissolved contaminants negatively as the DOM-contaminant complex gets too large to pass over biological membranes. Easily digested organic matter has a positive impact on dietary uptake (Gunnarsson et al. 1999), due to a higher ingestion rate of particles with higher organic matter quality (Granberg

& Forbes 2006). Also, feeding strategy of benthic invertebrates affects the uptake of organic contaminants (Kaag et al. 1997), where selectively feeding organisms increase their dietary uptake by feeding on fine organic particles onto which hydrophobic organic contaminants primarily sorb (Kukkonen & Landrum 1996). Hence, organic matter quantity and quality have an impact on uptake and bioavailability of organic contaminants to benthic invertebrates.

1.3 Organic Matter and Bioavailability in Aquatic Environments

Biofilms cover most surfaces in aquatic environments and are complex matrices that consist of algae, bacteria, fungi, detritus, and protozoa (Stevenson 1996). Biofilms also contain a large fraction of extracellular polymeric substances (EPS), which are microbial exudates, for example polysaccharides, proteins, nucleic acids, and lipids (Decho 1990; Battin et al. 2007). EPS are largely dominated by polysaccharides, which occur as tight capsules or dispersed slime surrounding microbial cells (Fig. 2), and as particles or colloids in the surrounding environment (Decho, 1990).

EPS contain abundant adsorptive ligands that sequester different types of DOM (Decho 1990), organic contaminants (Wolfaardt et al. 1994) and metals (Schlekat et al. 1998; Selck et al. 1999), either through binding or colloidal trapping. Thus, biofilms play an important role in the fate and partitioning of contaminants (Headley et al. 1998; Lawrence et al. 2001; Allan et al. 2004). Furthermore, biofilms also provide a food source and habitat for many invertebrate species that live in close association with biofilms (Decho & Lopez 1993; Peters & Traunspurger 2005). The easily digested EPS within biofilms can positively affect dietary uptake of associated contaminants, mediated by an efficient sorption of contaminants to EPS from overlying water, a rapid desorption during gut passage, and absorption into tissues (Decho & Lopez 1993).

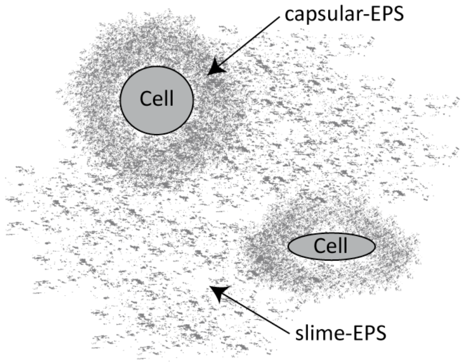


Figure 2 Extracellular polymeric substances (EPS) surrounding microbial cells, as tight capsular EPS or as slime-EPS between cells, forming biofilms (redrawn from Decho 1990).

By inhabiting aquatic biofilms, benthic invertebrates constitute a link between biofilm-associated contaminants and higher trophic levels. In streams, periphyton dominated biofilms make up a large proportion of the organic matter that directly interacts with running water, accordingly associations of pesticides in biofilm in agricultural streams after peak exposures are of high interest.

Humic substances (HS) are also important organic components of freshwater ecosystems and their sediments (Decho & Luoma 1994). They are considered to be recalcitrant and reduce the bioavailability of contaminants to benthic invertebrates (e.g., Decho & Luoma 1994; Selck et al. 1999). HS, however, although major constituents of the complex mixtures of DOM in natural waters, are not well-defined. DOM-quantity and quality in freshwater ecosystem varies as a result of their trophic status, hydrology, and characteristics of the catchment area (Thurman 1985). Forest lakes tend to have DOM of higher complexity than more productive lakes, due to the higher input of allochthonous organic matter of terrestrial origin, than the autochthonous organic matter origin from microbial exudates within the lakes (McKnight & Aiken 1998; Haitzer et al. 1999).

DOM-quality characteristics, for example the degree of aromaticity and molecular weight affects HOC sorption to DOM positively (Chin et al. 1994; Uhle et al. 1999; Akkanen et al. 2004). DOM with higher molecular weight have a higher ability to form hydrophobic domains, which are essential for associations with HOC (Chin et al. 1994; Cabaniss et al. 2000). Consequently, increased DOM-concentrations result in decreased passive uptake of DOM-associated HOC to aquatic organisms (Kukkonen & Oikari 1991; Haitzer et al. 1999; Akkanen & Kukkonen 2001). Furthermore, since DOM efficiently sorbs to EPS in biofilms, DOM can affect HOC associations with aquatic biofilms through rapid and efficient sorption (Carter & Suffet 1982), thereby also impacting the dietary uptake of HOC in benthic invertebrates, feeding on biofilms. Moreover, DOM-concentrations in freshwater ecosystems on the Northern Hemisphere are increasing (Monteith DT et al. 2007; Erlandsson et al. 2010), highlighting the importance of knowledge on how DOM affects the fate and bioavailability of contaminants, for example pesticides, in aquatic ecosystems.

2 Objectives of this Thesis

The main objective of the studies presented in this thesis was to further contribute to the knowledge on sorption and interaction of modern pesticides with different sources of organic matter and bio-films in aquatic ecosystems. In particular, to study how these interact-tions affect pesticide bioavailability to benthic invertebrates.

The specific objectives of each paper (I-IV) were to:

I study the effect of different organic matter qualities, for example microbial biofilm or humic substances (HS), on uptake, and hence bioavailability, of the insecticide chlorpyrifos to the sediment-dwelling midge larvae *Chironomus riparius*.

II estimate how chlorpyrifos bioavailability to *C. riparius* was affected by two common types of organic matter that dominate sediments, extracellular polymeric substances (EPS) and HS. We also tested the effects of quality (commercially available EPS or HS, or extracted at our laboratory) and quantity (low, medium and high) on pesticide uptake of these two organic matter.

III quantify the fate and bioavailability of insecticides with different hydrophobicity (carbofuran, lindane and chlorpyrifos) in a model freshwater ecosystem with primary producer (aquatic biofilms) and primary consumers (the snail *Theodoxus fluviatilis*) at three different concentrations of dissolved organic matter (DOM; low, medium and high).

IV evaluate the sorption behaviour of insecticides with different hydrophobicity (carbofuran, lindane and chlorpyrifos) to DOM of different origin and complexity, and also to quantify partitioning of insecticides to different molecular size-fractions within each DOM.

3 Materials and Methods

This is a summary of materials and methods used in all four studies included in this thesis. For more detailed information on each study, I refer to the specific paper in the appendix.

3.1 Test Organisms and Substances

The test organisms used (Papers I – III) are benthic invertebrates with different feeding behaviour, i.e., selective and non-selective feeding behaviour. Both organisms are common in European freshwater ecosystems and play an important role in the organic matter cycling (Skoog 1978; Pinder 1986). They also constitute an important food source for higher trophic levels.

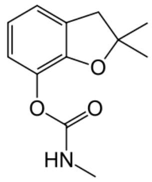
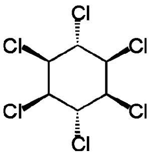
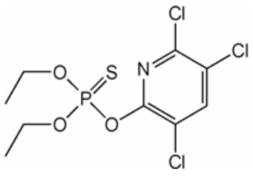
Chironomus riparius (Papers I-II) is a non-biting midge with a holometabolic life cycle of four different developing stages (egg, larvae [instar], pupa and adult [imago]). Furthermore, as larvae they have four additional developing stages, where the last three instars, live in close relation to the sediment (Pinder 1986). They construct tubes of sediment particles, in which they live (Pinder 1986; Berg 1995), and selectively feed on surface sediment as deposit feeders (Åkerblom & Goedkoop 2003). The larvae's close association to sediment, pore water within the sediment, and the overlying water leads to both passive and dietary routes of uptake of potential contaminants (as shown in Fig. 1). *Chironomus* larvae were selected as test organisms due to their ecological relevance and recommended use in toxicity tests (e.g., Organisation for Economic Co-operation and Development [OECD] 2004). For the studies in this thesis, 4th instar larvae were collected from a continuous culture in our laboratory.

Theodoxus fluviatilis (Paper III) is a gilled snail that lives on hard substrata in the upper littoral zone of lakes and brackish waters, and in running waters (Kirkegaard 2006). The snail is a non-selective grazer (Neumann 1961) that predominantly feeds on aquatic biofilms and associated diatoms or other algae. Similar to the *Chironomus* larvae, *T. fluviatilis* is exposed to potential contaminants both through passive and dietary uptake, i.e., from overlying water, and also biofilms that they feed on. The snail was selected as a test organism to represent grazing primary consumers, and due to its ecological importance. Snails were collected in Lake Erken, and allowed to acclimatise to laboratory conditions, prior to experiments, in an aquarium with aerated water and periphyton-covered stones from Lake Erken.

Three insecticides (carbofuran, lindane and chlorpyrifos) were used as model substances for HOC. In the two first studies only chlorpyrifos was used, but the two last studies also included carbofuran and lindane. All substances were ^{14}C-labeled, measured with liquid scintillation counting. Additions were consistently below sublethal concentrations for the test organisms. These three insecticides covered a broad range of log K_{OW}, from nearly hydrophilic to hydrophobic (Tab. 1). They were chosen as model substances since they have been extensively used all over the world both in agricultural and urban pest control (Ware 1993). Nowadays all three are banned in Sweden, but they are still detected in surface waters and sediments (Graaf et al. 2010).

All three insecticides are neurotoxins and affect the neurotransmitter acetylcholine of target organisms, leading to paralysis and

Table 1 Structural formulae, octanol-water partitioning constants (log K_{OW}) and molecular weights (Da) of the pesticides used in this thesis.

	Carbofuran	Lindane	Chlorpyrifos
Structural formula			
Log K_{OW}[a]	1.5	3.5	4.7
Molecular weight[a]	221.3	290.8	350.6

[a] Tomlin 2009

consequently death. Carbofuran and chlorpyrifos inhibit the enzyme acetylcholinesterase, thus increasing both the level and the duration of action of acetylcholine. Lindane increases the release of acetylcholine. These neurotoxins, of course, also have a negative impact on non-target aquatic organisms, and can affect freshwater populations, communities, and ecosystems (Liess & von der Ohe 2005; van den Brink et al. 2009).

3.2 Experimental Design (Papers I–IV)

3.2.1 Bioavailability of Chlorpyrifos in Bottom Substrates with Different Sources of Organic Matter (Papers I-II)

In the first study, bioavailability of chlorpyrifos to *C. riparius* was quantified in microcosms containing silica particles (40-63 µm, covering the size range that *Chironomus* larvae ingest; Davies et al. 1999) and 50 mL sterilised lake water (Lake Ekoln) with additions of four different types of organic matter:

1. dissolved humic acids,
2. sterile sediment,
3. sediment, and
4. microbial extract

These treatments represent organic matter that naturally occurs in freshwaters, except sterile sediment. The latter treatment was included to address the importance of living microbiota to pesticide assimilation and further uptake in benthic organisms. Controls lacking organic matter addition were included. Also, blanks, without chlorpyrifos addition, were run for controls, sediment and microbial extract treatments. Sieved fine sediment (Lake Ekoln) was used for sterile sediment, sediment and microbial extract treatments. In these treatments the sediment was autoclaved, untreated or used for the extractions of sediment microbes (Mermillod-Blondin et al. 2001), respectively. Untreated sediment and sediment microbes were added to microcosm, followed by six days of biofilm growth for sediment and microbial extract treatments, respectively. Artificial humic acids (Humic acid sodium salt, Sigma–Aldrich) were dissolved in sterile lake water (50 mg/L) and autoclaved sediment was added to the silica particles for humic and sterile sediment treatments, respectively. Total carbon, total nitrogen and EPS (quantified as polysaccharides) were measured in all treatments. Additionally, bacterial abundance in sediment and

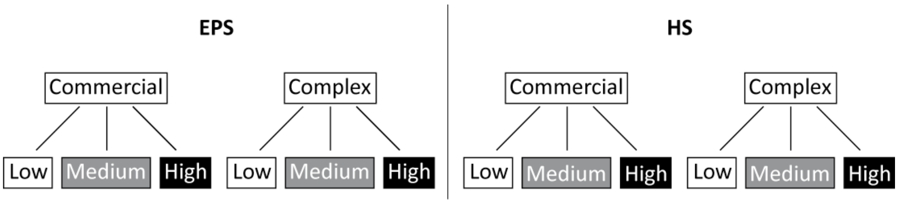


Figure 3 Schematic overview of the experimental treatments in the second study. Extracellular polymeric substances (EPS) and humic substances (HS) with different origin, commercially available or extracted at our laboratory (i.e., complex) were each used at three different concentrations (low, medium, and high).

microbial extract treatments were quantified by staining cells with SYTO 13 (as described in Liess & Haglund 2007).

Also in the second study, the bioavailability of chlorpyrifos to *Chironomus* larvae was quantified. However, this time two different types of organic matter (EPS or HS) of different qualities (commercial or complex) were used to address the impact of molecular complexity of EPS and HS on pesticide assimilation, at different concentrations (low, medium and high) (Fig. 3).

Microcosms were established, which contained silica particles (40-63 µm), artificial lake water (M7-medium; OECD 2004), and four different types of organic matter:

1. commercial EPS (Alginic acid sodium salt, Sigma–Aldrich),
2. complex EPS (see below),
3. commercial HS (Humic acid sodium salt, Sigma–Aldrich), and
4. complex HS (see below)

The three different concentrations (low, medium and high) resemble those found in natural sediments. Complex EPS were cultured from sediment microbes, cultivated for six days (see above), after which microbial EPS were extracted by centrifugation twice; first to separate cells and associated EPS from the overlying water (1,700×*g*, 15 min) then to separated EPS from cells (7,100×*g*, 30 min). The supernatant (mainly containing high-molecular polymeric chains, i.e., polysaccharides) was freeze-dried. Complex HS (i.e., humic and fulvic acids) were isolated from boreal lake water (Lake Vikasjön) with DEAE-cellulose according to Miles et al. (1983), and fulvic and humic acids were extracted according to Thurman & Malcolm (1981), and freeze-dried.

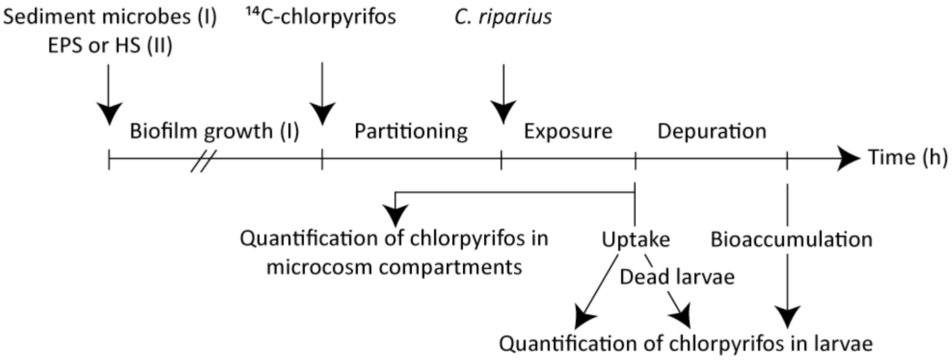


Figure 4 Schematic overview of experimental set up (Papers I-II) of *Chironomus riparius* larvae exposed to ^{14}C-labeled chlorpyrifos in microcosm with bottom substrate with different organic matter additions (see text for details).

After organic matter additions (Papers I-II), and biofilm growth (6 d; Fig. 4 [Paper I]), sublethal concentrations of ^{14}C-labled chlorpyrifos (0.7 µg/L) were added to the water phase of all treatments, and allowed to partitioning for 18 h (Paper I) or 24 h (Paper II). Then, sets of eight (Paper I) or ten (Paper II) 4th instar larvae were transferred to microcosms and exposed for 24-26 h (Fig. 4). After exposure, larval condition was scored as normal behaviour, impaired behaviour or dead (Paper I). Three (Paper I) or five (Paper II) larvae were taken for determination of chlorpyrifos uptake (Fig. 4). The remaining five larvae were depurated (21-24 h) in microcosms with uncontaminated water, sand and food (Tetraphyll®) (Fig. 4). After depuration, chlorpyrifos tissue accumulation was quantified (Fig. 4). Also, freshly formalin-killed larvae were exposed for 24 h to examine passive uptake of chlorpyrifos over the epithelium (Fig. 4). Water samples were taken prior to and after exposure, while bottom substrate samples were taken after exposure (Fig. 4). All samples were analysed for chlorpyrifos concentrations by liquid scintillation counting of ^{14}C.

3.2.2 Fate and Uptake of Insecticides along DOM-gradients (Paper III)

The fate, uptake and bioavailability of the insecticides carbofuran, lindane, and chlorpyrifos in microcosms with autotrophic biofilms and the snail (*Theodoxus fluviatilis*) were examined in this study. The experimental design was set up to mimic short-term peak exposures in agricultural streams (24-48 h). Surface waters from three boreal lakes were used, corresponding to three different DOM-treatments:

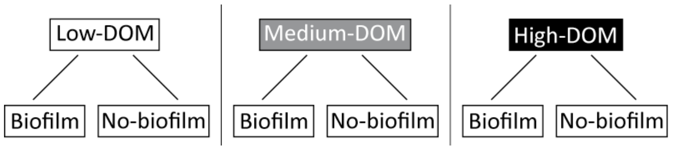


Figure 5 Schematic overview of the experimental treatments in the third study with three concentrations of dissolved organic matter (DOM; low, medium, and high) in microcosms with ("Biofilm") and without ("No-biofilm") biofilm.

1. Low-DOM (TOC 9.0±1.1 mg/L, Lake Björklinge-Långsjön),
2. Medium-DOM (TOC 19.6±0.35 mg/L, Lake Siggeforasjön), and
3. High-DOM (TOC 35.3±1.5 mg/L, Lake Vikasjön)

Biofilms were grown on marble tiles (10 × 10 cm) covered with lake water for 30 days in a climate room (20±2 °C) under a 16 h/8 h light/dark cycle of photosynthetically active radiation (PAR, 400-700 nm). Microcosms were established with filtered lake water and tiles with biofilms were randomly allocated to each DOM-treatment in microcosms ("biofilm treatments") (Fig. 5). Additional treatments lacking biofilm covered tiles ("no-biofilm treatments") were included to examine the passive uptake of insecticides in snails decoupled from feeding, i.e. reflecting the diffusion over epithelium and respiratory organs (Fig. 5). Also, blanks, lacking insecticide additions, were run for each biofilm DOM-treatment to quantify background radiation.

^{14}C-labeled insecticides were added at sublethal concentrations (3.8±0.05 nmol/L [carbofuran], 3.0±0.03 nmol/L [lindane], and 5.3±0.03 nmol/L [chlorpyrifos]) and allowed to partition for 24 h. Sets of three snails were exposed for 24 h in each replicate, after which they were taken for determination of insecticide uptake. Biofilm on tiles were photographed and analysed by image-analysis to estimate the grazing rate by snails, and collected for insecticide assimilation. Subsamples of biofilms were taken from blanks for total carbon and total nitrogen analysis. All four DOM-treatment waters were also analysed for TOC-concentrations and absorbance. Water samples were taken 5, 24 and 48 h after insecticide addition to quantify pesticide water concentrations. All samples were analysed for insecticide concentrations by liquid scintillation of ^{14}C.

Since pesticide additions differed among treatments, we calculated bioconcentration factors (BCF = [organism] / [surrounding media], at steady state), as these allow for straightforward comparisons of pesticides sorption to biofilms and uptake in snails.

3.2.3 Sorption of Insecticides to Size-fractions of Different DOM (Paper IV)

In the last study we (1) quantified pesticides associations of different hydrophobicity with different types of DOM, and (2) studied if the associations varied among different molecular size-fractions of DOM. Four different sources of DOM were used, two commercially available and two extracted from boreal Lake Vikasjön, to cover a wide range of different DOM:

1. Suwannee River fulvic acid (SR FA),
2. Suwannee River humic acid (SR HA),
3. Lake Vikasjön fulvic and humic acids (LV FAHA), and
4. Lake Vikasjön dissolved organic acids (LV DOA)

In contrast to the commercially available HS of terrestrial origin used in Paper II, the two commercial DOM were of aquatic origin, extracted from Suwannee River in Georgia, USA (International Humic Substances Society, IHSS). SR FA only contained fulvic acids and the SR HA contained only humic acids. The other two DOM-sources, extracted from Lake Vikasjön, contained both fulvic and humic acids (as described in Paper II; LV HAFA) and dissolved organic acids isolated by DEAE-cellulose and desalted by dialysis (LV DOA). Water from all four treatments were analysed for TOC-concentrations and absorbance.

High-performance size exclusion chromatography (HPSEC) with a UV-detector was used for chromatographic characterisation of DOM-samples (Fig. 6). Molecular size-fractions of DOM were fractionated by a TSKgel®-column (G2000SW, Tosoh Bioscience), using a phosphate buffer (NaH_2PO_4, pH 6.8, $I = 0.05$ mol/L) as the mobile phase at a flow rate of 0.7 mL/min. Void volume and total permeation volume was determined with blue dextran and acetone, respectively, and calibration curve was obtained using Polystyrene Sulphonates (PSS; 32, 17, 8.0, 4.3, 1.0 kDa).

Each DOM-sample was dissolved in 15 mL 1/1:MilliQ/phosphate buffer in Teflon-tubes (TOC 30 mg/L). Then ^{14}C-labeled carbofuran, lindane or chlorpyrifos were added to a final concentration of 30 µg/L, and allowed to associate with DOM for 48 h on a shaking table (100 revolutions per minute [RPM], dark, room temperature). Samples of DOM and pesticide (100 µL) were injected into the TSKgel®-column, and fractionated (Fig. 6) into nine molecular size-fractions: ">30", "30-25", "25-20", "20-15", "15-10", "10-5" (all kDa), "5 kDa<H_20", "H_20", "sorbed on column". Concentrations of pesticides in each size-fraction were measured by liquid scintillation. Blanks for each DOM-sample and ^{14}C-internal standard allowed correction for background levels and quenching.

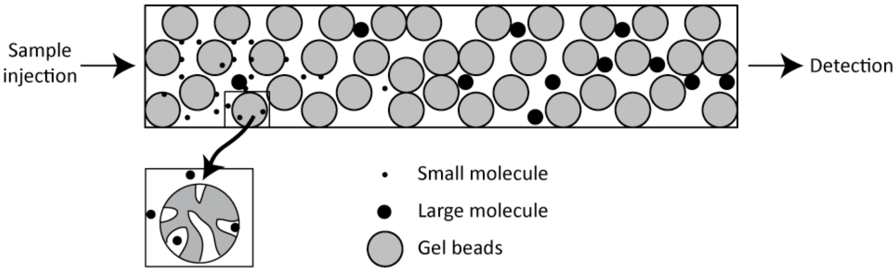


Figure 6 Schematic of a size exclusion chromatography column using HPSEC. The sample is injected and transported through the column with an eluent, i.e., a mobile phase. The molecules will be separated into molecular size-fractions by the gel beads within the stationary phase of the column, where small molecules will diffuse into the pores of the gel beads (shown in the enlarged square) and the larger molecules will not. Hence, the larger molecules will pass through the column faster, i.e., have a shorter retention time and will give a signal in the detector (e.g., UV-detector) first.

Due to low recoveries (%) of the insecticides, additional recovery measurements after HPSEC were performed with high-pressure liquid chromatography coupled with tandem mass spectroscopy (HPLC/MS/MS) for carbofuran and chlorpyrifos as described by Jansson & Kreuger (2010). We also studied the effect of DOM on carbofuran and chlorpyrifos recovery, by dissolving insecticides in SR FA. Carbofuran and chlorpyrifos (30 µg/L) were dissolved with 1/1: MilliQ/phosphate buffer or with SR FA (TOC 30 mg/L) dissolved in 1/1:MilliQ/phosphate buffer. The pesticides were allowed to partition to SR FA over night on shaking table (100 RPM, dark, room temperature). Both treatments were fractionated on the TSKgel® column. Five samples were collected: (1) pesticides dissolved in the columns void volume, i.e., prior to the largest size-fraction had passed the column, (2-3), pesticides dissolved in the permeation volume, divided in two equal samples, and (4-5) pesticides dissolved in the mobile phase after the permeation volume, divided in two equal samples. Subsamples (1.5 mL) were taken, followed by addition of 30 µL internal standard and injected into the HPLC/MS/MS. Additionally, sample prior to HPSEC-fractionation was analysed by HPLC/MS/MS to quantify the recovery of carbofuran and chlorpyrifos. This sample was diluted 50 times to compensate for the dilution of the mobile phase during the HPSEC-fractionation, and corresponded to 100% recovery.

4 Results and Discussion

4.1 Bioavailability of Chlorpyrifos in Bottom Substrates with Different Sources of Organic Matter (Papers I-II)

The bioavailability of chlorpyrifos to *Chironomus* larvae was affected by different organic matter additions to bottom substrate in our studies. However, different effects were seen, suggesting that not only the quantity but also the quality of organic matter affects the bioavailability of pesticides.

4.1.1 Sediment Microbes and Biofilms Positively Affect Bioavailability (Paper I)

Chlorpyrifos uptake in larvae was nearly twice as high in the presence of living sediment and microbial biofilms as in controls (Fig. 7). Conversely, larval uptake in treatments with additions of humic acids or sterile sediment did not differ from that in controls (Fig. 7). Different sources of DOM evidently affected uptake of chlorpyrifos in larvae. Also, after depuration, chlorpyrifos concentrations in larval tissue, i.e., bioaccumulation of chlorpyrifos, followed the same pattern with higher larval concentrations in treatments with sediment and biofilms than those with sterile sediment or humic substances, or controls (Fig. 7). Treatment-induced differences were even larger after depuration than when larval uptake was quantified directly after exposure (Fig. 7). Our results show that living microbes and biofilms strongly increase the bioavailability of chlorpyrifos to *C. riparius*.

The presence of living sediment microbes and biofilms probably contributed with an easily digested food source (i.e., EPS and microbes) onto which chlorpyrifos had sorbed. *Chironomus* larvae are known to ingest large number of bacteria (Johnson et al. 1989), and selectively feeding benthic invertebrates ingest EPS to a high extent (Decho &

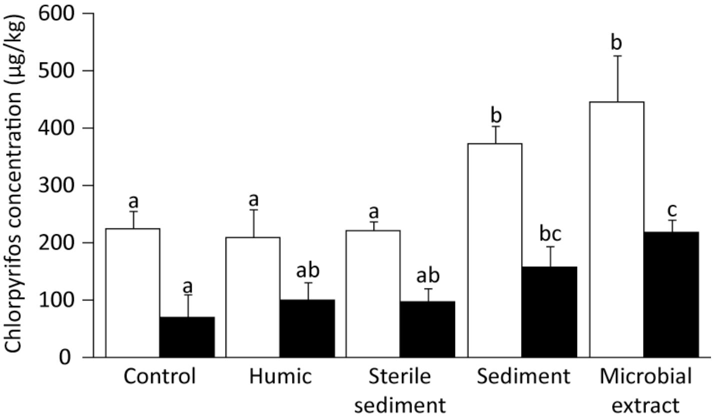


Figure 7 Chlorpyrifos concentrations in larvae (µg/kg, mean±1 SD) after exposure (white bars) and depuration (black bars) in controls and treatments with additions of different sources of organic matter. Bars with different letters are significantly differed (Bonferroni/Dunn).

Moriarty 1990). Easily digested food sources in the sediment increases the bioavailability of sediment-associated organic contami-nants to benthic organisms (Wolfaardt et al. 1994; Gunnarsson et al. 1999; Granberg & Forbes 2006; Thorsson et al. 2008). Thus, the higher chlorpyrifos uptake in sediment and microbial extract treatments may have been an affect of a more easily digested organic matter, with associated pesticide, compared to the other treatments.

Our results show that the organic matter quality affects chlorpyrifos bioavailability since uptake in sterile sediment treatment was nearly half of that found in the sediment and microbial extract treatments. EPS-concentration in the bottom substrate, i.e., the quantity, of the sterile sediment treatment was as high as in the two other treatments. The higher uptake in the two latter treatments is, presumably, an indicator of the higher food quality in these due to the presence of living microbes. *C. riparius* is known to selectively feed on high-quality organic matter (Åkerblom & Goedkoop 2003), as most deposit feeders (Taghon et al. 1978). Chlorpyrifos was probably taken up to a higher extent in treatments with higher food quality. Indeed, our results show that microbes and biofilms increased the uptake in *Chironomus* larvae through dietary uptake. Our results further support the importance of dietary uptake as an important route of uptake of organic contaminant to benthic invertebrates. However, larval concentrations after passive uptake in dead larvae was as high as 46% of that in controls, revealing that also passive uptake was of great importance. The presence of microbes and biofilms in the bottom substrate affected larval condition; no normal larval behaviour was observed, and the highest number of dead larvae was found in this treatment. These effects may be a consequence of the higher dietary uptake of this neurotoxin, but also that the larvae's close relation to surface sediment increased the passive uptake of chlorpyrifos due to associations of the pesticide with the biofilm surrounding the larvae. Thereby, the presence of biofilm resulted in a higher impact on the larvae, probably both through increased dietary and passive uptake.

Water concentrations of chlorpyrifos were higher in treatments with humic, sediment and microbial extract additions (ranging 0.65±0.09 to 0.67±0.04 μg/L) than in controls (0.47±0.02 μg/L), probably due to the higher concentrations of DOM (i.e., HS and EPS) that allowed for chlorpyrifos sorption.

Bioconcentration factors (BCF) ranged from 320±63 to 670±120 L/kg, with the highest BCF in microbial extract treatment, and the lowest in humic treatment. BCF in controls (480±67 L/kg) were 1.5 times higher than those in humic treatments, thus uptake of chlorpyrifos was decreased in presence of humic substances. This assumption is supported by earlier studies showing that the presence of DOM decreases the bioavailability of organic contaminants (Kukkonen & Oikari 1991; Haitzer et al. 1999; Akkanen & Kukkonen 2001).

Chlorpyrifos concentrations in the bottom substrate were higher only in the sterile sediment treatments than in controls. The major share of added chlorpyrifos was recovered in the bottom substrate (58±1.1–79±2.0%) showing a high sorption of this hydrophobic compound to the bottom substrate with organic matter of different origin.

4.1.2 EPS and HS Negatively Affect Bioavailability (Paper II)

Chlorpyrifos uptake in larvae was inversely correlated to the quantity of both EPS and HS (Fig. 8). The observed decreased in larval uptake of chlorpyrifos between low and high EPS-concentrations was as high as 90%. For HS-treatments the uptake decreased with 60%, showing a stronger negative effect on chlorpyrifos bioavailability in presence of EPS. The negative affect of EPS on chlorpyrifos uptake was contradicttory to what we expected, since EPS are found to be an important food source (e.g., Decho & Lopez 1993) and a vector for sediment-associated contaminants to benthic invertebrates (Harvey & Luoma 1985; Wolfaardt et al. 1994; Schlekat et al. 1998; Selck et al. 1999).

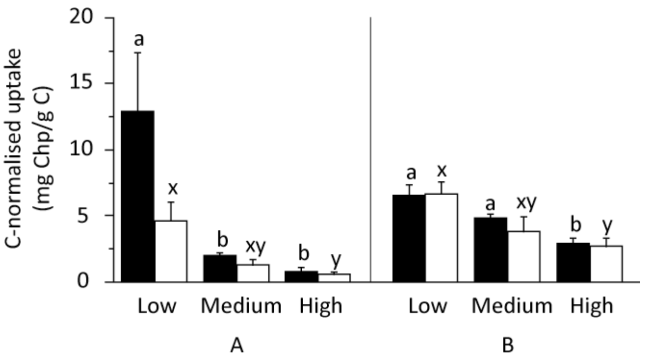


Figure 8 Carbon-normalised larval uptake of chlorpyrifos (Chp, mean±1 SE, mg /g) for commercial (black bars) and complex (white bars) EPS (A) and HS (B), respectively, added at three concentrations (low, medium and high). Bars within each treatment with different letters are significantly different.

The larger negative impact of EPS on chlorpyrifos bioavailability compared to HS, probably reveals a lower dietary uptake, likely due to a lower food quality of EPS. As benthic invertebrate's ingestion rates generally increases with increasing food quality (Taghon 1981; Granberg & Forbes 2006), larvae in EPS-treatments possibly ingested a lower amount of chlorpyrifos contaminated bottom substrate than in the HS-treatments. Bottom substrate concentrations of chlorpyrifos did not differ between commercial EPS and HS or between complex EPS and HS. However, C/N-ratios in commercial and complex EPS-treatments were between three and nine times higher, respectively than those in the HS-treatments. C/N-ratio is a commonly used as an indicator of food- or organic matter quality. Therefore we conjecture that chlorpyrifos bioavailability to larvae in EPS-treatments was likely lower due to a lower ingestion rate of EPS-substrate than of HS-substrate, due to the lower food-quality. The conclusion that food quality affected larval uptake is further supported by the results of Paper I. In Paper I larval uptake of chlorpyrifos was nearly twice as high in untreated sediment as in sterile sediment treatment (Fig. 7), despite the fact that these treatments were similar in their EPS- and chlorpyrifos concentrations (data not shown). The latter, being sterilised, had a lower organic matter quality since it was lacking living microbiota, probably resulting in a lower ingestion rate by *Chironomus* larvae and consequently lower dietary uptake.

Furthermore, the association of chlorpyrifos to EPS and HS in the second study probably also had an impact on the passive uptake of chlorpyrifos in the larvae, decreasing the bioavailability. *Chironomus* larvae have a highly permeable epithelia involved in the uptake of dissolved O_2 (Buchwalter et al. 2002), a tissue that readily allows passive uptake of dissolved contaminants. Passive uptake of chlorpyrifos in dead larvae was 40% of that in controls, showing that this route also was quantitatively important. The negative relationship between EPS/HS quantities and chlorpyrifos uptake further stresses the importance of passive uptake in larvae. Probably the higher TOC-concentrations in low than in high EPS-/HS-concentrations allowed a higher degree of chlorpyrifos sorption to dissolved EPS/HS, resulting in molecular complexes too large to pass epithelium membranes and subsequently resulting in a lower bioavailability. However, TOC-concentrations of the commercial HS treatment did not increase with increased addition of HS in bottom substrate. Thus, the decreased larval uptake of chlorpyrifos in commercial HS-treatments between low and high HS-concentrations, despite equal TOC-concentrations, shows that not only passive uptake was negatively related to EPS/HS-concentrations, but also the dietary uptake. Possibly the decreased chlorpyrifos uptake was due to dilution of chlorpyrifos in the bottom substrate with increasing amount of organic matter (Ballance et al. 2002), leading to lower dietary uptake.

Interestingly, the negative effect of EPS and HS on chlorpyrifos uptake was more pronounced for the commercial polymers. Consequently, not just the quantity of EPS and HS, but also their qualitative characteristics had an impact on the uptake of chlorpyrifos. The commercial HS consisted of humic acids extracted from soil, being more recalcitrant than the complex HS extracted from lake water, containing both humic and less complex fulvic acids. The commercial EPS contained homopolymeric chains of anionic polysaccharides ($[C_6H_8O_6]_n$) (Sigma-Aldrich, advise note), whereas the complex EPS contained polysaccharides produced by sediment microbes, probably of higher molecular diversity (Decho 2006, pers. comm.). EPS-quality can, however, vary within sediments and be either labile or refractory for consuming organisms (Decho & Lopez 1993). The different qualities among our organic matter additions probably altered the association with chlorpyrifos.

After depuration, chlorpyrifos concentration in larval tissue was close to zero. In Paper I approximately 60% of chlorpyrifos was lost through depuration. The high loss of chlorpyrifos during depuration suggests that the pesticide was strongly associated with gut material leading to low degree of absorption into tissue as the contaminated bottom substrate passed through the larval gut tract.

4.2 Fate and Uptake of Insecticides along DOM-gradients (Paper III)

DOM-concentrations affected the fate and the bioavailability differently among all three insecticides in our simple model ecosystem.

Uptake in snails was affected by DOM-concentrations depending on the insecticide hydrophobicity (Fig. 9). For example, carbofuran uptake in no-biofilm treatments was lower in medium- and high- DOM treatments than in low-DOM treatment (Fig. 9A). Conversely, chlorpyrifos uptake was higher in medium- and high-DOM treatments compared to low-DOM in no-biofilm treatments (Fig. 9C). Consequently, insecticide hydrophobicity plays an important role for the uptake and hence their bioavailability to benthic invertebrates.

Carbofuran BCF for snail (BCF_{snail}) was twice as high in low-DOM, than those in medium- and high-DOM treatments (Fig. 10B), probably due to a higher uptake of freely dissolved carbofuran in treatments with low-DOM concentration. Also, chlorpyrifos uptake was 50 and 30% lower in biofilm treatments for medium- and high-DOM treatments, respectively, than in no-biofilm treatments (Fig. 9C). We interpret this as a result of a lower amount of dissolved chlorpyrifos in

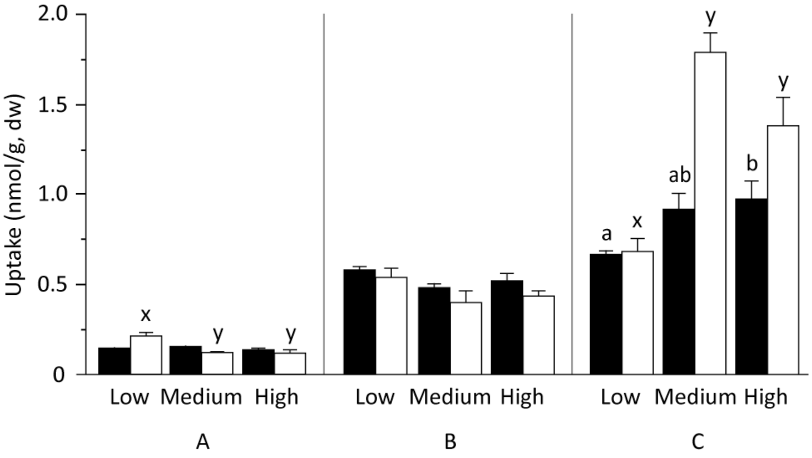


Figure 9 Uptake (nmol/g dw, mean±SE) of carbofuran (A), lindane (B) and chlorpyrifos (C) in snails exposed in water from low- (Low), medium- (Medium) and high- (High) DOM treatments. Black bars show biofilm treatments and white bars no-biofilm treatments. Bars with different letters within each pesticide are significantly different.

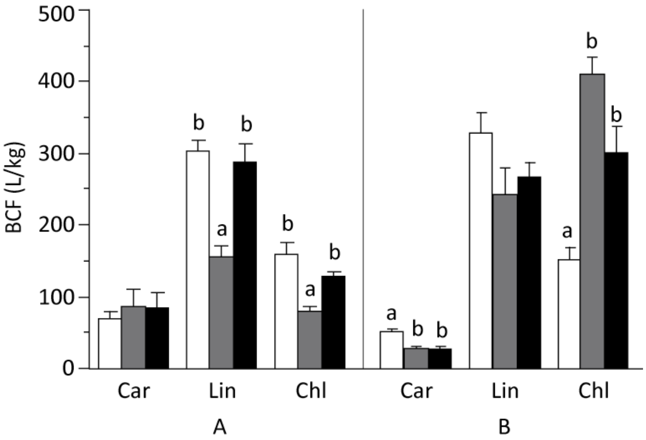


Figure 10 Bioconcentration factors (BCF, L/kg, mean±SE) of biofilms (A) and snails, (B) for carbofuran (Car), lindane (Lin), and chlorpyrifos (Chl) from low- (white bars), medium- (grey bars) and high- (black bars) DOM treatments. Bars with different letters within each pesticide are significantly different.

the overlying water due to efficient sorption to biofilms. The negative effect of DOM-concentrations of carbofuran uptake in no-biofilm treatments and the decreased uptake of chlorpyrifos in presence of biofilms reveals that passive uptake of dissolved insecticides over the snail's gills or other epithelium was an important route of uptake.

DOM-concentrations had an impact on insecticide sorption to biofilms. However, sorption of lindane and chlorpyrifos to DOM was not straightforward concentration-dependent (Fig. 10A), with lower BCFs for biofilm ($BCF_{biofilm}$) for medium-DOM than for the other two DOM treatments. The lack of unidirectional effects of DOM-concentration suggests that not only the DOM-quantity, but also DOM-quality properties was of importance for the fate of these insecticides,

Common DOM-quality characteristics are molecular weight and degree of aromaticity, i.e., the ratio of aromatic elements in the molecules, derived from various physicochemical analyses (Chin et al. 1994; Weishaar et al. 2003). As the molecular weight of DOM increases, the aromaticity and the hydrophobic character also increases. As a consequence, hydrophobic contaminants are sorbed to a higher extent (Kukkonen & Oikari 1991; Chin et al. 1994; Uhle et al. 1999; Akkanen et al. 2004). Estimated DOM-quality characteristics for our DOM-treatments resulted in weight-average molecular weights of 790, 1900, and 2000 Da, and aromaticities of 9.8, 29, and 31% for low-, medium-, and high-DOM treatments, respectively. These descriptors show that the medium- and high-DOM were rather similar in aromaticity and molecular weight. Thus these qualitative descriptors cannot explain the observed differences in $BCF_{biofilm}$ for lindane and chlorpyrifos (Fig. 10A). However, most studies on the association of HOC to DOM have been performed with nonpolar, planar contaminants (e.g., PAHs and PCBs; see earlier mentioned references), whereas lindane and chlorpyrifos are non-planar and have more bulky 3D-molecules. This may have affected the association with DOM due to steric hindrance, i.e., prevention of molecular interactions due to the spatial structure of the molecule. Furthermore, $BCF_{biofilm}$ for lindane was 14% higher than that of chlorpyrifos across DOM-treatments, showing that other chemical properties of pesticides than hydrophobicity (i.e., molecular weight, volume or connectivity) may effect their associations with DOM.

Water chemistry variables for the different boreal lakes could also have affected DOM-quality characteristics, hence affecting the sorption of insecticides and ultimately bioavailability. The alkalinity in our low-DOM (2.93±0.04 meq/L) was 24- and 10-times higher than those of medium- and high-DOM treatments, respectively. The higher ionic strength in the low-DOM treatment may have coiled the DOM molecules to a higher extent, making them smaller (Her et al. 2002), and more hydrophobic (Carter & Suffet 1982), likely increasing the association of organic contaminants (Chin et al. 1994). Therefore, the higher sorption of lindane and chlorpyrifos in the low-DOM than in the other DOM treatments may have contributed to a proportionally high $BCF_{biofilm}$ and the non-unidirectional trend of $BCF_{biofilm}$ of lindane and chlorpyrifos. In conclusion, even though we could not pinpoint one specific characteristic of DOM-quality, or a chemical variable affecting DOM-quality, our results indicate that both the DOM-quantity and quality had an impact on the fate and bioavailability of insecticides across a wide range of log K_{OW}.

As expected, water concentrations of the two more hydrophobic insecticides, chlorpyrifos and lindane, were affected by presence of biofilms. Chlorpyrifos water concentrations decreased by up to 10±1.0% in the presence of biofilm, whereas lindane concentrations, somewhat surprisingly, increased by 17±0.7% across DOM-treatments. We speculate that the biofilm may have altered parts of the DOM-pool, reducing the number of specific chromophores of importance, why a higher signal of excited light was detected by the scintillation counter than in no-biofilm treatments. Alternatively, the presence of a biofilm created a thin surface film in the water/air interface of DOM and/or EPS released from the biofilm, prohibiting volatilisation of this relatively volatile insecticide (Tomlin 2009) resulting in higher water concentrations (McLachlan 2011, pers. comm.).

Although the major share of the added insecticides was recovered in the water phase (ranging from 112±1.7, to 57±1.6%), and the sorption to biofilms was relatively low in all treatments (<1%), their biofilm concentrations (as pmol/g) of carbofuran, chlorpyrifos and lindane, respectively, were on average 75-, 130- and 380-times higher than those in water (as pmol/ml). Therefore, the rapid sorption of pesticides to biofilms may dampen initial toxic effects of pesticides to many aquatic organisms after peak exposures. However, negative effects on the micro- and meiofauna of biofilms may instead be strong.

4.3 Sorption of Insecticides to Size-fractions of Different DOM (Paper IV)

Our previous studies (Papers I-III), with a high resolution of organic matter, showed that, not only the DOM-quantity or presence of organic matter in sediment, but also the quality of the different DOM and organic matter (i.e. bottom substrate coatings and biofilms) mediates pesticide fate and bioavailability. Consequently, in this study we aimed to unravel the interactions among various natural DOM and insecticides used in previous studies. Most studies reporting DOM sorption of HOC have been done with PAHs and PCBs. Accordingly, we wanted to broaden the knowledge of HOC sorption to DOM, studying modern pesticides and how these non-planar more bulky molecules interact with DOM.

To do this, a non-destructive DOM-fractionation approach was tested to separate DOM with associated insecticides into different size-fractions. We used an HPSEC method, unfortunately without success due to sorption of insecticides to the HPSEC-column. The endeavour to contribute to further knowledge on how DOM interacts with contaminants in aquatic ecosystem failed. Nonetheless, this method development is of importance, and possibly the failure can encourage further development of a non-destructive method.

To fractionate our DOM by HPSEC we used a silica-based stationary phase (TSKgel®), commonly used to separate natural DOM (for example, Peuravuori & Pihlaja 1997; Alberts et al. 2002; Wu et al. 2007;

Table 2 Recovery (%, mean±1 SE) of pesticides after HPLC-fractionation for each DOM-treatment (Suwannee River fulvic acid [SR FA] Suwannee River humic acid [SR HA] Lake Vikasjön fulvic and humic acids [LV FAHA] and Lake Vikasjön dissolved organic acids [LV DOA]).

Pesticide	SR FA	SR HA	LV FAHA	LV DOA
Carbofuran	19±11	33±13	32±14	51±1.5
Lindane	1.9±0.75	50±8.3	36±7.7	35±6.5
Chlorpyrifos	5.1±0.20	8.9±2.6	5.2±0.56	6.7±1.1

Temnerud et al. 2009). The TSKgel® column separated our DOM samples efficiently, and there were some differences among DOM treatments. SR FA had a molecular size range of 3.7–22 kDa, whereas SR HA had 3.7–81 kDa. Our DOM-samples from Lake Vikasjön, LV FAHA and LV DOA showed the same size range as SR HA. However, SR HA had a signal intensity of the largest size-fraction that was seven and three times higher than LV FAHA and LV DOA, respectively. The higher signal in SR HA reveals a higher complexity of Suwannee River DOM than that in Lake Vikasjön (McKnight & Aiken 1998).

Insecticide recoveries after HPSEC-analysis were very low ranging from 1.9 to 51% (Tab. 2). Chlorpyrifos almost consistently showed the lowest recovery, less than 10% across DOM-treatments. Also, measured insecticide concentrations showed high variability (data not shown), and a cumulative increase in concentration was observed for carbofuran and lindane. Hence, the results revealed some kind of systematic error within the analysis.

4.3.1 Insecticide Sorption to the Column

HPLC/MS/MS was used to quantify carbofuran and chlorpyrifos recoveries after the HPSEC-analysis. For both insecticides, no recovery was found after one permeation volume (Fig. 11), i.e., the sorption of insecticides to the stationary phase was as high as 100%. After 60 min, i.e., corresponding to three injections in the HPSEC-analyses, carbofuran was detected, and presence of DOM (SR FA) did not effect the retention time, or recovered amount of carbofuran (Fig. 11). Chlorpyrifos recovery, on the other hand, was 0% after 80 min, i.e., after four permeation volumes (Fig. 11), showing that all chlorpyrifos injected into the column sorbed to the stationary phase. However, in presence of DOM, chlorpyrifos was detected 30 min after the first injection (Fig. 11), revealing lower sorption efficiency to the stationary phase, possibly due to stronger associations with DOM. The TSKgel® column contains slightly negatively charged silica groups (SiOH,

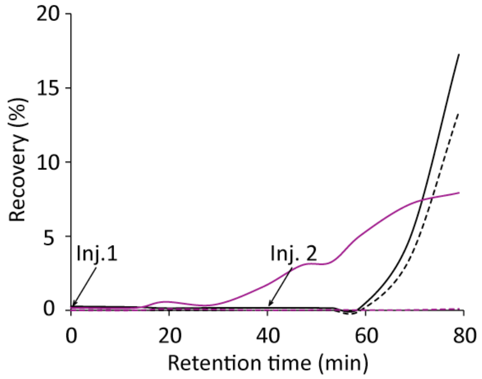


Figure 11 Recover (%) of carbofuran (black lines) and chlorpyrifos (purple lines), in presence (continuous lines) and absence (dotted lines) of Suwannee River fulvic acid measured during two injections (Inj. 1 and 2).

Berdén & Berggren 1990). The slight negative charge on the stationary phase may have altered the interaction between DOM and pesticides as the complex passes through the column, possibly by altering the conformation of the DOM. The pesticides have probably desorbed from the DOM and sorbed to the stationary phase, leading to a large retention during passage through the column and low pesticide recoveries. Alternatively, there was a direct sorption of the pesticides on the stationary phase even without any conformational change of the DOM. Our results show that silica-based columns are not suitable to study the interaction between pesticides and different size-fractions of DOM.

We aimed to study pesticide sorption to DOM, a process that occurs in nature when pesticides are released in aquatic ecosystems after runoff-events. Enabling the development of a widely valid model, we included aquatic DOM of varying origin (Suwannee River, USA, and Lake Vikasjön, Sweden) and complexity (FA, HA, FAHA, and DOA), and insecticides with different hydrophobicity (as well as other physico-chemical properties) in the experimental design. We intended to fractionate DOM as non-destructively as possible, and also to allow the insecticides to sorb prior to separation, providing a highly realistic natural scenario. Unfortunately, our approach was not successful due to methodological constraints. However, knowledge of how pesticides, as well as other organic contaminants, associate with different sources of and molecular sizes DOM is of crucial importance for the understanding of their fate and bioavailability in aquatic ecosystems.

4.3.2 Alternative approaches

An alternative approach for this study would be to use HPSEC-columns with another stationary phase, using the same experimental set up. DOM has successfully been fractionated by columns with organic polymers as stationary phases, for example Sephadex®, also using PSS-standards for calibration (Müller et al. 2000; Hoque et al. 2003). However, functional groups of the organic polymers may have acidic properties, allowing electrostatic interactions with the analyte molecule (Her et al. 2003). Thus, the problem with pesticide sorption to the stationary phase may remain.

Another approach would be to fractionate DOM prior to insecticide addition, using TSKgel® column, after which insecticides would be added to each molecular size-fraction, allowed to partition, followed by concentration quantification of dissolved vs. sorbed insecticides, for example with a semipermeable membrane device (SPMD). SPMD was developed to sample HOC in contaminated aquatic environments (Huckins et al. 1990), and passively accumulates non-ionic HOC by membrane-lipid-water partitioning from the water (molecular weight cut-off 1 kDa). SPMD has been efficiently used in the presence of DOM (Gourlay et al. 2005). Such an experimental set up would exclude the sorption problem of pesticide sorbing to the HPSEC-column. However, the scenario would not be as realistic as we intended in our experimental set up, since the whole DOM molecular size range would not be accessible for pesticide sorption, but instead already separated fractions. Hence, some size-fractions might give under- or overestimates of pesticide DOM-sorption. Nevertheless, such an experimental set up might provide results, not “contaminated” by method errors, and it would therefore contribute further to our knowledge of the pesticide interactions with DOM and ultimately their fate in aquatic environments.

5 Conclusions and Future Perspectives

This thesis shows that uptake and bioavailability of pesticides to benthic invertebrates is affected by many different factors (as summarised in Fig. 12). The source and quality of sediment organic matter to which pesticides interact may have strong consequences on the environmental fate and effect of these compounds. In particular, the complex interactions of pesticides with different sources of organic matter and biofilms in aquatic environments govern the fate and bioavailability of pesticides.

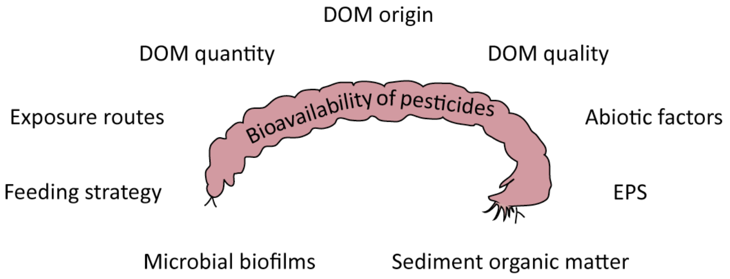


Figure 12 Bioavailability of pesticides to benthic invertebrates is mediated by many different parameters, depending on the benthic organisms living strategies as well as the surrounding environment and its physicochemical characteristics.

The studies included in this thesis show that:

- microbes and biofilms in bottom substrates increase pesticide bioavailability to benthic invertebrates (Paper I)
- microbes and biofilms in sediments are important for pesticides initial incorporation in aquatic food webs (Paper I)
- EPS, separated from its microbes, and HS decrease pesticide bioavailability to benthic invertebrates (Paper II)

- organic matter with high food-quality enhance dietary uptake in deposit feeding benthic invertebrates (Papers I-II)
- regardless of feeding strategy, uptake of pesticides in benthic invertebrates are mediated both trough passive and dietary routes of uptake (Papers I-III)
- DOM-quality and not just DOM-quantity is important for the fate and bioavailability of pesticides in aquatic environments (Papers I-III).
- pesticides may rapidly sorb to aquatic biofilms following runoff-events in agricultural landscapes, and concentrations in biofilm can be many times higher than those in water (Paper III)
- moderately hydrophobic to hydrophobic insecticides associate with DOM since presence of DOM resulted in higher recoveries in fractionation method used (Paper IV). However, pesticide interactions with specific DOM size-fractions were not successfully quantified.

In conclusion, when pesticides are released into the environment potentially having toxic effects on benthic invertebrates and other aquatic organisms, not just the pesticide's concentrations are of importance. The physicochemical properties of the aquatic environment are of importance since they regulate the organic matter quantity and quality, which in this thesis are shown to have great impact on pesticide fate and bioavailability in freshwater ecosystems. Organic matter quantity and quality are seldom included in experimental protocols for toxicity tests on which pesticide regulations and legislations are based upon, why regulators potentially miss important information to evaluate toxic effects in natural environments.

Pesticide usage is increasing worldwide, why more knowledge of this group of contaminants is needed. Pesticides have different physicochemical characteristics than most studied POP, thus predictions of pesticide effects cannot be derived directly from the behaviour of traditional POP in aquatic environments. Additionally, DOM concentrations have been found to increase in aquatic environments on the Northern hemisphere, presumably as a result of climate change. Increasing DOM concentrations due to global warming would have an impact on pesticide fate and bioavailability in aquatic ecosystems. Therefore, more research within this field is highly required.

6 Svensk sammanfattning

Biotillgänglighet av bekämpningsmedel till bottenlevande djur: betydelsen av akvatiska biofilmer och humösa ämnen

Bekämpningsmedel, till skillnad från andra miljögifter (t.ex. PCB och dioxiner), sprids avsiktligt i vår miljö och de är dessutom framställda för att vara giftiga. Bekämpningsmedel används bl.a. på åkrar för att minska de negativa effekterna av ogräs, eventuell svamptillväxt eller skadeinsekter på jordbruksproduktionen. Som en negativ effekt av besprutningen, t.ex. vid avrinning efter kraftiga regn i samband med besprutning eller utdikning av åkrar, når bekämpningsmedel sjöar och vattendrag i närliggande områden. När bekämpningsmedlen når vattenmiljöerna kan de tas upp av de organismer som lever där, och därefter transporteras upp i näringsväven. Detta kan ge effekter på hela ekosystem och t.o.m. slå tillbaka med effekter på viktiga ekosystemfunktioner, både terrestra eller akvatiska, som t.ex. insektspollinering, eller tillgång till rent dricksvatten.

När bekämpningsmedel mot skadeinsekter når vattenmiljöer har de mycket stor effekt på de bottenlevande djuren p.g.a. att denna grupp till största del består av vattenlevande insekter, eller insekter med vattenlevande utvecklingsstadier. Insektsmedlen är dessutom mycket biologiskt aktiva, och har verkningsmekanismer på t.ex. nervsystemet som leder till kraftiga spasmer vilka direkt eller indirekt leder till död.

Studierna jag presenterar i denna avhandling handlar om hur insektmedel tas upp av bottenlevande djur och hur biotillgängliga de är, d.v.s. hur effektivt de tas upp i organismerna. Jag har även studerat hur biotillgängligheten påverkas beroende på vilken typ av sjö eller vattendrag som djuren lever i.

Bottenlevande djur exponeras för otaliga miljögifter genom att många av dem ansamlas på botten, i sedimenten, som en följd av att de binder till partiklar i vattnet som sedan sjunker till botten. Miljögifter som har låg vattenlöslighet, d.v.s. är fettlösliga, ansamlas till stor grad i sedimenten och halterna blir därför mycket högre där än vad de är i vattnet. Bottenlevande djur exponeras för miljögifter på tre sätt: (1) de som finns lösta i det överliggande vattnet, (2) de som finns lösta i porvattnet mellan sedimentpartiklar, och (3) de som finns bundet i sedimenten (se figur 1). Bottenlevande djur får i sig föroreningarna passivt genom upptag över hud eller andningsorgan, eller aktivt via födointaget.

I denna avhandling har jag studerat biotillgängligheten av bekämpningsmedel i två vanligt förekommande bottenlevande djur: fjädermygglarven *Chironomus riparius* och den schackmönstrade gälsnäckan *Theodoxus fluviatilis*. Dessa två djur har två olika strategier för att få i sig föda, vilket kan påverka upptaget av miljögifter via födan. Fjädermygglarven lever i sedimenten i små rör byggda av partiklar, och de äter små partiklar med högt näringsvärde som de selektivt väljer från omgivande sediment. Gälsnäckan å sin sida, betar alger i biofilmer som växer på stenar och andra hårda ytor. Med sin ”skraptunga” skrapar de av biofilmerna. En metod som till skillnad från fjädermygglarverna gör dem mindre selektiva. Många fettlösliga miljögifter binder till partiklarna med högt näringsvärde, och därmed får fjädermygglarverna i sig större mängd miljögifter, som en bieffekt av sitt selektiva födointag.

Miljögifter som innehåller kolatomer kallas för organiska föroreningar. Beroende på de fysikaliska och kemiska egenskaperna hos dessa beter de sig olika när de förorenar vatten. Är de svårlösta i vatten binder de snabbt till organiskt material i vattnet, antingen partiklar i vattnet eller på botten, eller till organiskt material som är löst i vattnet, d.v.s. som inte sjunker till botten. Jag har i denna avhandling undersökt två olika organiska material som är mycket vanliga i sötvattensmiljöer: (1) akvatiska biofilmer, vilket är den slemmiga hinna som täcker stenar och andra hårda ytor i sjöar och vattendrag, och (2) humusämnen, vilket är de gula organiska syrorna som ger skogssjöar dess bruna karaktär. Dessa båda organiska material har en påverkan på upptaget, d.v.s. biotillgängligheten, av miljögifter i akvatiska organismer, beroende på det organiska materialets sammansättning.

Akvatiska biofilmer består av olika mikroorganismer, t.ex. alger, bakterier, och svampar, men också av nedbrytningsmaterial. Algerna och mikroorganismerna utsöndrar s.k. EPS (eng., extracellular polymeric substances, se figur 2) för att öka sin överlevnad. EPS består till största delen av polysackarider, d.v.s. långa sockerkedjor, och dessa inbinder näringsämnen som algerna och mikroorganismerna behöver för sin överlevnad. Som en följd av detta kan EPS även binda in miljögifter i biofilmerna. Genom att äta biofilm med inbundna föroreningar fungerar bottenlevande djur som inkörsportar för föroreningar, vilka sedan kan transportera sig vidare upp i näringsväven, och på så sätt påverka hela akvatiska ekosystem. Akvatiska biofilmer är ansedda att öka biotillgängligheten av inbundna miljögifter p.g.a. att biofilmerna är relativt lättnedbrytbara för botten-levande djur.

Humusämnen, å andra sidan, är nedbrytningsprodukter av växter och de är stora, svårnedbrytbara, komplicerade molekyler med mycket varierande kemisk sammansättning. Humus finns i stort sätt alla vattenmiljöer och är den största beståndsdelen av det lösta organiska materialet som kallas DOM (eng., dissolved organic matter). Precis som EPS, har humus och DOM stor förmåga att inbinda andra molekyler och därmed även miljögifter. När en förorening är bunden till humus eller DOM minskar biotillgängligheten till de vattenlevande organismerna. Komplexet blir helt enkelt för stort för att kunna tas upp via huden, andningsorganen, och/eller mag- och tarmkanalen.

I denna avhandling har jag studerat tre olika insektsmedel (karbofuran, lindan och klorpyrifos) med olika vattenlöslighet: från relativ vattenlöslig till fettlöslig. Exponeringstiderna i försöken har varit korta (24–48 h) för att efterlikna så kallade pulser, d.v.s. situationer då höga halter av bekämpningsmedel förekommer i vatten-miljön under en kort tid (exempelvis då ett kraftigt regn har samman-fallit med besprutning).

I studierna som ingår i denna avhandling fann vi att det organiska materialet har stor effekt på biotillgängligheten av bekämpningsmedel för bottenlevande djur.

I den första studien (Paper I) fann vi att närvaro av organiskt material påverkar upptaget av klorpyrifos i fjädermygglarver, där födokvaliteten har en betydelse för upptaget. I närvaro av sediment och biofilm producerat från mikroorganismer i sedimenten ökade upptaget av klorpyrifos i larverna kraftigt.

I den andra studien (Paper II) fann vi att närvaro av humus eller EPS minskade upptaget av klorpyrifos till larverna – en minskning som blev kraftigare vid högre halter av humus och EPS. Vi fann alltså att endast

EPS – separerad från den levande biofilmen – inte ökar biotillgängligheten av insektsmedel till bottenlevande djur.

Den tredje studien (Paper III) visade att upptaget av insektsmedel i gälsnäckor och inbindningen i biofilmer påverkas av olika halter och ursprung av DOM. Dessutom påverkar DOM biotillgängligheten av insektmedel olika beroende på dess kemiska egenskaper, t.ex. vattenlöslighet. Vi fann också att biofilmer kan ansamla relativt höga halter bekämpningsmedel efter en s.k. pulsexponering, och att det verkar som de på så sätt minskar exponeringen för bottenlevande djur.

För både larverna och snäckorna var det passiva och det aktiva upptaget av föroreningar av betydelse, oavsett födointagsstrategi (Papers I-III).

Eftersom vi fann så stor effekt av både DOM-halter (Papers II-III), och dess ursprung (Paper III) på biotillgängligheten ville vi studera inbindningseffektiviteten av insektsmedel till olika molekyl-storlekar av DOM med olika ursprung (Paper IV). Tyvärr lyckades vi inte med denna studie, p.g.a. problem med analysmetoden som vi hade valt. Däremot fann vi att DOM inbinder pesticider i så stor grad att de kan påverka analyser av miljögifter i förorenat vatten med DOM.

Sammanfattningsvis fann vi att det organiska materialet i akvatiska ekosystem har betydelse för biotillgängligheten av bekämpningsmedel till bottenlevande djur när de når akvatiska ekosystem. Vi fann framförallt att både kvantiteten och kvaliteten av det organiska materialet är av stor betydelse för biotillgängligheten.

Det har visat sig att halterna av DOM i sjöar och vattendrag på norra halvan av jordklotet ökar, och att detta troligtvis är en följd av den globala uppvärmningen. Skulle det vara så, att DOM-halterna ökar succesivt p.g.a. klimatförändringar, samtidigt som vi använder större mängder bekämpningsmedel i jordbruket, är fortsatt forskning inom detta område av stor vikt i framtiden.

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Tack!