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MicroNAC
Advanced tools for assessment of the Microbial Natural Attenuation Capacity for Micropollutants in the water cycle

Agrotechnology & Food Sciences Group

DATE
November 30, 2017

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Executive Summary

The increasing presence of organic micropollutants in different segments of the water cycle will or can threaten future water resources. These micropollutants are currently being detected at low concentrations in groundwater in areas that will be used for drinking water production. While current monitoring (chemical analyses) gives an indication of the presence of these micropollutants, little is known about the fate and transformation of these compounds in groundwater. Thus, there is little knowledge on the potential for natural attenuation of micropollutants under in situ conditions. There is a lack of information on biodegradation potential and rates under environmental conditions and a necessity for more tools to assess natural attenuation capacity. This information is required to improve models used to assess and predict the long term risks of contamination of drinking water intakes.

MicroNAC thus aims at providing knowledge to improve the prediction of pesticide fate and transformation in groundwater systems. This research is divided into three research lines:

- Research Line 1: In situ assessment of microbial diversity related to micropollutants and subsurface geochemistry
- Research Line 2: Role of DOC in biodegradation of Chloridazon and Chloridazon-desphenyl
- Research Line 3: Understanding fate and degradation of commonly researched contaminants: MCPP, 2,4-D, dichlobenil/BAM, bentazon

Research Line 1 is executed in the field to understand actual in situ field conditions. Research Lines 2 and 3 are performed under controlled laboratory conditions to understand the influence of specific parameters on degradation.

Research Line 1 research is performed at a drinking water location in The Netherlands by sampling and analyzing groundwater. Two monitoring wells are sampled at 5 depths per well in consecutive years and water is analyzed for geochemical composition, micropollutant concentration, and microbial community composition. Results indicate a high level of reproducibility between duplicate analyses, indicating a robust sampling and analysis method. Also, results show a high level of stability in the microbial community between years, which provides a proof-of-principle for this approach to understanding in situ micropollutant degradation. Furthermore, analysis of the microbial community composition in relation to the water composition data provided some insight into natural attenuation. Statistical analyses indicate that the presence of micropollutants was negatively correlated with the presence of electron acceptors. This result suggests that electron acceptor availability may limit micropollutant removal in groundwater. A variety of microbes with known degradation capacity for recalcitrant organic pollutants are observed; however these could not be directly linked to any of the micropollutants measured in groundwater samples.

Research Line 2 investigates the degradation of chloridazon-desphenyl, the highly recalcitrant degradation product of the pesticide chloridazon. The research focuses on providing insight into the role of supplemental dissolved organic carbon (DOC) on the degradation of chloridazon-despheyl by selectively adding supplemental carbon with different characteristics. Results show that while chloridazon is biodegraded in all conditions, chloridazon-desphenyl is recalcitrant to degradation. The quality of DOC determines whether or not chloridazon-desphenyl is degraded, with more labile DOC stimulating degradation. For example, in the presence of DOC extracted from compost, chloridazon-desphyl is completed degraded; in contrast, in the presence of DOC extracted from groundwater, chloridazon-desphenyl is not degraded. Overall these results indicate that chloridazon-despheyl can be biodegraded, however degradation depends highly on the quality of DOC present.

Research Line 3 investigated the transformation of 2,4-D, MCPP, BAM, and bentazon, a selection of priority compounds of particular interest. The role of electron acceptor availability

is investigated in columns filled with aquifer material and run under different redox conditions, namely nitrate-reducing, sulfate-reducing, and methanogenic conditions, and without addition of an additional. No significant degradation of pesticides is observed after 200+ days of the column experiment, indicating that electron acceptors, nutrients, temperature, and pesticide concentration probably are not limiting degradation. Following DOC addition, 2,4-D biodegradation is observed, indicating that electron donor or carbon availability may have limited degradation activity. Molecular analysis of community size and composition shows no significant increase in the microbial community size and only limited enrichment of a redox-specific community. Together, these results indicate that carbon or electron donor availability may be limiting 2,4-D degradation, but not other factors such as community size, nutrient availability or degradation capacity. As no degradation is observed of MCPP, BAM, and bentazon, further research is required to understand the transformation of these compounds.

Overall, MicroNAC provides a number of important conclusions that are relevant for future practice. The research clearly shows that in situ microbial communities are stable, indicating that molecular tools can be utilized to monitor and predict degradation. Furthermore, electron acceptor availability appears to be negatively correlated with the presence of micropollutants, pointing towards a potential focus of future research. Laboratory experiments particularly highlight the importance of DOC availability and quality in the biodegradation of micropollutants. Further investigations into the role of DOC in micropollutant biodegradation is crucial to translating these results into a viable prediction and remediation technique. These major findings form a basis for follow-up projects in the development of natural attenuation-based remediation techniques for groundwater systems.

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Acknowledgements

The report summarizes the findings of the TKI Water-technology project “MicroNAC: Advanced tools for assessment of the Microbial Natural Attenuation Capacity for Micropollutants in the water cycle”.

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Aspects of the reported research can be found in the following thesis:

Michael Lim: Biodegradation of pesticides from groundwater in aquifers under different redox conditions influence of supplemental DOC (dissolved organic content) (2017)
Robin Meijer: Biodegradation of 2,4-D & BAM in groundwater (2017)
Spencer Crook: Using molecular tools to understand and predict pesticide biodegradation (2016)
Carolin Riegraf: The change in herbicide toxicity upon biodegradation under different redox conditions (2016)
Martina Antonucci: The role of dissolved humic and fulvic Acids in microbial degradation of herbicide chloridazon(2015)
Laura Tabernero Cogul: Environmental conditions for microbial degradation of pesticide chloridazon (2015)

General Introduction

The widespread application of herbicides especially in agricultural settings, misusing such as careless application, high application rates as well as spilling accidents led to an increased amount of herbicide residues detected in groundwater in the past decades (de Lipthay et al., 2007; Cabral et al., 2003; Thrasher et al., 2004). According to the intended purpose of pesticides, it is reasonable that contamination of these compounds can constitute a threat to non-target organisms as well as to nontarget settings such as groundwater (Junghans et al., 2006; Pimentel, 1995). In areas where drinking water supply is mainly ensured by groundwater, pesticide residues reaching adjoining drinking water resources and thereby causing environmental contaminations are of major concern (Boesten and Van der Linden, 1991; Kolpin et al., 1998). In Europe, an average of 70% of the drinking water supply is covered by groundwater resources. In the Netherlands, about two third of the drinking water is provided through groundwater resources (Navarrete et al., 2008).

Not only pesticides residues but also their metabolites can leach into adjacent water bodies, especially because some of these metabolites show a higher mobility and are less degraded than their parent compounds. Therefore, even higher amounts of metabolites compared to their parent compounds are present in aquifers (Holtze et al., 2008). This is especially of concern, since degradation processes can also lead to persistent metabolites. Despite their environmental significance, these metabolites are often neglected (Kolpin et al., 1998).

Pesticides are degraded and transformed in the environment through a variety of biotic and abiotic reactions and transformations. While abiotic transformations such as photolysis, can play an important role in the degradation of pesticides in some environments, the effect is often limited by physical barriers. Conversely, microbial biodegradation plays a key role in the degradation of pesticides in soil as well as ground and surface waters (Fenner et al., 2013; Helbling, 2015). Not only does biodegradation represents the major route of removal of pesticides from the environment, in a practical sense, biodegradation is often the only viable method of pesticide removal from contaminated environments (Fenner et al., 2013). This can be partially attributed to the manner in which pesticides are applied and become transported to the environment (Fenner, et al., 2013). Compared to other contaminants or pollutants which often have a single point source and high relative concentrations, pesticides are applied sporadically to a broad area (K. Fenner et al., 2013; Helbling, 2015). The result is diffuse, low concentrations of pesticides in the nanogram to low microgram per liter of ground or surface water (Byer, et al., 2011; Loos et al., 2009; Loos et al., 2010; Smalling, et al., 2012). This presents a significant barrier to traditional physical or chemical means of removal and remediation.

While pesticides are commonly encountered in groundwater systems, the fate, transformation, and natural degradation of these compounds is largely unknown. Due to the low concentrations and heterogeneous distribution, it is nearly impossible to draw conclusions on pesticide degradation based on chemical analysis of groundwater. Furthermore, groundwater is largely anaerobic. While pesticides are often tested for their aerobic fate prior to admittance to the market or in studies investigating their biodegradation in surface soils, the fate of compounds under anaerobic conditions is unknown. Finally, microbial community composition and microbial-mediated processes in generally oligotrophic groundwater have been largely unexplored. To this end, MicroNAC aims at developing a new insights into natural attenuation processes in groundwater systems. This research is divided into 3 research lines, namely:

- Research Line 1: In situ assessment of microbial diversity related to micropollutants and subsurface geochemistry
- Research Line 2: Role of DOC in biodegradation of Chloridazon and Chloridazon-desphenyl
- Research Line 3: Understanding fate and degradation of commonly researched contaminants: MCPP,2,4-D, dichlobenil/BAM, bentazon

In Research Line 1, field investigations are used to understand the interplay between microbial community composition, groundwater geochemistry and micropollutants. This research is explorative, in the sense that monitoring is performed to explore the natural processes occurring in groundwater. Whereas other research lines focus on a selection of pesticides and degradation products, Research Line 1 produces a data set in which all micropollutants are considered. Research Lines 2 and 3 are laboratory investigations in which the fate and transformation of specific pesticides and their transformation products are studied under controlled conditions. Research Line 2 focuses on Chloridazon and Cloridazon-desphenyl, the latter of which is a transformation product regularly encountered in groundwater (Loos et al., 2010). Research Line 3 examines the fate and stimulated biodegradation of a selection of more commonly researched pesticides, namely 2,4-D, MCPP, and bentazon, as well as the transformation product BAM from the pesticide dichlobenil.

Research Line 1: In situ assessment of microbial diversity related to micropollutants and subsurface geochemistry

1. Introduction

While biodegradation has been identified as the major route of removal of compounds from the environment, the typical method of pesticide application presents several hurdles, making biodegradation difficult to predict and monitor. Biodegradation occurs most efficiently when the pesticide is the major electron donor or acceptor. For this condition to be met, a relatively high concentration of pesticide must be present. Conversely, in the environment, particularly in groundwater, pesticides are typically present in extremely low concentrations. Diffuse applications result in pesticide concentrations being several orders of magnitude lower than other dissolved organic carbon (Helbling, 2015). Because of low relative concentration, pesticide degradation is not the microbial process which dominates. Further, because spraying is done sporadically depending on the growing season and crop variety, pesticides are transported to the environment with spatial and temporal heterogeneity (Helbling, 2015). This lowers selection pressure to retain or upregulate specific genes related to pesticide degradation and minimizes degradative capacity.

The aforementioned challenges which exist related to biodegradation serve to further strengthen the argument that molecular tools are necessary to understand, monitor, confirm and predict biodegradation and biotransformation of pesticides in environmental conditions. Traditional chemical analysis can be “tricked” by heterogeneity. That is, because pesticides occur sporadically, the presence or absence of a pesticide as detected by current chemical techniques says nothing about the presence of degradation. As well, due to the problem of low concentrations, monitoring geochemical parameters to confirm microbial activity may speak more to dissolved organic carbon (DOC) turnover than to pesticide degradation (Fenner et al., 2013). Additionally, improving biodegradation through biostimulation (BS) and bioaugmentation (BA) requires methods to assess and monitor the effect of treatment, which can be accomplished through the use of molecular tools.

For molecular tools to be an effective method of detecting and monitoring pesticide biodegradation, several criteria must be met. The tools themselves must be accurate, replicable, and reproducible. Further, bacterial communities within contaminated soil or water resources must be stable. If bacterial communities are constantly changing, it is challenging to use molecular tools to monitor degradation in the field. Finally, because ground water resources are anaerobic, studies to detect correlations between specific pesticide presence, redox conditions, and microbial community composition must be conducted to bridge knowledge gap in anaerobic microbial pesticide metabolism and provide a clearer picture into real world conditions.

Previous experiments have shown molecular tools, especially the direct detection of pesticide degradation genes through the use of q-PCR, to be relevant in predicting and understanding pesticide degradation. However, these experiments have been conducted using pure cultures in aerobic conditions (Devers, et al., 2004; Baelum et al., 2012; Monard et al., 2010; Monard et al., 2013; Rodríguez-Cruz et al., 2010; Sagarkar et al., 2013). As these conditions do not dominate environmental groundwater samples, molecular tools are applied in a more environmentally relevant context. Because degradative genes have not yet been elucidated in anaerobic conditions, q-PCR detection of degradative genes is not possible. Instead, observations are used to elucidate connections between microbial community composition, redox conditions, and pesticide presence. In this way, the current understanding of pesticide biodegradation in the environment is furthered.

The aim of this study is to assess the feasibility of using molecular tools to monitor, predict, and better understand environmental pesticide degradation. To accomplish this, two contaminated groundwater monitoring wells were selected. Water samples were extracted at a variety of depths and microbial DNA was extracted from the water samples. 16s rRNA genes were amplified and sequenced. Sequencing data was compared to geochemical data from the same wells. These results give insight into the microbial communities present in groundwater resources, and the relation between these communities and the extant geochemical and redox conditions over a variety of depths.

2. Materials and Methods

Site Description

Two ground water monitoring wells near Emmen, Netherlands were selected for this study. Well 22 was located adjacent to a canal which was subject to runoff from agricultural production and effluent from an upstream wastewater treatment plant, while well 23 was located approximately 500m east adjacent to residential houses. These wells serve as water quality monitoring sites for a drinking water production company in the area. The groundwater at both sites is anaerobic.

Ground water Sampling

Ground water was extracted from two ground water monitoring wells. Following an initial purging, 10L water samples were extracted in duplicate at depths of approximately 13, 21, 28, 40, 46 m and 13, 27, 37, 47, and 54m for wells 22 and 23 respectively. In both wells, 2m screens were present at the sampling depth to filter larger particles. Identical sampling procedures were carried out approximately one year apart. Samples were labeled according to well identity, depth, and age. Samples were stored at 4C in alcohol sterilized HDPE jugs prior to filtering. Additional samples were collected for chemical analysis.

Filtering, DNA extraction and DNA purification

Water samples were vacuum filtered using cellulose nitrate membrane filters with a 0.2 um pore size. At least 3L of water from each sample was filtered to ensure sufficient biomass was present on each filter. As few filters as possible were used to filter the samples. The majority of samples used only 1 filter with the exception of sample 22-1b, 22-2a, 22-2b, 22-3a, 22-4a, 23-3a, and 23-5a which required 2 filters. As well, sample 22-1a required 3 filters to be used. Filters were stored at -20°C prior to DNA extraction. Total microbial community DNA was extracted from the filters by substituting the filters themselves in place of soil in the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, USA). Subsequent steps were carried out according to the manufacturer's instructions. DNA quality and quantity was assessed through the use of 1% agarose gels and Nanodrop spectrophotometry (Thermo Scientific, Wilmington, USA). Community DNA was stored at -20°C for downstream applications.

Amplification of 16s rRNA gene and Sequencing

A two-step PCR protocol which first selects the 16s rRNA gene of interest, then subsequently adds tags and adapters was used. The initial PCR amplification was carried out using universal forward and reverse primers 27F (GTT[TC]GAT[TC][AC]TGGCTCAG), and 338R (GC[AT]GCC[AT]CCCGTAGG[TA]GT). These primers selected a fragment of the V1-V2 hypervariable region of the 16s rRNA gene. An additional PCR amplification was carried out as previously described with the exception that primers included adapters and barcode tags. PCR products were visualized on a 1% agarose gel to ensure the presence of an appropriate size band with sufficient DNA concentration. Amplicons were quantified through Qubit fluorometric

quantification (Thermo Scientific, Wilmington, USA) and samples were pooled in equimolar concentrations.

Analysis of raw sequencing data

Pyrosequencing data were analyzed using the NG-Tax pipeline (Ramiro-Garcia, 2016). Paired end libraries were combined and sequences which did not have an identical match to both the barcode and primer sequence were discarded. An OTU picking strategy which looked only at reverse reads was adopted. Reverse reads were selected in order to give a lower number of NA OTUs. OTUs which made up less than 0.01% of relative abundance were discarded. Chimeras were removed via the standard method described in NG-Tax. Taxonomy was assigned to OTUs through the use of the uclust algorithm and the SILVA 111 database at a 97% confidence level.

Statistical Analysis

Reproducibility of 16sRNA diversity was assessed by comparing Unifrac unweighted distances between duplicates. Bacterial community similarity between samples was analyzed through Canoco 5.0 (Biometris, Wageningen, The Netherlands). Principle component analysis (PCA) of class level taxonomy and UniFrac principle coordinate analysis of beta diversity. Beta diversity variation between samples was analyzed via students T-test. Unconstrained PCA analysis through Canoco 5.0 was used to determine the degree of correlation between environmental geochemical data and sequencing data. Redundancy analysis was performed through Canoco 5.0 to determine the percentage of variation in bacterial community composition at class level explained by geochemical data.

3. Results and Discussion

Geochemical and Micropollutant Data

Historical geochemical data was analyzed. Each line represents one year of data. Nitrate, Sulfate, dissolved Iron (Fe^{2+}) and dissolved organic carbon (DOC) remained relatively stable in each well over an 16 year sampling time 2000 to 2016 (Figure 1.1). Large differences between well 22 and well 23 were evident. No nitrate can be detected in well 22. Conversely, well 23 displays an inverse relationship between nitrate concentrations and sampling depth, with higher concentrations being detected at shallow sampling points. Transitions between redox conditions as depth increased were evident as electron acceptor concentrations changed with depth. At shallow depths in well 22, iron reducing conditions predominated. As sampling depth increased in well 22, sulfate reducing conditions were observed. Conversely, well 23 displayed high nitrate concentrations at shallow depths, indicating nitrate reducing conditions. Within well 23, sulfate concentrations increased rapidly with depth, reaching a peak at approximately 27m. Sulfate levels then quickly decreased with depth. Iron concentrations in well 23 increased with depth indicative of redox conditions within well 23 transitioning towards iron reduction. In each well, DOC levels decreased as depth increased. Similar levels of sulfate, nitrate, iron and DOC can be observed in the deep sampling points of both well 22 and well 23, indicative of a potential hydraulic connection between these wells.

Similar trends between transitioning redox conditions with depth were observed in each well. This result can be explained by the energetic favorability of the electron acceptors present. As nitrate reduction is more energetically favorable, nitrate reducing conditions are expected in more superficial sampling depths. This trend is visualized in well 23. However, because well 22 is bereft of nitrate, iron reducing conditions predominate at shallow depths. As the nitrate is consumed in well 23, a transition is observed from nitrate reducing conditions to iron(III) reducing conditions similar to the superficial samples in well 22. Finally, in both well 22 and well 23, sulfate reducing conditions are observed in deep samples. In essence, energetically favorable electron acceptors are being consumed in shallow samples, transitioning to less

energetically favorable electron acceptors as depth increases. These reductions are accompanied by the oxidation of DOC which decreases in concentration over depth.

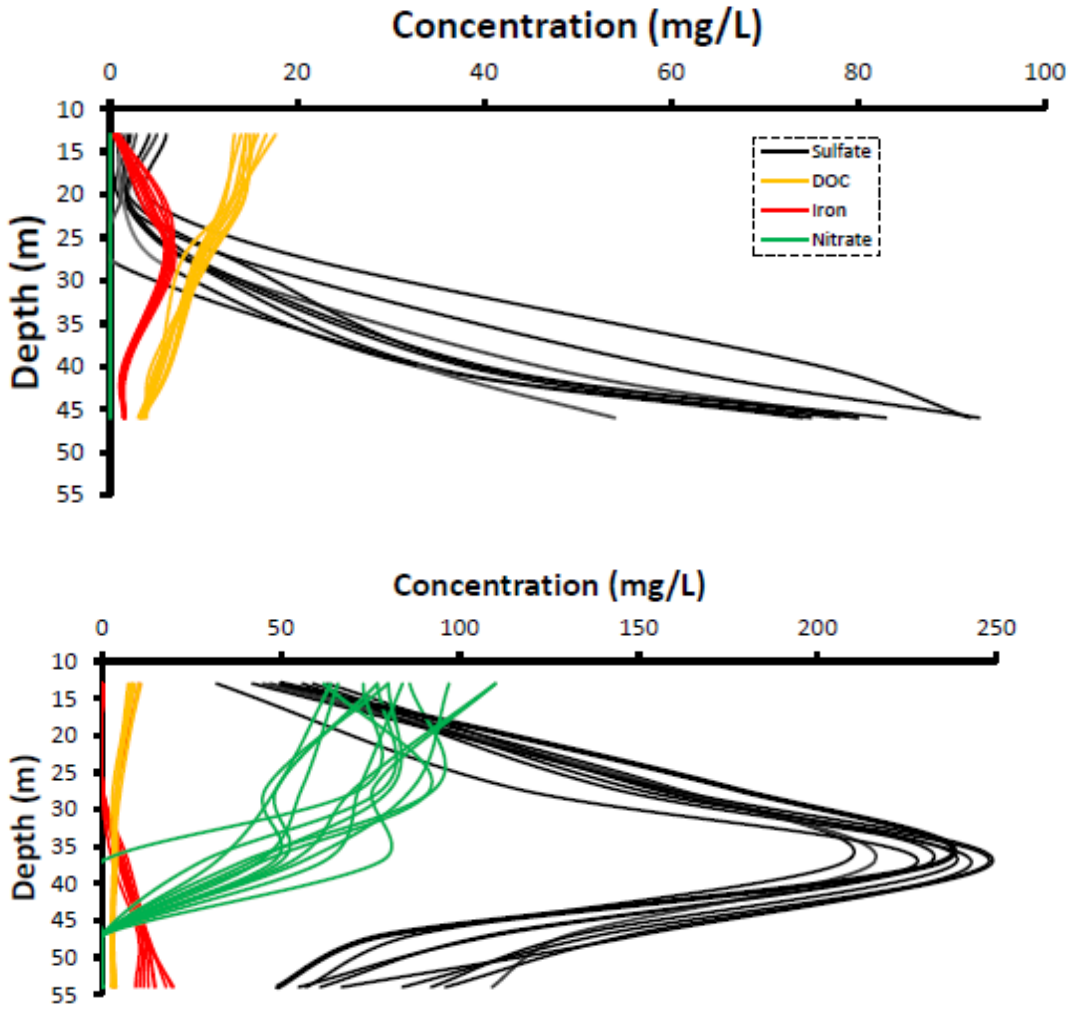


Figure 1.1. Sulfate (mg/L sulfate), DOC (mg C/L), Iron (mg/L iron) , and Nitrate (mg/L nitrate) concentration in well 22 (above) and well 23 (below) between 2000 and 2016. Note the differing x-axis scales between well 22 and well 23.

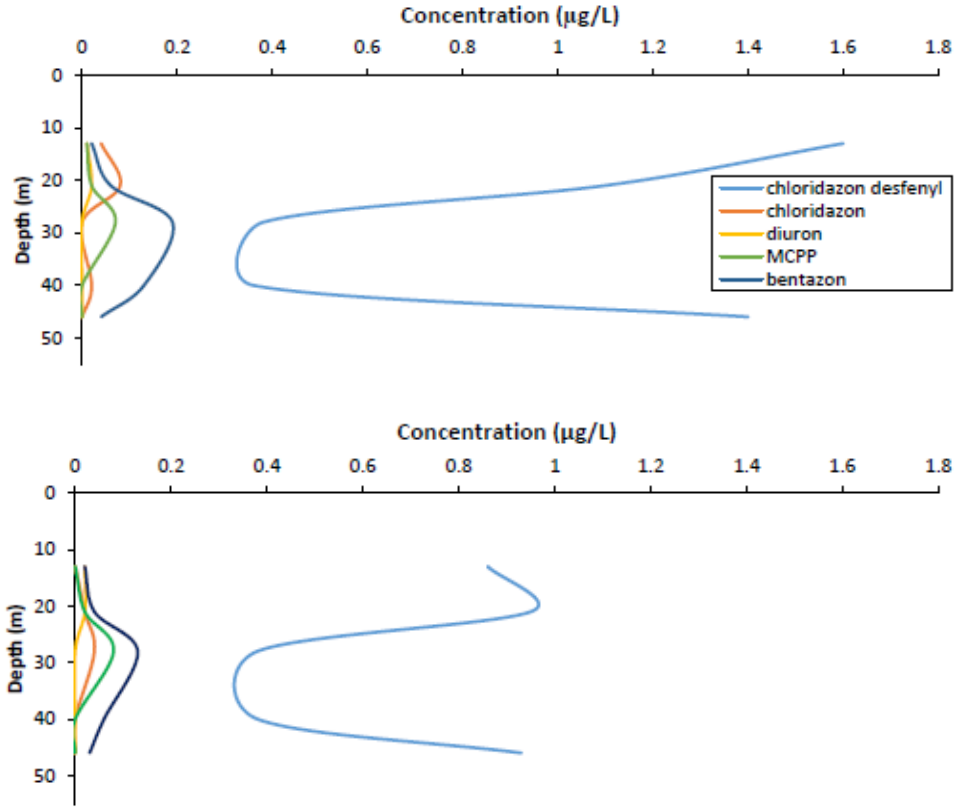


Figure 1.2. Micropollutant data from well 22 as provided by the drinking water production company. The top graph displays data from 2015 samples, while the bottom graph displays data from 2016 samples.

Pesticide presence and concentration within well 22 was consistent from 2015 to 2016 (Figure 1.2). Chloridazon desfenyl as the predominant pesticide metabolite present in well 22. All pesticides are present in sub microgram per litre concentrations. Pesticide composition was consistent from 2015 to 2016 in well 23 (Figure 1.3). Comparatively fewer pesticides were present in well 23 compared to well 22. Pesticides were not detected to any appreciable degree in shallow samples of well 23. This is a notable difference between well 22 and well 23. All pesticides are present in sub microgram per litre concentrations (Figure 1.3).

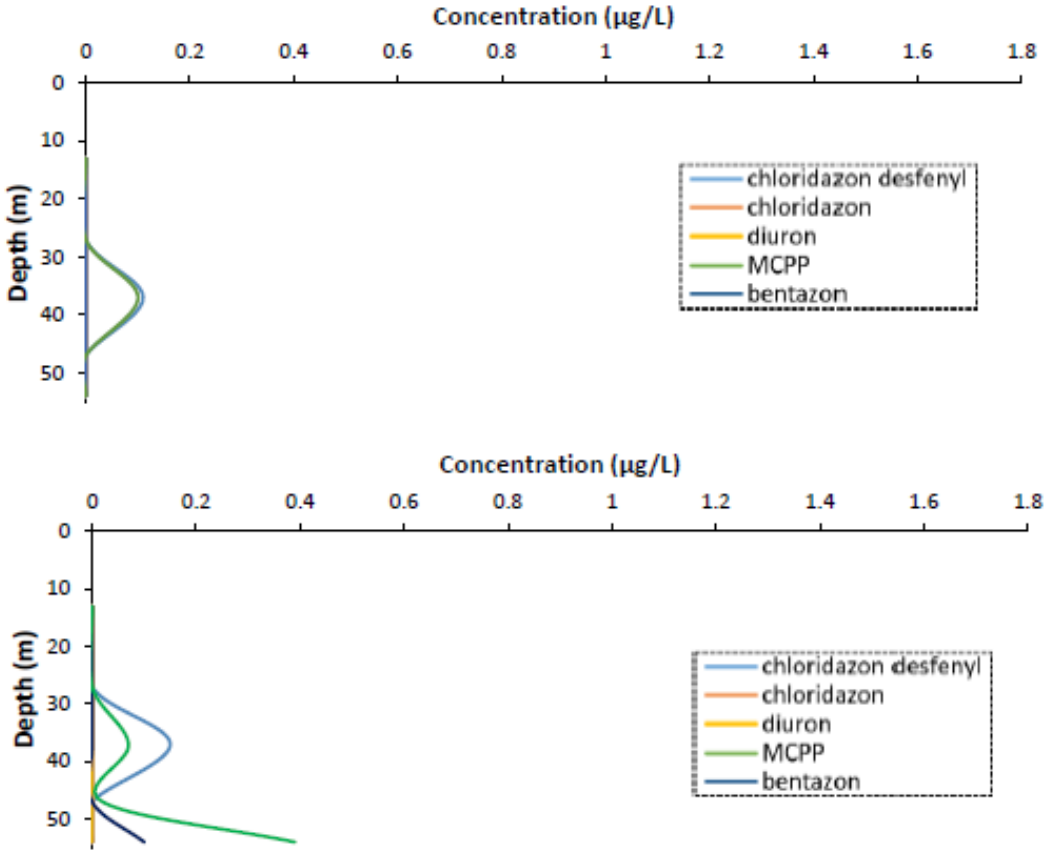


Figure 1.3. Micropollutant data from well 23 as provided by the drinking water production company. The top graph displays data from 2015 samples, while the bottom graph displays data from 2016 samples.

Community Composition

Samples were composed of 2144000 total reads with an average read number of 53600. The maximum number of reads was 137483, present in sample 22-2c. The minimum number of reads was 1021 found in sample 22-5b. In general, good sampling depth was observed with rarefaction curves of each sample approaching a slope of 1.

The microbial community composition at the phyla level (Figure 1.4) was made up by Proteobaceria, Candidate division OD1, Candidate division OP3, and Chloroflexi. These phyla made up 29.6% (±12.6%), 14.0% (±9.8%), 13.3% (±9.1%), and 12.4% (±6.7%) of the total microbial population on average respectively. Major differences in community composition at the phyla level were evident between well 22 and well 23. Proteobacteria made up 33% (±15%) of well 22 and 26% (±8.5%) of well 23. Similarly, Bacteroidetes were more prevalent in well 22, making up 4% (±3%) of the total community while comprising only 1.2% (±1.5%) of well 23. As well, bacteria of the phyla Firmicutes contributed 1.7% (±2.6%) to the total community in well 22 and only 0.4% (±0.5%) to that of well 23. Nitrospirae abundance was similar (approximately 7%) in both wells. Non assigned (NA) OTUs which could not be assigned to a kingdom made up 1.9% of samples on average. However, the distribution of NA OTUs was not uniform. Sample 22-1a and 22-1b were comprised of a disproportionately large percentage of NA OTUs (24% and 14% respectively).

The microbial community at class level as visualized in Figure 1.5 is made up primarily of unculturable bacteria belonging to candidate divisions 63.2% (±11.8%). Non assigned OTUs made up 2.9% of the total microbial community. The most abundant class was

Deltaproteobacteria making up 27.3% (±17.2%) of the microbial community. Nitrospira, Alphaproteobacteria and Ignavibacteria made up 7.4% (±10.0%), 3.2% (±4.4%), and 2.2% (±5.3%) of the total community respectively. Similarity between duplicates, as well as samples within the same depth separated temporally could be visualized. In general, the class level community appears stable within a one year time scale. As well, duplicate samples did not significantly differ from one another ($P<0.01$).

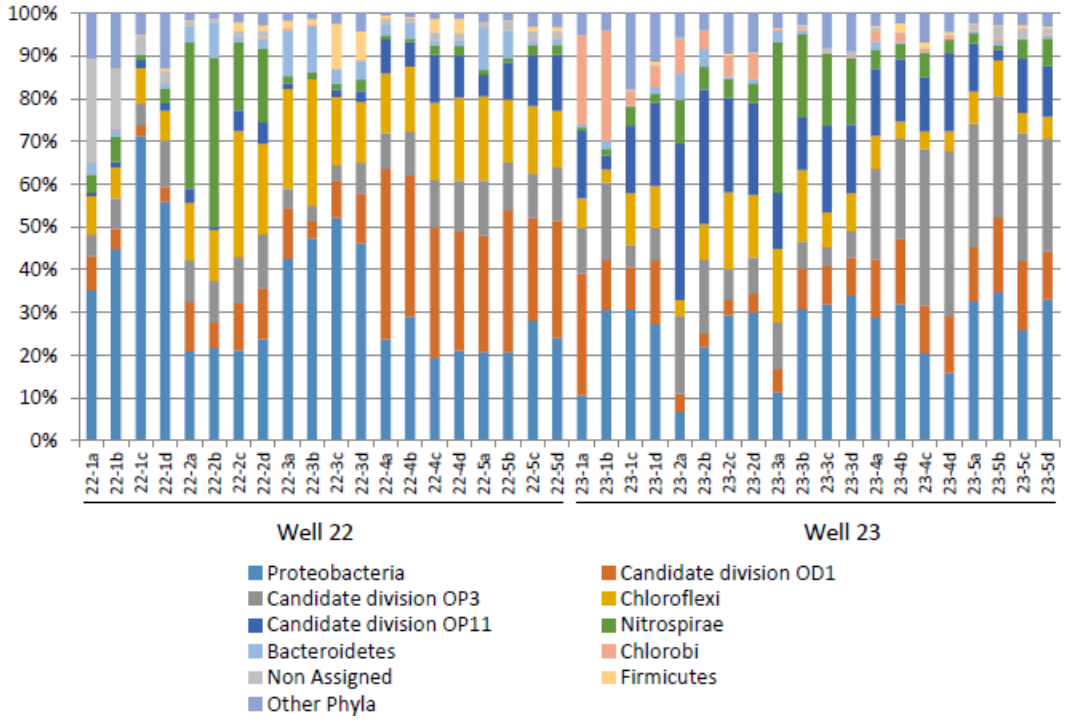


Figure 1.4. Bacterial community composition at phyla level based on Illumina Miseq sequencing of 16s rRNA. All phyla making up less than 1% of the total community were combined into the category “Other Phyla”.

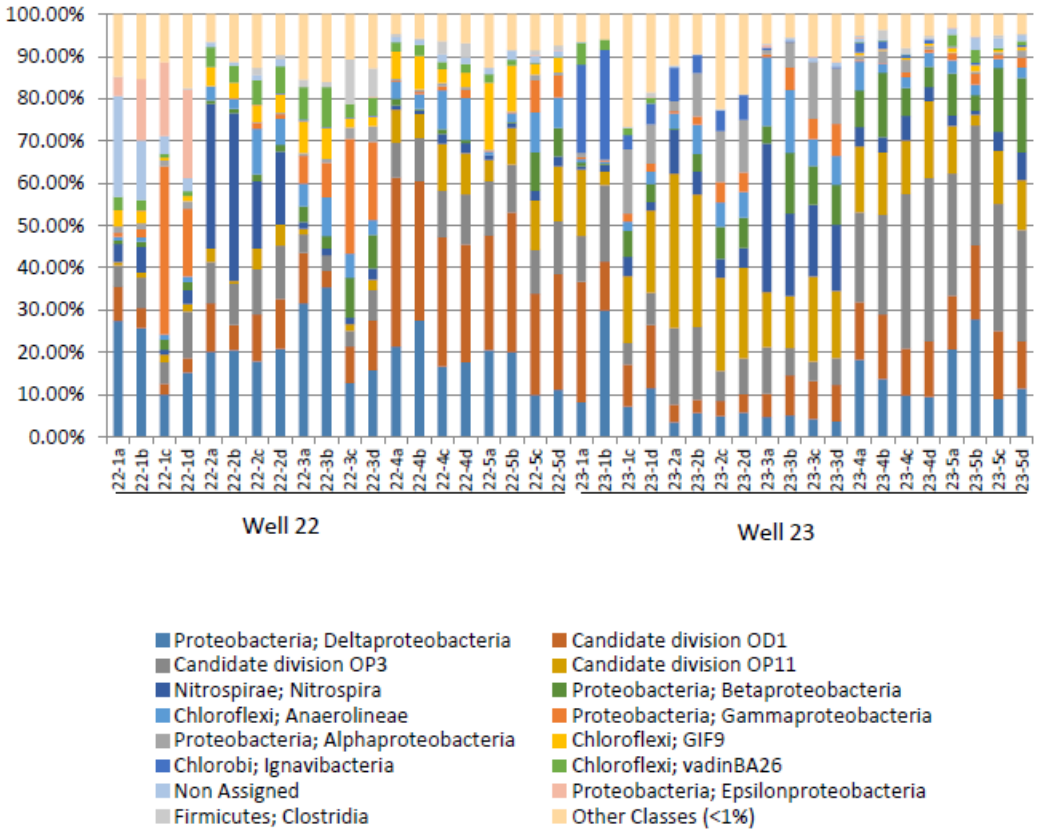


Figure 1.5. Bacterial community composition at class level based on Illumina Miseq sequencing of 16s rRNA. All classes making up less than 1% of the total community were combined into the category “Other classes”.

Stability of the Microbial Community

Community diversity between duplicates (Ie. 22-1a and 22-1b; 23-5c and 23-5d) were highly similar. As well, samples taken one year apart appeared to form clusters. These results show similarities within the microbial communities present in samples at each depth. Microbial communities did not appear to change to a high degree over time. No significant difference between community composition among duplicates was observed (P>1.00) indicating high replicability. In general, samples from well 22 were found the cluster to the left side of the PCA while samples from well 23 were found on the right side (Figure 1.6). Samples 22-2, 22-3, 22-4, 22-5, as well as 23-4, and 23-5, clustered close to the center of the PCA. Samples 23-2, and 23-3 clustered closely on the far right of the PCA (Figure 1.6). Samples from 22-1 grouped away from all other samples toward the bottom left of the PCA.

Beta diversity was visualized via principle coordinate (PCoA) analysis of unweighted Unifrac distances (Figure 1.7). Distance between samples is indicative of microbial community similarity. Duplicates clustered with minimal spatial separation indicating high replicability. Additionally, samples from well 22 at depths of 13m, 21m, and 28m clustered together, as did samples from well 23 at 13m, 27m, and 37m. As well, samples from both well 22 at depths of 40m and 46m, and well 23 at depths of 47m and 54m exhibited low spatial separation from one another. There are strong similarities between the first three sampling depths, in well 22. Similarly, there are similarities between the first three sampling depths of well 23. However, well 22 and well 23 at these shallow sampling depths are dissimilar. Conversely, there is a high degree of similarity between the deepest 2 sampling regions of both well 22 and well 23. For this reason, samples 22-1, 22-2, and 22-3 are grouped into “well 22 shallow samples”. Similarly, samples 23-1, 23-2, and 23-3 are grouped into “well 23 shallow samples.” Samples

22-4, 22-5, 23-4, and 23-5 are grouped into “deep samples” due to the high similarity observed within microbial community composition (Figure 1.7).

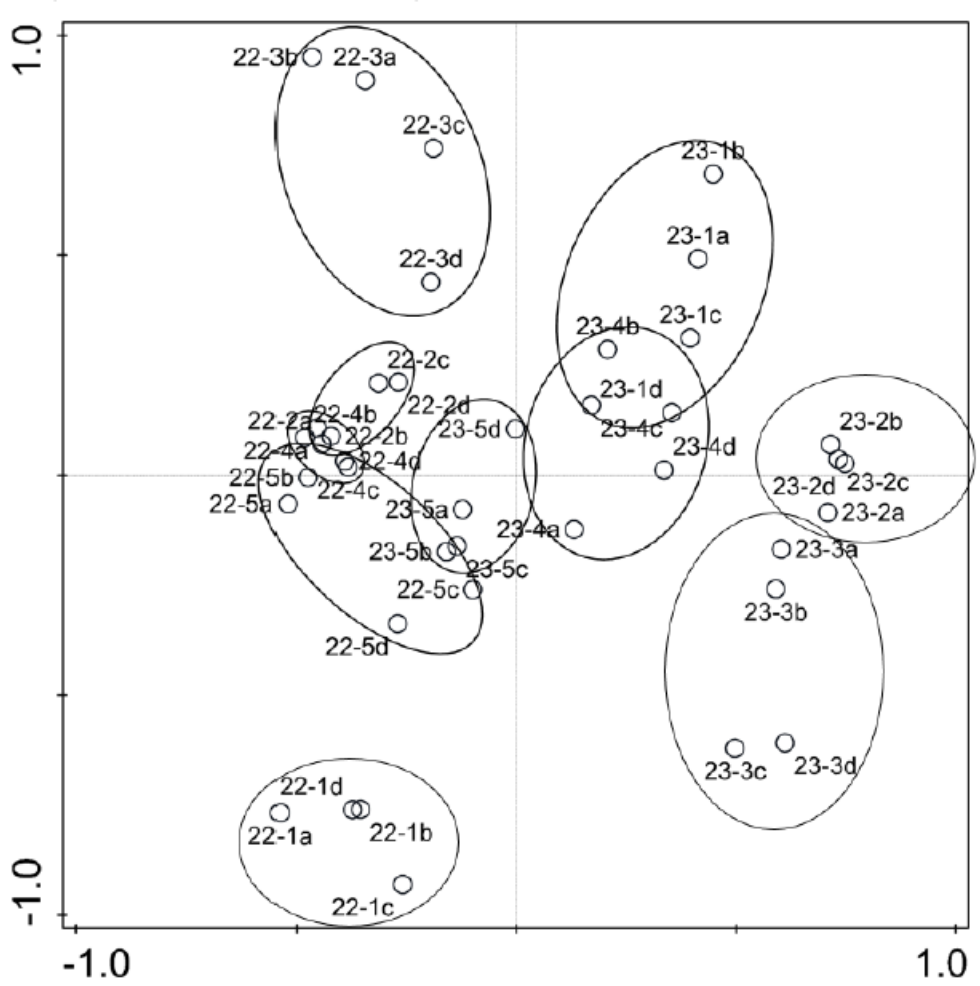


Figure 1.6. PCA of microbial community at class level performed on Canoco 5.0. Samples from the same well and sampling depth are circled.

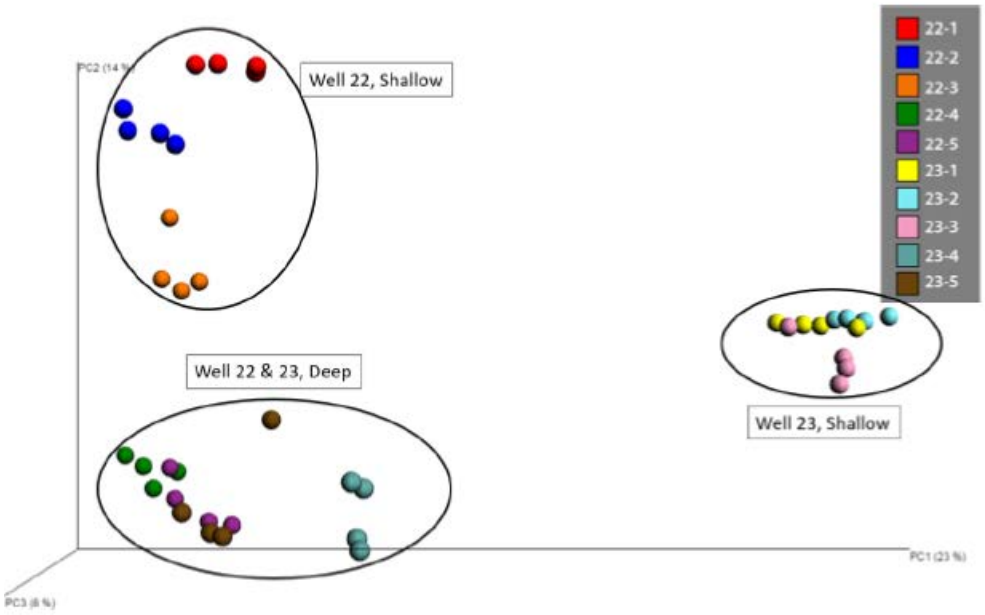


Figure 1.7. PCoA analysis of unweighted Unifrac distances.

To visualize the effects of environmental variables on class level taxonomy, a PCA was conducted. The electron acceptors nitrate and sulfate, as visualized in Figure 1.8, clustered towards the middle right of the PCA. Conversely, carbon sources including the pesticides MCPP and bentazon clustered toward the upper left quadrant. This suggests a negative correlation between prominent electron acceptors and carbon sources. Dissolved iron was found to be correlated weakly with carbon sources. This result is in opposition to the trend visualized with other electron acceptors. However, additional analysis determined MCPP had a greater degree of correlation to 22-3 samples than both bentazon and DOC. Nitrate and sulfate exhibited similar magnitudes of correlation but were correlated with different samples. Nitrate was more correlated with samples in 23-2 and 23-1. In these samples, Anaerolineae, OP3, and Betaproteobacteria were the predominate classes however the magnitude of correlation was fairly low. NA-OTUs were highly correlated with samples 22-1. Epsilonproteobactera were also associated with 22-1 but to a lesser degree than NA-OTUs (Figure 1.8).

The environmental variables and geochemical data was able to explain much of the differences in microbial community composition between samples (Figure 1.9). Sulfate, nitrate nitrite, DOC, Chloridazon Desfenyl, explained 65.6% of the variation observed in class level taxonomy. Diuron, MCPP and Bentzon were not significant explanatory variables on class. Sulfate explained 21.7% of the variation in class taxonomy ($P<0.01$). Nitrite explained 15.7% of the variation in class ($P<0.001$) and nitrate explained 13.4% of the variation observed in class ($P<0.01$). Chloridazon was responsible for explaining 7% of the variation in class while DOC was responsible for explaining 14.6% of the variation in class ($P<0.01$). Bentazon, MCPP, and Diuron explained 4.9%, 9.2% and 12.3% of the variation in class but were not statistically significant explanatory values ($P>0.05$) (Figure 1.9). This result displays the high degree of effect that geochemical data has on the microbial community composition within this study. The presence of absence of specific microbes appears highly dependent on the chemical composition of the environment (Figure 1.9).

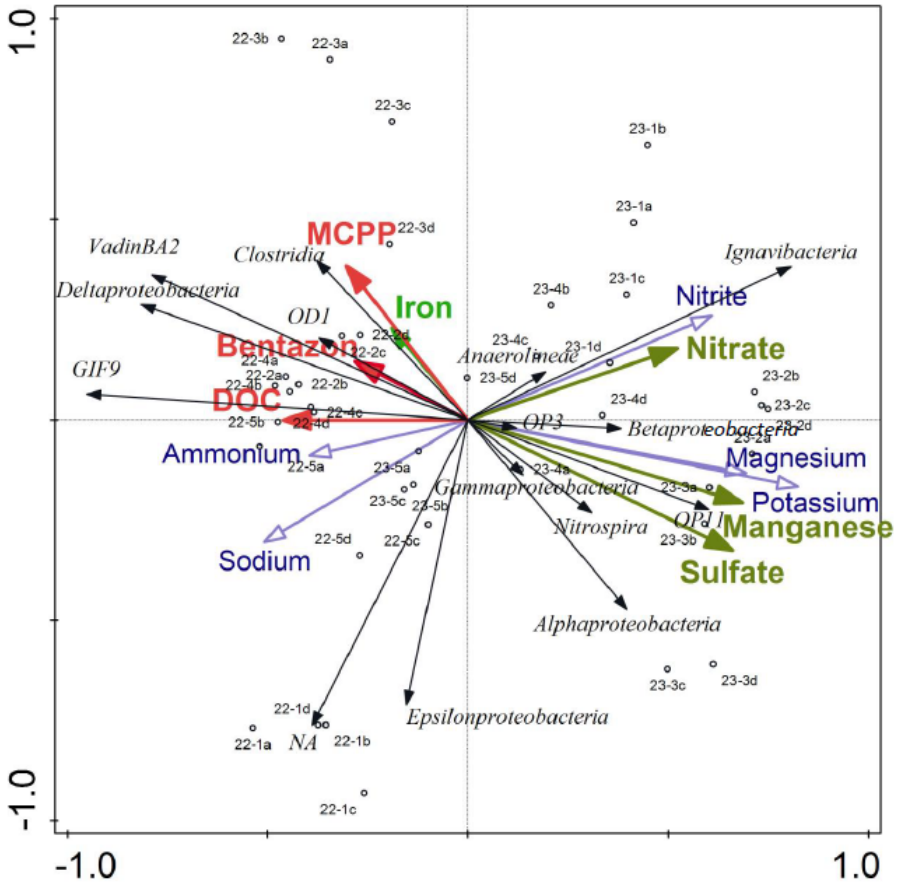


Figure 1.8. Unconstrained PCA analysis displaying class level taxonomy in black italics, electron acceptors in green text, and carbon sources in red text. Other environmental variables are displayed in blue text. Arrow direction indicates correlation between samples in the same direction. Arrow magnitude indicates degree of correlation.

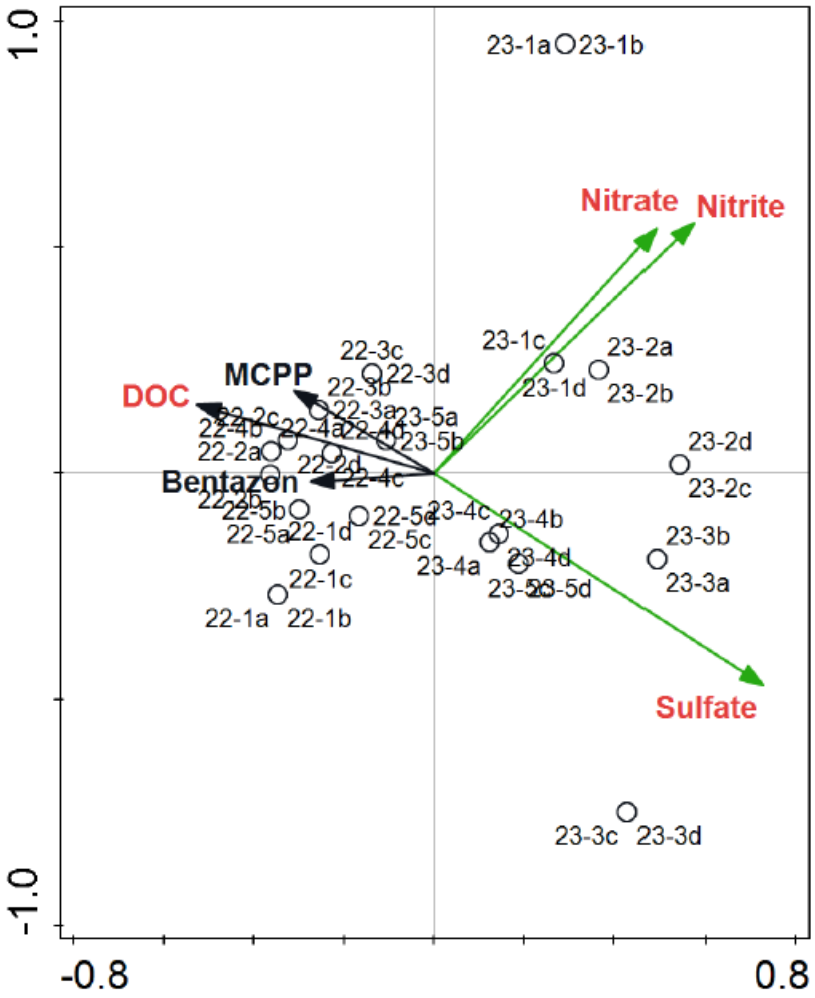


Figure 1.9. RDA analysis of electron acceptor, carbon source, and micropollutant effect on explaining variation in class level taxonomy. Arrow direction shows correlation between environmental variable associated with the arrow, and sample community composition. Arrow length shows amount of explanation the environmental variable has on predicting class level taxonomy. Red labels are statistically significant (P>0.05)

General Discussion

There appears to be a difference between the hydrological composition of shallow and deep samples. Firstly, large differences within the geochemical data (Figure 1.1), especially in regards to sulfate and nitrate concentrations within shallow samples of well 22 and well 23 are evident. Further, the unweighted UniFrac data shows that the microbial community differs greatly between shallow samples of well 22 and well 23 (Figure 1.7). This difference in geochemical data and microbial community composition may be due to surface water intruding into shallow samples, especially within the first 3 sampling depths, influencing the geochemical data which has a direct effect on the microbial community composition. Conversely, within deep samples, well 22 and well 23 display high similarity in both the geochemical data and the microbial community composition. Due to the congruency between deep samples of well 22 and well 23 including high similarity in microbial community composition as measured through unweighted UniFrac distance between deep samples of both well 22 and 23 (Figure 1.6 and 1.7), and similar geochemical data (Figure 1.1), indicating hydraulic connection exists between well 22 and well 23 within “deep sampling depths”. Ground water at depths of greater than 40 meters flows horizontally toward drinking water

production wells located east of the two sampling wells. This flow may be minimizing the role surface water plays in regards to influencing the composition of these deep samples.

Despite the spatial proximity of well 22 and well 23, drastic differences in both geochemical, and microbial community composition were observed (Figure 1.1, 1.4 and 1.5). As previously mentioned, this result can potentially be explained by surface water infiltration into these shallow samples. Expanding further, the lack of nitrate in well 22, as well as the low levels of sulfate at shallow sampling depths compared to well 23 may be explained by the well location. As well 22 was located directly adjacent to a canal downstream from a wastewater treatment plant. This could be caused by eutrophication of the surface water within the canal. Runoff rich in DOC, as well as nitrate and phosphate rich fertilizers typically applied to crops may be washed into the canal, causing a spike in microbial activity. This eutrophication may deplete oxygen, leading to the total consumption of nitrate within the canal or in very shallow groundwater. If this is the case, it may explain why no nitrate is present within well 22. As well, the relatively high concentration of pesticides within well 22 compared to well 23 supports the hypothesis of agricultural runoff within the canal adjacent well 22 infiltrating the shallow samples of well 22. However, pesticides within well 22 are still low when compared to other carbon sources.

The pesticides present within the wells are present in extremely low concentrations, often below 1ug/L (Figure 1.3). Conversely DOC is often more than a thousand times more abundant. These pesticides are often persistent in the environment due to their chemical structure. As these molecules are recalcitrant and present in such low concentrations, it follows that more labile carbon sources would be preferentially degraded by the bacterial communities present (Helbling, 2015). This explains why pesticides exert minimal effect on the microbial community composition when compared to DOC within the RDA analysis (Figure 1.9). Further, general difficulties were encountered in correlating microbial communities and their associated metabolic capabilities with specific geochemical data.

Microbial Community Composition

The microbial community composition in deep groundwater samples are largely unexplored, presenting difficulties in interpreting a vast amount of the data generated in this study. Microbial community composition within deep samples of well 22 and 23 were composed mainly of Candidate Division OD1, OP3, and OP11. These phyla made up 22%, 20%, and 11% of the total data set respectively (Figure 1.4). In comparison to culturable phyla, relatively little is known about these bacteria. These candidate divisions consist of bacteria which have not yet been cultured. Therefore the metabolic capabilities of these bacteria are largely unknown and unexplored. The high prevalence of these candidate divisions is expected, as research shows the ubiquity of unculturable phyla within environmental ground water samples(Rinke et al., 2013). However, few conclusions regarding the impact these phyla play within the samples, or the environment in general can be made.

Recently, advances in genomics and single cell sorting has allowed for genomic sequencing of several candidate division phyla (Rinke et al., 2013). This development has allowed for candidate division OD1 (proposed name *Parcubacteria*), to be classified as phylogenetically similar to candidate division OP11 (proposed name *Microgenomates*). These two unculturable bacteria are part of a larger superphyla called *Patescibacteria*. The similarity between these phyla may explain the high relative abundance of both OD1 and OP11 in all sampling depths. Interestingly, these bacteria lack the highly conserved purH1 gene used in purine synthesis and instead possess a purine biosynthesis gene more closely resembling that of eukaryotes (Rinke et al., 2013). This novel finding helps illustrate the potential wealth of genetic diversity locked away within these candidate phyla. As these phyla were highly associated with samples in well 22, the well which was found to harbor a greater concentration of pesticides, perhaps

these candidate phyla may contain members which hold a key to understanding pesticide biodegradation.

The present study focuses on the abundance of various microbes present in samples. However, bacteria within a given phyla or class are often diverse with respect to their metabolic capabilities. To address this, we attempt to look at class and order level taxonomy to gain deeper insight into relevant metabolic capabilities of the microbial communities present (Figure 1.4 and 1.5). While general metabolic trends within these taxonomic classifications can be pointed out and connected with geochemical data, it is difficult to make concrete conclusions as to which metabolic processes are being carried out by these classes or phyla. We can only speculate based on previous studies, what metabolic processes may predominate within a given sample given the relative abundances of various community members and the corresponding geochemical data present.

Another hurdle encountered in analyzing this data set, is the sheer diversity of the microbial community encountered. With 38 phyla making up the total microbial community (Figure 1.4), and each phyla potentially consisting of metabolically diverse members, inferring which members are performing which processes became problematic. Previous research has shown that the most abundant members within a community may not be the most active or relevant (Campbell, et al., 2011). Similarly, rare or under represented taxa within a community may be highly active and important members despite their low abundance. As well, microbes may be more transcriptionally active when present in low relative abundance (Campbell et al., 2011). This paradoxical attribute of microbial metabolism makes interpreting 16s rRNA sequencing data such as those found in this study difficult. These problems are compounded by the fact that pesticides are present within samples and the environment at low concentrations. The scarceness of pesticides as a carbon source means detecting the likely small effect pesticides exert on a given microbial community using 16s rRNA analysis is difficult.

Some highly abundant members within the microbial communities were found in some cases, to be associated with geochemical data explaining their presence. Orders of bacteria associated with sulfate reduction were found in both well 22 and well 22. Deep samples from both well 22 and 23 displayed a high percentage of *Deltaproteobacteria*, *Syntrophobacterales* with approximately 5.4% relative abundance (Figure 1.5). This order is composed of strictly anaerobic sulfate reducing bacteria (SRB). *Syntrophobacterales* are able to reduce sulfate through the use of the dissimilatory sulfite reductase enzyme encoded by the *dsrAB* gene. However, previous research has displayed the ability D. *Syntrophobacterales* to degrade DOC in the absence of sulfate, sulfite and other organosulfonates in coculture (Plugge, et al., 2011). It may be that these bacteria are utilizing sulfate containing intermediates produced by other members of the consortia. Due to the high levels of sulfate present in deep samples of well 22 and 23 (Figure 1.1), sulfate reducing bacteria are expected. However, due to the flexibility of the metabolic requirements of *D. Syntrophobacterales*, it is difficult to say whether these microbes are actively participating in sulfate reduction without additional experiments to detect gene expression. We can say however, that deep samples from both well 22 and well 23 differ significantly from shallow samples of well 22 and well 23.

Shallow samples from well 23 included some unique members not found in other wells and sampling depths to an appreciable degree. Shallow samples in well 23 exhibited *Alphaproteobacteria*, *Rhizobiales* at 6% relative abundance. *A. Rhizobiales*, is known best as a nitrogen fixing order of bacteria which are symbiotic with plant roots. The enzyme nitrogen reductase encoded by the *nifH* gene confers the ability of *A. Rhizobiales* to fix nitrogen (Coelho et al., 2008). Bacteria belonging to this order were not found to any appreciable degree in other sampling groups in this study. The presence of *A. Rhizobiales* in shallow samples of well 23 may partially explain why high levels of nitrogen containing compounds are observed in this well as opposed to well 22 which did not contain nitrate. However, as

nitrogen is likely not present at depths of 12m and below, it is unlikely that nitrogen fixation is happening to an appreciable degree. Instead, these nitrogen fixing bacteria may be infiltrating into deeper samples from surface water where nitrogen fixation associated with plant roots could potentially be a relevant process. Additionally, previous studies examining microbial groundwater composition has detected the presence of *A. Rhizobiales* at depths of 100m. When grown in pure culture, these bacteria exhibited the ability to utilize ammonium and nitrate while growing heterotrophically in the absence of plant roots (Kutvonen, et al, 2015). Therefore, the *A. Rhizobiales* may be utilizing the nitrate found in shallow samples of well 23.

As well, in shallow samples of well 22 and well 23, as well as deep samples of well 22 and 23, *Nitropirae*, *Nitrospirales* were detected with relative abundances of 2%, 11% and 12% respectively. This order is composed of facultative anaerobic gram-negative cells which oxidize nitrate forming nitrite. However, research has displayed the ability of these organisms to survive in the absence of nitrogen containing compounds in co-culture (Garrity et al., 2001). The higher relative abundance of *N. Nitrospirales* in shallow samples of well 23 may be explained by the high levels of nitrate in these samples. Due to the lack of nitrate in well 22, bacteria which rely on nitrate oxidation are not expected. However, due to the metabolic flexibility of *N. Nitrospirales*, bacteria belonging to this genera in shallow samples of well 22 are likely persistent through co-culture or are simply present, but not active members. While *N. Nitrospirales* was abundant in shallow samples of both well 22 and well 23, relative abundance of other phyla helped differentiate shallow samples of well 22 and well 23.

Bacteria associated with denitrification were found with higher abundance in samples containing high levels of nitrate. Bacteria belonging to the order *Ignavibacteriales*, were found with high relative abundance in shallow depths of well 23. This order was not found to be substantially prevalent in other sampling groups. Within PCA analysis (Figure 1.8), *Ignavibacteriales* correlated with nitrate, nitrite, and shallow samples of well 23. Research shows this order, belonging to the phyla *Chlorobi*, is often found alongside sulfur reducing, denitrifying bacteria in waste water treatment reactors (Zhang, et al., 2015). Further, these consortia are associated with high levels of nitrate removal. *Ignavibacteriales* may be so prevalent in shallow samples of well 23 due to the high concentrations of nitrate also in these samples. These bacterium may be metabolically active in a syntrophic or symbiotic relationship with active nitrate degraders to facilitate the removal of high concentrations of nitrate found in shallow samples of well 23.

With respect to the electron donor most likely utilized by the aforementioned bacteria, DOC predominates. This assumption is based on the high relative abundance of DOC and the labile nature of DOC compared to the pesticides present (Aislabie, et al., 1995). As well, the RDA and PCA analyses point toward DOC exerting a more substantial effect on explaining the variation observed within the community composition (Figure 1.8 and 1.9). While this may be the most likely scenario, it is possible that some degradation of recalcitrant compounds is taking place within groundwater samples. This assertion is based on past research showing the ability of several bacterial members found within the samples, to degrade recalcitrant compounds. Bacteria belonging to the order *N. Nitrospirales* were found to be associated with the removal of hydrocarbons from oil-contaminated soil environments (Abbasian et al., 2016; Schulze-Makuch et al., 2011). Similarly, *Ignavibacteriales* has been associated with the degradation of several recalcitrant compounds. A recent study has implicated *Ignavibacteriales* within a community which exhibits benzene degradation. Benzene, being a cyclic, recalcitrant molecule shares many characteristics with pesticides. As well, benzene rings are found with ubiquity within the structures of many pesticides and other POPs. Members belonging to the order *Ignavibacteriales* have also been found to degrade cellulose (Podosokorskaya et al., 2013). Finally *Ignavibacteriales* have been found to be associated with the degradation of alkenes and other oil refinery products (Embree, et al., 2014; Xie et al., 2016). The ability of members belonging to the order *Ignavibacteriales* to degrade a

diverse set of recalcitrant compounds lends credence to the hypothesis that some biodegradation may be occurring within the samples despite the low concentrations of contaminants. Finally, a specific species belonging to the order *Rhizobiales*, has been found to degrade 4-fluorocatechol and catechol in pure culture (Carvalho, et al., 2006). While it is unlikely this specific strain is found within the environmental samples, it reinforces the idea that *Rhizobiales* may be degrading DOC, or potentially using other carbon sources in the absence of plant roots and nitrogen, two factors which are unlikely to be found. These data provide evidence for the hypothesis that the microbial communities present may possess the ability to degrade recalcitrant compounds including pesticides.

These observations, while not hard and fast, may in part explain the differences observed in community and geochemical data among samples. In some instances, the dominant microbial community members are found in samples containing geochemical data which correspond well to past research concerning their metabolism. In other cases, microbes are found within geochemical environments that seem not to suit their known metabolic properties. These results underline both the flexibility of bacteria, and the difficulty presented in using 16s rRNA data alone to predict the metabolic capabilities of bacteria in the environment.

4. Conclusion

Overall, there was a high degree of replicability between duplicates in this experiment. Further, the geochemical data was able to explain a substantial portion (65.6%) of the variability between microbial community composition. Finally, community stability and geochemical stability is observed in both wells at each sampling depth. Stability within the microbial community composition is important in the context of identifying biodegradation within this study because environmental conditions were also stable over time. To detect pesticide biodegradation through the investigation of microbial community composition, microbial communities that intrinsically vary according to environmental conditions and pesticide presence are required. That is, if a microbial community does not respond to differences in redox conditions or pesticide presence, molecular tools, specifically 16s rRNA genomic data, will not be useful to detect or understand pesticide degradation. These results, along with the community stability over time, help provide a foundation for the use of molecular tools to understand pesticide biodegradation in the environment.

Furthermore, analysis of the microbial community composition in relation to the water composition data provided some insight into natural attenuation. Statistical analyses indicate that the presence of micropollutants was negatively correlated with the presence of electron acceptors. This result suggests that electron acceptor availability may limit micropollutant removal in groundwater. A variety of microbes with known degradation capacity for recalcitrant organic pollutants are observed; however these could not be directly linked to any of the micropollutants measured in groundwater samples.

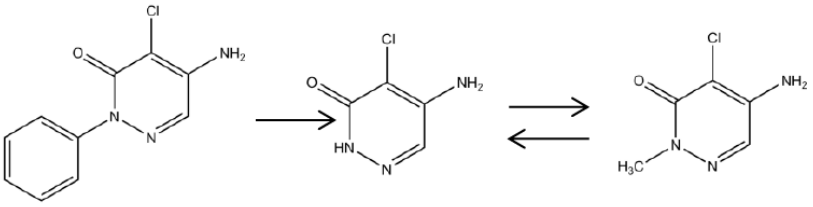
Research Line 2: Role of DOC in biodegradation of Chloridazon and Chloridazon-desphenyl

1. Introduction

Chloridazon [5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone] is the active ingredient of the herbicide Pyramin (EFSA, 2007). It is a photosynthesis inhibitor that has been used for more than 20 years in several countries in Europe for preventing the occurrence of weeds in sugar beet and beet root culture. It is applied during the pre-plant, pre-emergence, and early post-emergence of weeds. Although in the literature contradicting claims are found about the environmental fate of Chloridazon, it can be stated that the compound is highly mobile in different types of soils (EFSA 2007, Buttiglieri et al., 2009). As a consequence, it can potentially leach through the soil profile and enter surface and groundwater, becoming a risk for water quality. Buttiglieri et al. (2009) reported several studies showing that Chloridazon and its two metabolites Chloridazon-desphenyl and Chloridazon-methyldephenyl are detected in natural waters in many countries in Europe, in particular in agricultural areas. In the Netherlands, although the concentration of Chloridazon currently does not exceed the acceptable concentration of 0.1 µg/L in groundwater (ESFA, 2007), it has been listed among the substances that can cause potential problems for drinking water by Vewin, the association of Dutch water companies (Vewin, 2014). This has been done because Chloridazon degradation metabolites, which are even more persistent in water than the parent substance, are expected to exceed the tolerable level of 0.1 µg/L in groundwater (EFSA, 2007). Chloridazon-desphenyl, in fact, was the most frequently detected compound above the limit of 0.1 µg/l in the Pan-European survey of Loos et al. (2010), which analysed 164 ground-water samples from 23 European Countries (Loos et al., 2010). Additionally, Chloridazon-methyldesphenyl was the eighth most frequently detected chemical above the threshold value of 0.1 µg/L (Loos et al. 2010). Understanding the degradation dynamics of Chloridazon is therefore of vital importance to be able to predict its occurrence in the environment and to preserve water quality.

Microbial degradation is the most important process for Chloridazon removal (EFSA, 2007; Ligens et al. 1985). In soils, Chloridazon can be degraded in 13 days to 8 weeks (half-life), depending on soil type, moisture content and temperature (Buttiglieri et al., 2009). Biodegradation of Chloridazon starts with the breakdown of its phenolic ring, which can be rapidly mineralized to CO_2, forming Chloridazon-desphenyl. Through a reversible reaction Chloridazon-desphenyl can form Chloridazon-methyldesphenyl (Table 2.1). Both the degradation products of Chloridazon contain the pyridazinone ring, composed by two nitrogen heteroatoms and one carbonyl, one chlorine and one amino group. The pyridazinone ring shows slow to moderate microbial mineralization rates (EFSA, 2007), and makes the metabolites of Chloridazon being more persistent and mobile in water than the parent substance (Buttiglieri et al., 2009). Even though some Chloridazon degradation experiments have been already carried out (Buttilgieri et al., 2009), a lot is still unknown about Chloridazon degradation mechanisms. In particular, natural dissolved organic matter, which might enhance its degradation process, has not be taken into account in the existing studies.

Table 2.1. Properties of Chloridzaon as well as the degradation products Chlordazon-desphenyl and Chloridazon-methyldesphenyl.

	Chloridazon	Chloridazon-desphenyl	Chloridazon-methyl-desphenyl
Chemical structure			
IUPAC name	5-amino-4-chloro-2-phenylpyridazin-3(2*H*)-one	5-amino-4-chloro-4H-pyridazin-3-one	5-amino-4-chloro-2-methylpyridazin-3-one
Molecular formula	$C_{10}H_8ClN_3O$	$C_4H_4ClN_3O$	$C_5H_6ClN_3O$
Molar mass (g/mol)	221.6	145.5	159.6
Solubility in water at 20°C (mg/l)	340 (BCPC 2011) 422 (Hertfordshire 2014a)	No data	No data
K_{ow}	15.5(BCPC 2011, Hertfordshire 2014a)	No data	No data
K_{oc}	120 (Hertfordshire 2014a) 89-340 (BCPC 2011)	No data	No data
DT_{50} in aerobic soil (days)	31 (Hertfordshire 2014a) 124 (PAN 2014)	108 (Hertfordshire 2014b)	145 (Hertfordshire 2014c)
DT_{50} in anaerobic soil (days)	489 (PAN 2014)	No data	No data

1.1 Role of Dissolved Organic Matter

Dissolved organic matter (DOM) is degraded by microorganisms similarly to other fractions of soil organic matter (Kalbitz et al., 2003). From the literature it appears that the quality of DOM released from soil carbon amendments of different origins may have different effects on soil microbial activity (Amon and Benner, 1996; Kalbitz et al., 2003; Marschner et al., 2003; Straathof et al., 2014). In particular, the biochemical composition of natural DOM can control DOM biodegradability (Sun et al., 1997). Generally, DOM with higher H:C and N:C ratios is thought to be preferably degraded by microorganisms, leading to a higher microbial growth (Sun et al., 1997). On the other hand, the degree of aromaticity, the degree of complexity and condensation of the molecules and their hydrophobicity seems to negatively influence microbial DOM degradation (Kalbitz et al., 2003). These statements suggest that humic substances (HS) are generally very recalcitrant upon microbial degradation, as they are usually seen as long, coiled, refractory macromolecules. As a consequence, it can be thought that humic (HA) and fulvic (FA) will not play a role in fostering microbial degradation of Chloridazon. However, these assumption are refused by the a new view of the HS structure proposed by Piccolo et al.,(2001) and Sutton and Sposito (2005). According to this new view, HS should not be considered anymore as refractory polymer, but as aggregates of diverse and low-molecular mass organic molecules including biomolecules that could provide energy and nutrients to microorganisms. These (bio)molecular moieties are intimately associated with the humic fractions through bounds that protect them from microbial utilization. However, the interactions that hold together the molecules can be disrupted, making the biomolecules readily available and decomposable by microorganisms. This suggests that HS, even with a high aromatic and complexity degree can sustain microbial growth and activity as well as play a role in enhancing Chloridazon biodegradation. Microbial utilization of HA and FA and its

effects in Chloridazon degradation will therefore be influenced by the presence and amount of biomolecules associated with the humic fractions and by the possibility to disrupt the interactions that hold them together.

Several studies indicate that generally natural organic matter can positively affect microbial degradation of different types of pesticides. For example, McCormick et al. (1966) and Roeth et al. (1969) showed that the microorganisms that assimilate and metabolize natural organic carbon (OC) accounted for a more efficient degradation of herbicides 2-chloro-4-ethylamino-6-isopropylamino-1Ls-triazine (atrazine) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron). McCormick et al. (1966) reported a positive relationship between microbial degradation of OC and removal of the pesticides, with correlation coefficients ranging from 0.94 to 0.99. Roeth et al. (1969) showed that atrazine was degraded two to three times faster in the in a soil containing 4.8% of organic matter (OM) than a soil containing only 0.8% OM. Harris et al. (1967) found similar results for the herbicides simazine and triazine, suggesting that natural OC stimulates microbial activity. Therefore, natural DOM could enhance the growth and activity of Chloridazon-degrading microorganisms by serving as a supplemental carbon and energy source. As a result, the degradation of the pesticide could be accelerated.

Natural DOM could also be seen as primary substrate for co-metabolic biodegradation of Chloridazon, whereby microorganisms degrade a compound (in this case Chloridazon) without being able to use it as a growth-substrate (Suthersan, 1996; Dalton, 1982). As a consequence, they need another substrate (DOM) as C and energy source to support cell replication and growth (Horvath, 1972). Horvath (1972) showed that many pesticides, including DDT, are degraded via co-metabolisms. However, Ligens (1985) showed that only few aromatic or heterocyclic compounds structurally related to Chloridazon and few simple carbon compounds could enhance the growth of Chloridazon-degrading bacteria and increase the degradation of the herbicide. However, it must be noted that for this study Ligens isolated bacteria with the ability to grow on Chloridazon as a sole carbon source. Possibly, other bacteria with different properties than those studied by Ligens can degrade Chloridazon by co-metabolism.

DOM could also enhance Chloridazon biodegradation by inducing the production of Chloridazon-degrading enzymes. The presence of natural DOM in fact can preferentially stimulate the growth of microorganisms that produce the enzymes necessary to break down the more recalcitrant and aromatic fraction of DOM, namely dissolved HA and FA. These same enzymes can possibly be effective also for the degradation of Chloridazon, thanks to structural similarities between Chloridazon and HA and FA. As a results, DOM and in particular dissolved HA and FA, can be seen as structural analogues that catalyse Chloridazon degradation.

1.2 Role and Selection of a Structural Analogue

The presence of a structural analogue can enhance the biodegradation of a pesticide. A structural analogue is a chemical with a similar chemical structure to the substance that is to be biodegraded. The structural analogue should be biodegradable. The addition of such a structural analogue promotes the activity or growth of the microorganisms in order to be able to biodegrade the target compound (Brunner et al. 1985). In order to find an appropriate structural analogue several things need to be considered: it should be an available chemical substance with a similar structure to the original compound and it should be known to be biodegradable and easily measurable.

In this study, a structural analogue is considered for Chloridazon-desphenyl, the highly recalcitrant degradation product. Chloridazon-desphenyl's chemical structure consists of a heterocyclic ring containing two nitrogen atoms, two double bonds and several substituents

(carbonyl group, amino group, and chlorine). This structure gives certain stability to the molecule which makes it difficult to degrade. In order to stimulate biodegradation, two possible structural analogues are suggested based on their structure and biodegradability: pyridine and hydroxypyridine.

Pyridine: a conjugated heterocyclic ring (Figure 2.1). It is known to be biodegraded under aerobic conditions. It is chosen in order to tackle the heterocyclic ring of Chloridazon-desphenyl. Under aerobic conditions, degradation of N-heteroaromatic compounds is usually initiated by a hydroxylation adjacent to the N-heteroatom (Fetzner 1998). This feature applies to pyridine and some of its derivatives. The biodegradation of pyridine involves two general strategies: hydroxylation reactions followed by reduction (Figure 2.2, pathways G and H) or reductive pathways not initiated by hydroxylation (Figure 2.2, pathways A-F) (Fetzner 1998).

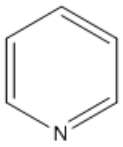


Figure 2.1. Pyridine structure.

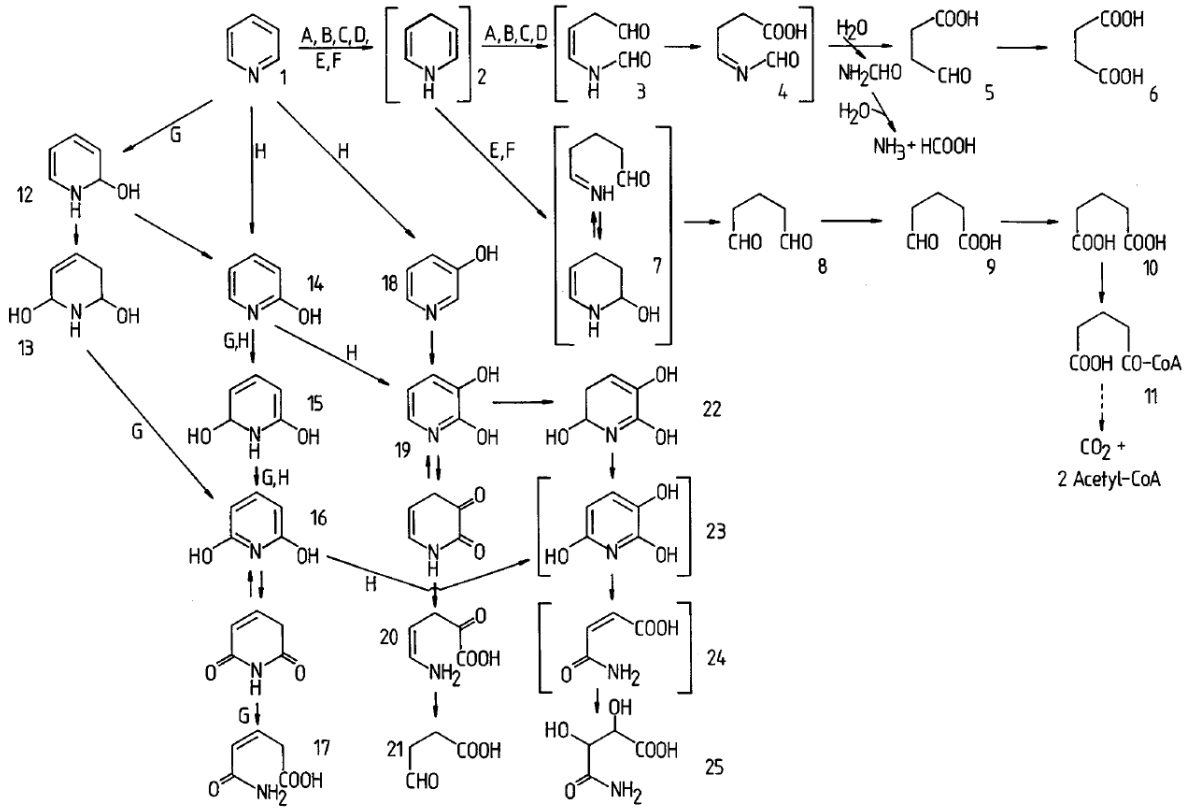


Figure 2.2. Hypothetical pathways for the aerobic microbial degradation of pyridine (Fetzner 1998).

Hydroxypiridine: a pyridine ring with a hydroxyl substituent. Biodegradation occurs via hydroxylation of the ring yielding a di- or tri-hydroxypyridine intermediate, followed by ring cleavage (Fetzner, 1998). The pathways for 2-hydroxypyridine and 3-hydroxypyridine are shown in Figure 2.2 (starting at 14 and 18, respectively). Another possible pathway for 2-hydroxypyridine and 3-hydroxypyridine is shown in Figure 2.3. Finally, a pathway for 4-hydroxypyridine is shown in Figure 2.4.

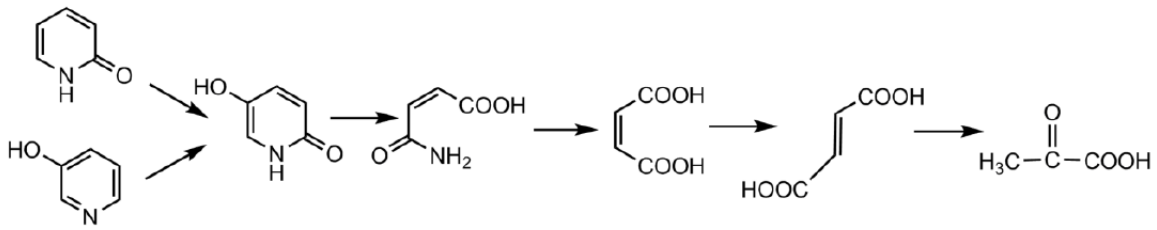


Figure 2.3. Bacterial degradation of 2-hydroxypyridine and 3-hydroxypyridine (Ratledge, 1994).

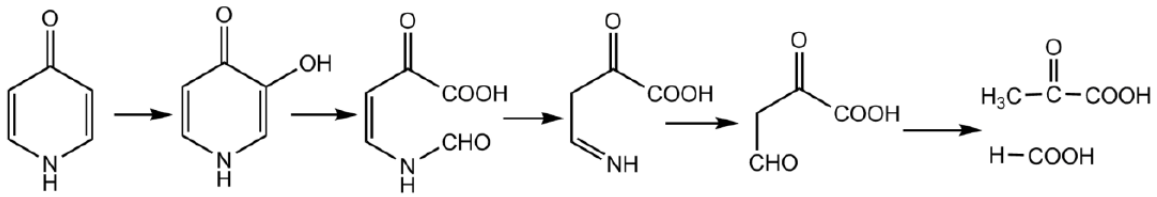


Figure 2.4. Bacterial degradation of 4-hydroxypyridine (Ratledge, 1994).

Both pyridine and hydroxypyridine are interesting as structural analogues because their biodegradation breaks the N-heterocyclic ring, which is also present in Chloridazon-desphenyl. Pyridine has no substituents, while hydroxypyridine has a substituent, which could be interesting to enhance the degradation capacity of the bacteria towards the Chloridazon-desphenyl substituents. However, some of the biodegradation pathways of pyridine involve hydroxylation, yielding hydroxypyridine as an intermediate, so a substituent will be present during biodegradation.

Finally, it is decided to use pyridine because of its availability, potential for aerobic degradation, and possibility to be measured analytically. The concentration of pyridine in the batches is selected to be 200 mg/l based on a literature research and practical reasons. The research included studies where structural analogues were used to promote biodegradation of a substance. Table 2.1 shows the concentration of substance and its structural analogue used in three studies. It is observed that the concentration of analogue is much higher than that of the substance. Furthermore, several pyridine biodegradation studies show that pyridine is degraded by pure cultures between 50 and 2000 mg/l (Li et al. 2009, Lin et al. 2010). A pyridine concentration within this range was chosen. According to Li et al. (2009), the incubation time needed for a complete degradation of 250 mg/l of pyridine by a pure culture is around 7 days. Since in our experiments a mixed culture was chosen, the time needed is expected to be higher.

Based on this literature search, keeping in mind that the concentration of chloridazon in the experiments will be 1 mg/l and avoiding too high concentrations, which could be toxic for the microorganisms, the concentration of pyridine is chosen to be 200 mg/l.

Table 2.1. Concentration values from biodegradation studies.

Substance	Analogue	Source
0.2 mg/l DCA	1400 mg/laniline	(You and Bartha 1982)
250 mg/l DDE	1000 mg/lbiphenyl	(Aislabie et al. 1999)

2. Materials and Methods

Experimental Setup

Inoculum

All the experiments held under aerobic conditions contained the same aerobic enrichment culture as inoculum. This enrichment culture was prepared in several steps, initially using aerobic soil from a site where chloridazon has been used as a pesticide for a long time, and adding aerobic media and 1 mg/l chloridazon. This enrichment was performed in triplicates (old aerobic inoculums). After about three months, where some degradation was observed, a new aerobic inoculum was prepared. This new aerobic inoculum contained 10% of a mix of the three old aerobic inoculums, 1 mg/l chloridazon and aerobic media. This enrichment was used for biodegradation experiments.

Media

The media for the aerobic and structural analogue experiments consisted of Sørensen buffer, macro nutrients and trace elements. The composition of the macro nutrients and the trace elements of the available stock solution is shown in Table 2.2 and Table 2.3. The preparation of 1 litre of aerobic media needs: 50 ml of buffer from a stock of 10 mM, six ml of the macro nutrient stock (Table 2.2) and 1.2 ml of the stock of trace elements (Table 2.3). The final concentrations in the media are shown in Table 2.2 and Table 2.3. The concentration of buffer in the final media is 0.5 mM.

Table 2.2. Macronutrients

Substance	Stock concentration (g/l)	Media concentration (g/l)
NH_4Cl	170	1.02
$CaCl_2.2H_2O$	8	0.048
$MgSO_4.7H_2O$	9	0.054

Table 2.3. Micronutrients

Substance	Stock concentration (g/l)	Media concentration (mg/l)
$FeCl_2.4H_2O$	2	2.4
$CoCl_2.6H_2O$	2	2.4
$MnCl_2.4H_2O$	0.5	0.6
$CuCl_2.2H_2O$	0.03	0.036
ZnCl2	0.05	0.06
HBO3	0.05	0.06
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.09	0.108
$Na_2SeO_3.5H_2O$	0.1	0.12
$NiCl_2.6H_2O$	0.05	0.06
EDTA (tripex 2)	1	1.2
Resazurin	0.5	0.6
HCl 36%	1 ml/l	1.2 µl/l

Batch Setup

All experiments were performed in glass serum bottles which were closed with Viton stoppers and an aluminium cap. They were kept in a 20°C controlled climate room, on a shaker (at 120 rpm) and they were covered with aluminium foil to prevent any photo-catalytic reactions (Buttiglieri et al. 2009). The experiments were fully aerobic, meaning that the headspace contained O_2 and that shaking was performed to ensure sufficient dissolution of molecular oxygen into the liquid media. Furthermore, regular monitoring of the headspace with GC was performed to monitor headspace O_2 concentrations; if O_2 was below 10%, the headspace was flushed with pressurized air.

Batches were prepared with various DOM sources, DOM fractions, pyridine as a structural analogue, and without an additional carbon source (Table 2.4). Furthermore, for all experimental setups, abiotic controls were prepared to ensure that observed degradation was actually due to biological processes. Abiotic controls were inoculated and subsequently inactivated with NaN_3 and $HgCl_2$. A summary of the batch experimental setups is provided below. All experimental setups were executed in triplicate.

Table 2.4. Overview of experimental setup. Batches are either biotic or abiotic, and may or maynot contain chloridazon ("–C").

	Inoculum (10% of batch volume)	Chloridazon (1 mg/L)	Compost: Total DOM (15 mg C/L)	Compost: Humic Acids (15 mg C/L)	Compost: Fulvic Acids (15 mg C/L)	Groundwater DOM (15 mg C/L)	Pyridine (200 mg/L; 152 mgC/L)	Toxic Agents (2 mM NaN_3 1 mM $HgCl_2$)
Biotic Tot DOM	X	X	X					
Biotic Tot DOM -C	X		X					
Abiotic Tot DOM	X	X	X					X
Biotic HA	X	X		X				
Biotic HA -C	X			X				
Abiotic HA	X	X		X				X
Biotic FA	X	X			X			
Biotic FA-C	X				X			
Abiotic FA	X	X			X			X
Biotic GW	X	X				X		
Biotic GW-C	X					X		
Abiotic GW	X	X				X		X
Biotic Pyr	X	X					X	
Biotic Pyr-C	X						X	
Abiotic Pyr	X	X					X	X
Biotic No Add	X	X						
Abiotic No Add	X	X						X

DOM

DOM characterization

Two different sources of DOM were used to obtain 4 different types of DOM. One DOM source was a DOM sample taken from a groundwater monitoring well. This DOM was collected from a filter of an anion exchange column used for the purification of drinking water. This sample was provided by the drinking water company Vitens and it was characterized by the Soil Quality Lab of Wageningen University in April 2013. The results of this fractionation, expressed in percentage of HA, FA, hydrophilics (Hi) and hydrophobic neutrals (HON) are shown in Figure 2.5. It is noted that the DOM of this sample was made up mostly by FA,

which made up the 85% of its total organic carbon (OC) (Figure 2.5). The sample contained also a small fraction of HA and Hi, which represented respectively the 2% and 10% of its total OC. A second fractionation of the sample was performed to see if any variation occurred. From the results it is seen that its DOM composition is nearly the same, with a slight decrease of FA content and an increase of HA and HON (Figure 2.5). Since for this project we wanted to focus on the role of the most common fractions of DOM found in groundwater, the natural composition of the DOM of groundwater sample made the fractionation not needed. Therefore, this sample was used for the experiments without previous fractionation.

The second source of DOM was a forest (leaf) litter compost collected from the Van Iersel compost facility in The Netherlands. This compost was chosen based on its properties which were investigated and characterized in a previous study of Straathof et al. (2014). According to this study, this compost showed to be a mature substrate, rich in FA and HA (Figure 2.6). In order to have more updated and reliable data about the DOM used for the experiments, also for this sample a second fractionation was performed.

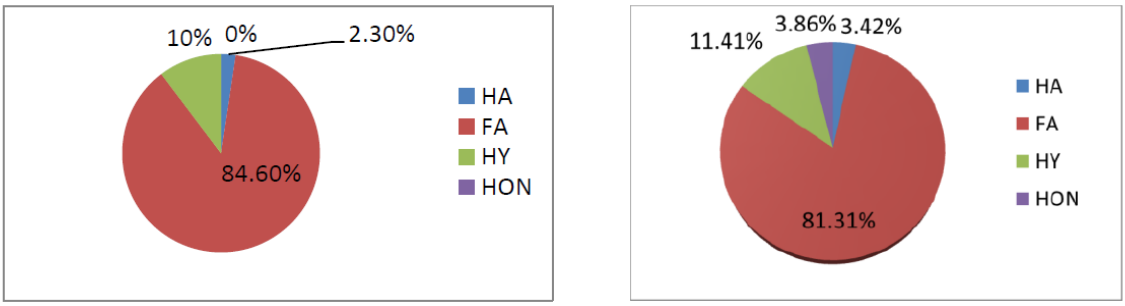


Figure 2.5. Characterisation of the Vitens' DOM sample, fractionated in 2013 (2.a) and in 2014 (2.b). The fractions are expressed as a percent of total DOC.

It can be observed that the results of the two fractionations show some differences between each other's. The latest fractionation indicates a higher percentage of FA in comparison with the fractionation of Straathof et al. (2014). Moreover, it shows an higher the percentage of HA, and a slight decrease of the Hi pool. This can be due to many reasons. One possible explanation is that the fractionation of the sample was done 4 weeks after its collection from the compost facility. Even though the compost was kept in a conditioned room at 4 °C, during this period microbial degradation might occurred, resulting in less Hi and HA content and an increase of FA fraction. Nevertheless, the differences in DOM properties could be caused by some variations in the compost production process, maturity level or in the raw material used to produce the compost. This hypothesis is also suggested by the fact that the organic matter extractable in ultrapure water from the compost that was used in this project, a few days after its collection was much lower than that extracted by Straathof et al.(903 mg/kg), even though the extraction ratio was higher (1:10 done by Straathof et al. and 1:2 done for this experiment). It cannot be excluded however than the differences in fractions composition was due to different dilution ratios used for these DOM samples. The dilution ratio has in fact an impact on the aggregation and of HS molecules, which can be classified as different DOM pools depending on their aggregates size (Sutton and Sposito 2005).

From the compost 3 types of DOM were extracted and used for the experiments. Isolated HA and FA were extracted and used as single samples. Moreover, a sample of total DOM was used for the experiment without previous fractionation of its C pools. This was done to investigate whether the utilization of HA and FA by microorganism and their effects on Chloridazon biodegradation showed some differences in the case they are singularly bioavailable or together with other DOM fractions.

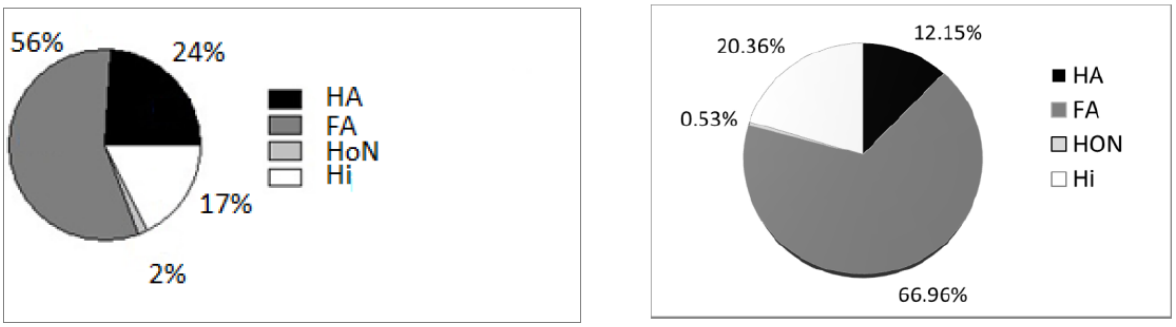


Figure 2.6. Characterisation of the forest leaf litter compost fractionated by from Straathof et al. (2014) (left) and the second time, this project (right). The fractions are expressed as a percent of total DOC.

DOM Extraction

To maximise the DOM concentration obtainable from the compost, a 1:2 (soil to water) extraction in ultra-pure water (UPW) was performed. Extraction with UPW was preferred because it allows to extract the directly bioavailable fraction of DOM (reader Applications in Soil and Water Chemistry (SOQ-34806), 2014). In total 3.5 kg of compost were used and extracted with 7 litres of UPW. The compost was firstly equilibrated with UPW (ratio 1:2) for 1 hour by horizontal shaking at 180 rpm. Thereafter, the solution was centrifuged at 3750 rpm for 15 min. The supernatant was taken and put in an ultra-speed centrifuge at 10000 rpm for 20 minutes to separate better the clean solution from the bigger fraction of organic matter. Subsequently, the supernatant solution was filtered through a 0.2 um membrane filter (cellulose acetate; OE676, Schleicher & Schuell) previously washed with UPW. A vacuum filtration system was used to filtrate the extraction solution. DOM is defined as the fraction of organic matter which passes through a 0.45 um filter (Perdue, 2003). However, for this project the 0.2 um filter was preferred in order to exclude the microorganisms from the extraction solution. Two subsamples were taken from the DOM extracted from compost. One was fractionated to isolate HA and FA, and the other was used for the total DOM experiments, without previous fractionation. The DOC concentration of the solution obtained by a 1:2 extraction was 170 mgC/L.

DOM Fractionation

A rapid-batch fractionation procedure (van Zomeren and Comans, 2007) was used to first isolate HA and FA fractions from the total DOM extracted from the compost. The starting solution containing DOM (in total 4.8 liters) was first acidified to pH 1 using 6M HCl. In this way the HA precipitated, since this fraction is not soluble at pH<2 (van Zomeren and Comans, 2007). The acidified solution was allowed to stand on a bench overnight and then was centrifuged at 3750 rpm for 20 minutes to recover the HA fraction. Subsequently, the HA were redissolved in a minimum volume of base solution of 0.1 M KOH (80 ml) and the concentration was measured with a Sievers™ 900 Series Total Organic Carbon (TOC) Analyser at the Soil Quality Department. The concentration of organic carbon in the HA solution was 493.5 mg/L. The solution was therefore diluted 3.1 times with UPW to reach the desired concentration of 170 mg/L. However, the final concentration was a bit lower than expected: 142 mg/L.

In order to lower the K concentration in the solution of HA intended to the experiments, cation-exchange Amberlite resin was used (1:40 resin to solution ratio). The resin on the one hand bound the K present in the solution, on the other hand it released H+ to the solution. In this way not only the HA solution was free from K, but also the pH was lowered to pH 7 without the addition of HCl. This was done to prevent the presence of supplementary salts in the experiments.

DAX-8 resin was added to the remaining supernatant composed of FA+HY+HON. A ratio of 1:5 resin to solution was used. The resin is non-ionic and hydrophobic so the partially aromatic FA and HON fractions bound on its surface, while the Hi of DOM remained in solution (van Zomeren and Comans, 2007). After having put for 1 h the solution with the resin in a horizontal shaker at 180 rpm, the solution was separated from the resin with a vacuum filtration system. In order to re-dissolve the FA pool, the resin was equilibrated in 0.1 M KOH (in total 200 ml) using a horizontal shaker at 180 rpm for an hour. This step was repeated two times, until the resin was clear in colour. Amberlite resin was added again to the FA solution in order to remove the K present and lower the pH to 7. Thereafter, the TOC concentration in the solution was measured on the TOC Analyser. The concentration of TOC in the FA solution was 404 mg/L. For the experiments the FA solution was diluted 2.5 times in UPW. The final concentration was 153 mg/L.

Analytical Methods

Chloridazon, Chloridazon-desphenyl, Chloridazon-methyldesphenyl

Chloridazon, Chloridazon-desphenyl, and Chloridazon-methyldesphenyl were measured in an HPLC from an external lab. The samples, which had an initial chloridazon concentration of approximately 1 mg/l, were diluted 100 times before sending them. This way, the external lab received samples which initially had 10 µg/l of chloridazon. The external lab provided bottles where the samples could be diluted and sent. The dilution process consisted of pipetting 1 ml of sample from the Eppendorf and adding ultra-pure Millipore water up to 100 ml.

Pyridine

The analysis of pyridine was performed in an HPLC in the ETE lab. Before analysing any samples, standard solutions at known concentrations of pyridine were analysed in the HPLC in order to obtain a proper calibration curve. Furthermore, one of the biotic samples from the structural analogue experiments was analysed, to make sure that pyridine could be clearly analysed in the samples. After analysing standard solutions of 50, 25, 12.5 and 6.25 mg/l pyridine, and a blank sample, it was observed that a linear calibration curve occurred between the concentrations of 0 and 25 mg/l pyridine. Therefore, this was the concentration range where the samples should be when analysed.

Microbial Respiration

The oxygen and carbon dioxide levels analysed by the GC showed the percentages of these compounds in the headspace of each of the sampled bottles. During the incubation time, the respiration of aerobic microorganisms was measured regularly to follow microbial activity. To measure the oxygen consumption and CO_2 production, a gas sample of 2-2.5 ml from the headspace of the experimental bottles was taken with a syringe of 2.5 ml volume and measured with a gas chromatograph (GC-2010 Gas Chromatograph Shimadzu). Loop injection was performed. The percentage of oxygen present in the headspace of the bottles was subtracted from the initial oxygen percentage (21%). The result is expressed as percentage of oxygen consumed, which was recalculated to mmol/L.

TOC analysis

TOC was measured at the beginning of the experiment (day 1) and at the end (day 63), to assess the degradation of DOM by microorganisms. Segmented Flow Analysis (SFA) analysis was performed by the Soil Quality Department Laboratory. Before the analysis, 5 ml of each sample were filtered with a 0.2 um filter to exclude the microbial biomass from the C measurements. Cellulose acetate filters were prewashed with 240 ml of UPW to prevent C contamination.

Aromaticity

For each DOM sample, the aromaticity was assessed at the beginning (day 1) and at the end of the experiment. Specific ultraviolet adsorbance at 254 nm was used as a parameter for estimating the aromaticity of the samples. SUVA254 values divided by the DOC concentration of the solution served as an indicator of overall DOM concentration and aromatic carbon contents (Leenheer, 2003). For this purpose a spectrophotometer (Genesys 10S UVeVIS, Thermo Fisher Scientific Inc., Waltham MA, USA) of laboratories of Soil Quality Department was used. UPW was used as a blank. Moreover, the data obtained from the SUVA measurements were correlate to the percent aromatic carbon contents determined by 13C NMR spectroscopy. This was done by using the equation found in the study of Weishaar et al. (2003). Weishaar et al. (2003) show in fact that there is a strong correlation between the SUVA254 and the percentage or aromatic carbon determined with ^{13}C NMR spectroscopy ($R^2=0.97$).

In addition, to follow the changes in the DOM structure after microbial degradation, a fractionation was performed at the end of the experiment period (day 63) to the solution of the experimental bottles containing HA, FA and total DOM. Both the experimental sets with and without Chloridazon have been used for the final fractionation in order to observe possible differences in the microbial degradation mechanisms of the DOM with the presence of the pesticide. Before starting the fractionation procedure the samples were filtered with 0.2 um acetate cellulose membrane filters to remove microbial biomass.

3. Results and Discussion

Chloridazon and Chloridazon-desphenyl Biodegradation

Comparing the results of abiotic controls with the biotic experiments it is seen that microbial degradation of Chloridazon occurred (Figure 2.7). The concentration of Chloridazon in abiotic experiments remains stable during the whole experiment, while Chloridazon-desphenyl is not formed in any of the abiotic experiments. Only in the abiotic experiments with HA a slight decrease of Chloridazon concentration is observed, probably caused by analytical uncertainties.

The no-DOM controls in absence of DOM show that Chloridazon-desphenyl is formed as a result of Chloridazon degradation and Chloridazon-desphenyl it is not further removed, indicating accumulation of Chloridazon-desphenyl. On the other hand, in the biotic experiments containing total DOM, HA, FA and groundwater sample, Chloridazon is completely removed within 14 days. At the same day, in the biotic batches with Total DOM Chloridazon-desphenyl is detected with a concentration around 0.007 mmol/L. This concentration is slightly higher than that expected if all the Chloridazon is broken down and transformed into Chloridazon-desphenyl, probably because of an analytical uncertainties in this measurement. From day 14, in the bottles with Total DOM the concentration of Chloridazon-desphenyl decreases to 0.001 mmol/L and stays constant from day 37 till the end of the experiment. In the case of the biotic experiments containing HA and FA Chloridazon-desphenyl is degraded faster. In fact, the concentration of this compound in the presence of HA and FA is already around 0.001 mmol/L at day 14 and it stays constant at this value till the end of the experiment. Contrary to the results of the DOM samples from compost, the Groundwater sample seems not to enhance microbial degradation of Chloridazon-desphenyl. Its concentration is fact stays constant at around 0.006 mmol/L from day 14 till the end of the experiment.

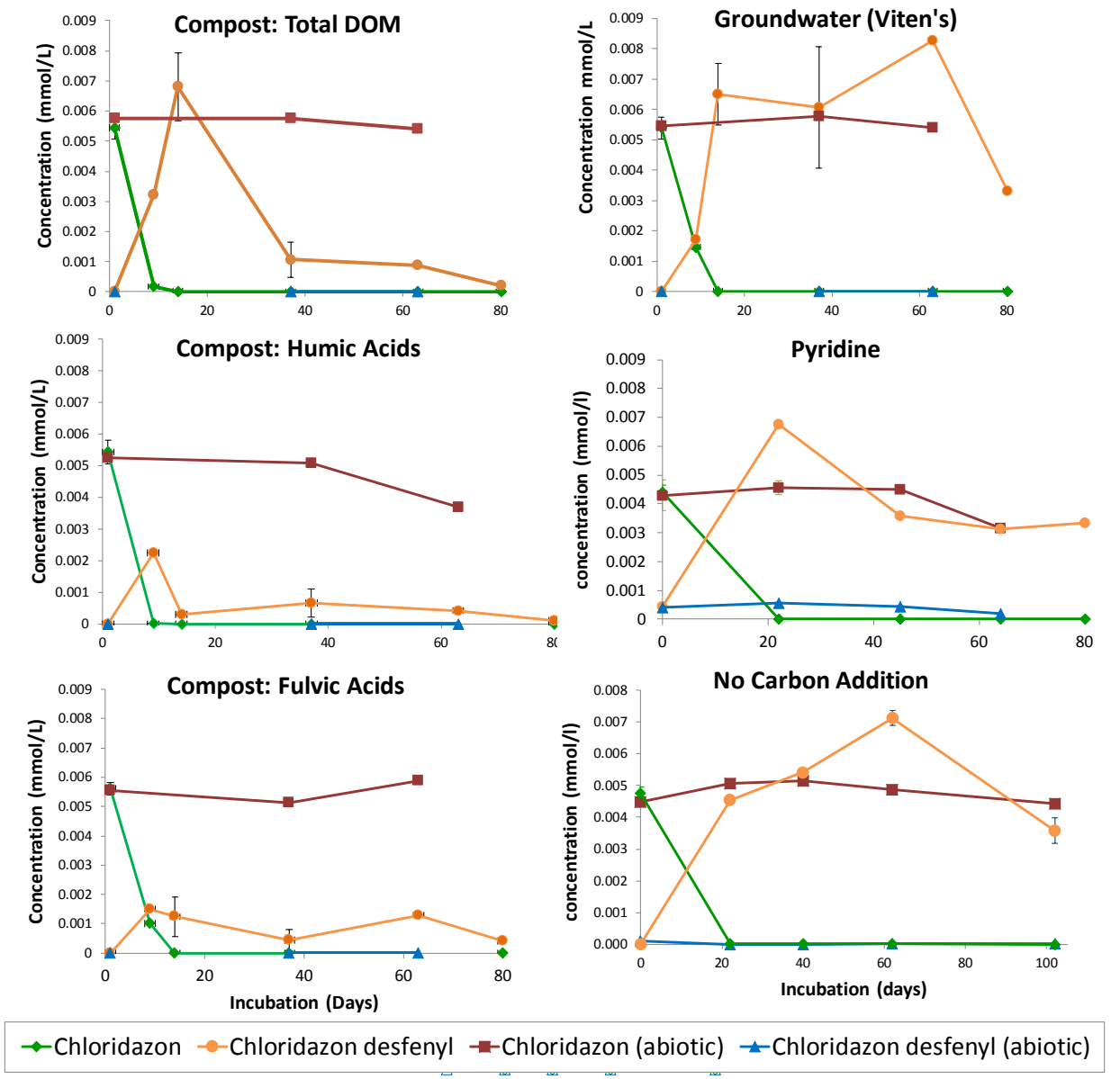


Figure 2.7. Biodegradation of Chloridazon and Chloridazon-desphenyl under aerobic conditions with and without DOM addition.

Microbial Respiration

In the graphs below (Figure 2.8), the respiration of aerobic microorganisms during the experimental period is shown. These graphs were normalized for the abiotic data. This was done because, although in the abiotic batches Chloridazon and DOM degradation were not observed, some variations of CO_2 and O_2 content were detected. This was probably due to abiotic processes, so it was decided to exclude these variations also from the biotic experiments and no-Chloridazon controls.

The results show that the biotic experiments (green lines) and in the no-Chloridazon controls (blue lines) containing total DOM, HA, and FA have similar O_2 consumption. At day 9 between 2.3 and 3 mmol/L of O_2 (averaged value) are consumed in the bottles with FA and HA. In the biotic batches and no-Chloridazon controls with total DOM, the same O_2 consumption is noted with one week delay (day 16).

Comparing the O_2 consumption with the CO_2 production, it can be seen that the high consumption of oxygen is not coupled with a similar CO_2 production, which stays much lower during the whole experimental period. The highest production of CO_2 is reached in the biotic bottles with total DOM (Figure 2.8) and in those with HA (Figure 2.8), where it is approximately 0.19 mmol/L for the former and 0.16 mmol/L for the latter (averaged values). In the presence of FA the CO_2 produced is equal to 0.06 mmol/L (averaged value) for the biotic experiments while no CO_2 is detected in the no-Chloridazon controls.

In contrast to the compost-derived DOM, the addition of pyridine resulted in significantly higher CO_2 production, both overall and relative to O_2 consumption. In batches with pyridine, the ratio of O_2:CO_2 is approximately 2:1. These results suggest full mineralization of pyridine, as opposed to compost DOM fractions, which were not fully mineralized.

In the groundwater sample and the experiments without additional carbon sources, different results are observed. In these experiments, the sharp O_2 consumption detected in the other bottles is not seen and the CO_2 production remains very low during the entire experiment period. While limited respiration in the bottles without DOM addition was expected, it is notable that no significant respiration was observed in the groundwater sample. These results indicate a significant difference in biodegradability of compost-derived DOM from groundwater DOM.

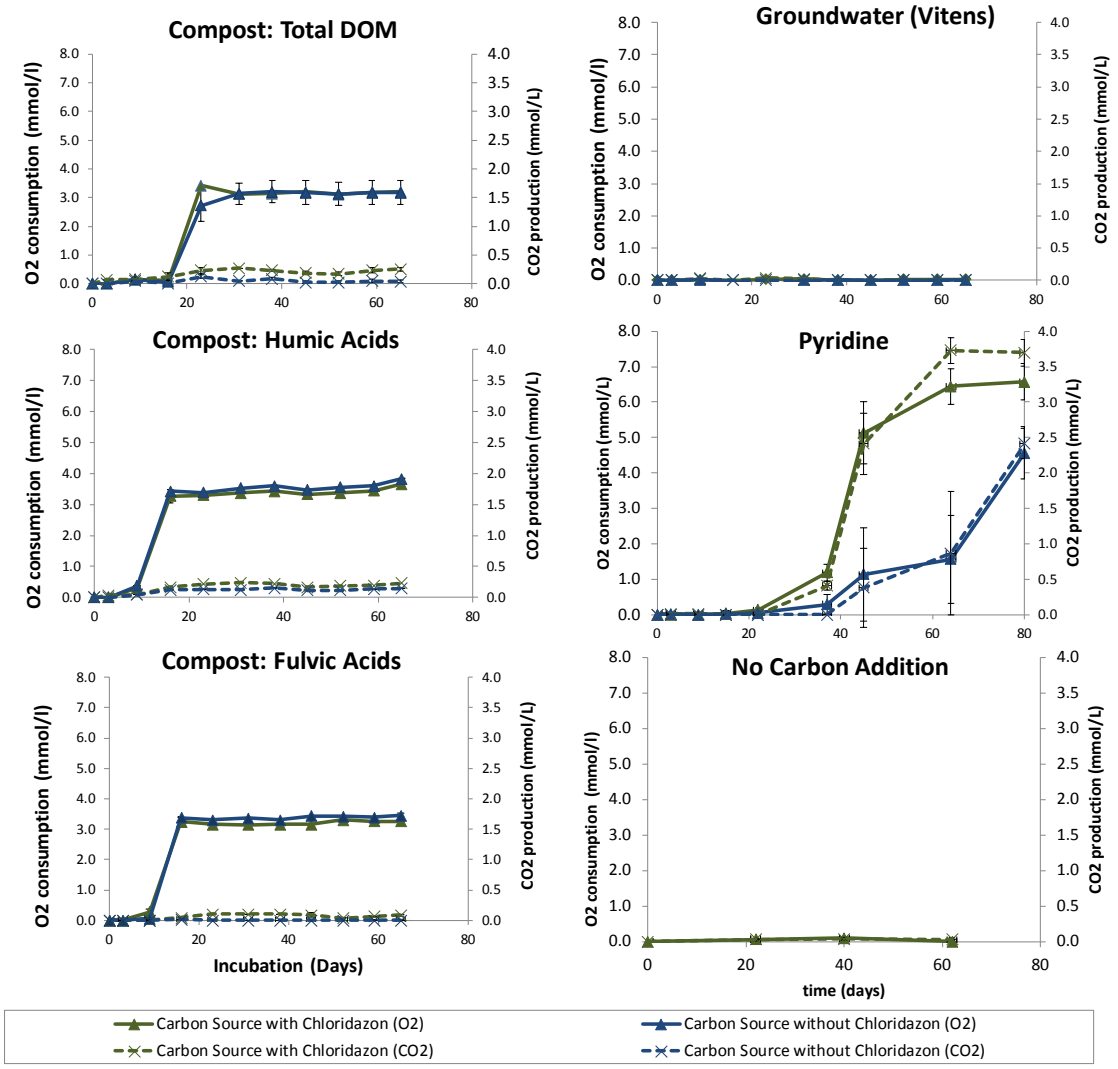


Figure 2.8. Respiration of batch experiments with and without addition of Chloridazon.

DOM and Pyridine Biodegradation

The total organic carbon (TOC) concentrations in the biotic experiments and no-Chloridazon controls were measured at the beginning (day 1) and at the end (day 63) of the experiment. From Table 2.5, it is observed that the values of TOC consumption in the biotic experiments and in the no-Chloridazon controls are very similar for all the types of DOM, suggesting that the presence of Chloridazon does not influence microbial utilization of DOM. In both the experimental setups, HA decreases the most in concentration, by as much as 25%, which is much higher in comparison with the other types of DOM. The TOC of the Total DOM decreased by 11%, followed by the TOC of Groundwater sample, which underwent a decrease of 7-8%. FA is the only sample in which a small difference between the TOC degradation in biotic experiment and no-Chloridazon controls is observed (4% of TOC removed in the biotic experiments versus 6.5% in no-Chloridazon controls). These results indicate that DOM samples are degraded at relatively low rates by the inoculated microorganisms.

Table 2.5. TOC degradation in biotic experiments with and without Chloridazon addition.

Biotic experiments	**Day 1**	**Day 63**	**Removal (%)**
	TOC (mg/L)	TOC (mg/L)	
Compost: total DOM	16.90	15.05 (±0.25)	10.9%
Groundwater	17.70	16.30 (±0.1)	7.9%
Compost: Humic Acids	14.20	10.70 (±0.1)	24.6%
Compost: Fulvic Acids	15.50	14.85 (±0.05)	4.2%
Compost: total DOM (No Chloridazon)	16.90	15.05 (±0.25)	10.9%
Groundwater (No Chloridazon)	17.70	16.35 (±0.15)	7.6%
Compost: Humic Acids (No Chloridazon)	14.20	10.6 (±0.2)	25.4%
Compost: Fulvic Acids (No Chloridazon)	15.50	14.5 (±0.0)	6.5%
	Pyridine (mg/L)	Pyridine (mg/L)	
Pyridine	170	0	100%
Pyridine (No Chloridazon)	167	123	27%

Aromaticity values of the DOM samples at the beginning (day 1) and at the end (day 63) of the experiment are reported in Table 2.6. Considering the data from day 1, it can be noted that HA is the sample with a highest aromaticity value (28%), as expected by reading literature (Gondar et al, 2005; Chen et al., 1977). Second are FA and groundwater samples, which show closely the same percentage of aromatic C content (respectively 25% and 24%). This result reflects the nature of groundwater, which is mainly constituted of FA. The sample with the lowest aromaticity value is Total DOM (not fractionated) (21%). Also this sample is made mostly by HA and FA, however the presence of also hydrophilic fraction (Hi) could be the reason for the lower aromaticity of its DOM.

At the end of the experimental period, the aromatic fraction becomes enriched in the HA microcosms, both in biotic experiments (from 28% to 32%) and no-Chloridazon controls (from 28% to 31%). In contrast, the other types of DOM show only a very minor variation in aromaticity degree throughout the experiment, both in biotic experiments and no-Chloridazon controls. This reflects the low microbial degradation of these types of DOM, shown in Table 2.5. The similarity of the aromaticity results of DOM in biotic experiments and no-Chloridazon controls indicates that the presence of Chloridazon does not have an effect on the microbial utilization of DOM.

Table 2.6. Aromaticity of the DOM types at the beginning (day 1) and at the end (day 63) of the experiment. The aromaticity is expressed as SUVA and as percentage of aromatic carbon calculated with the equation of Weishaar et al (2003). The data refer to the biotic experiments under aerobic conditions. Values are the average of duplicates.

Biotic batches	**$SUVA_{254}$ (L/m-mg) Day 1**	**% Aromaticity day 1**	**$SUVA_{254}$ (L/m-mg) Day 63**	**% Aromaticity Day 63**
Compost: total DOM	2.66 (±0.03)	21%	2.80 (±0.07)	22%
Groundwater	3.17 (±0.04)	24.3%	3.25 (±0.04)	25%
Compost: Humic Acids	3.74 (±0.02)	28%	4.22 (±0.04)	31%
Compost: Fulvic Acids	3.31 (±0.02)	25%	3.31 (±0.04)	25%

In Figure 2.9 the results from the fractionation of the total DOM, HA and FA samples at the beginning (day 1) and at the end (day 63) of the experiment are shown. All the DOM samples show that as microorganisms degrade TOC, the contribution of their fractions (HA, FA, Hi, HoN) to the total DOC undergoes some changes. The results in Figure 9.a indicate that total DOM experiences a complete depletion of its HA pool. Moreover, as the HA are depleted, the relative amount of Hi and FA increases (Figure 2.9).

Considering Figure 2.9, which refer to the HA sample, it is noted that the percentage of HA, which was 100% at the beginning of the experiment, undergoes a decline during the period, reaching 33% at the end of the experiment. On the other hand, the pools of FA and Hi, which at the beginning were not present, increased up to 48-51% for FA and 19-15% for Hi, respectively in biotic experiments and no- Chloridazon controls. This suggests that HA are not fully mineralized by microorganisms, but they are partly converted in smaller molecules that (re)supply supplied the Hi and FA pools.

The DOM sample initially made up only by FA (Figure 2.9), during the experimental period experienced a decrease of the original fraction (from 100% to 83%), resulting in an increase of Hi up to 17% both in biotic experiments and no-Chloridazon controls. Also from these results, it is seen that the presence of Chloridazon did not influence the changes in fractions of DOM following the microbial utilization of OC.

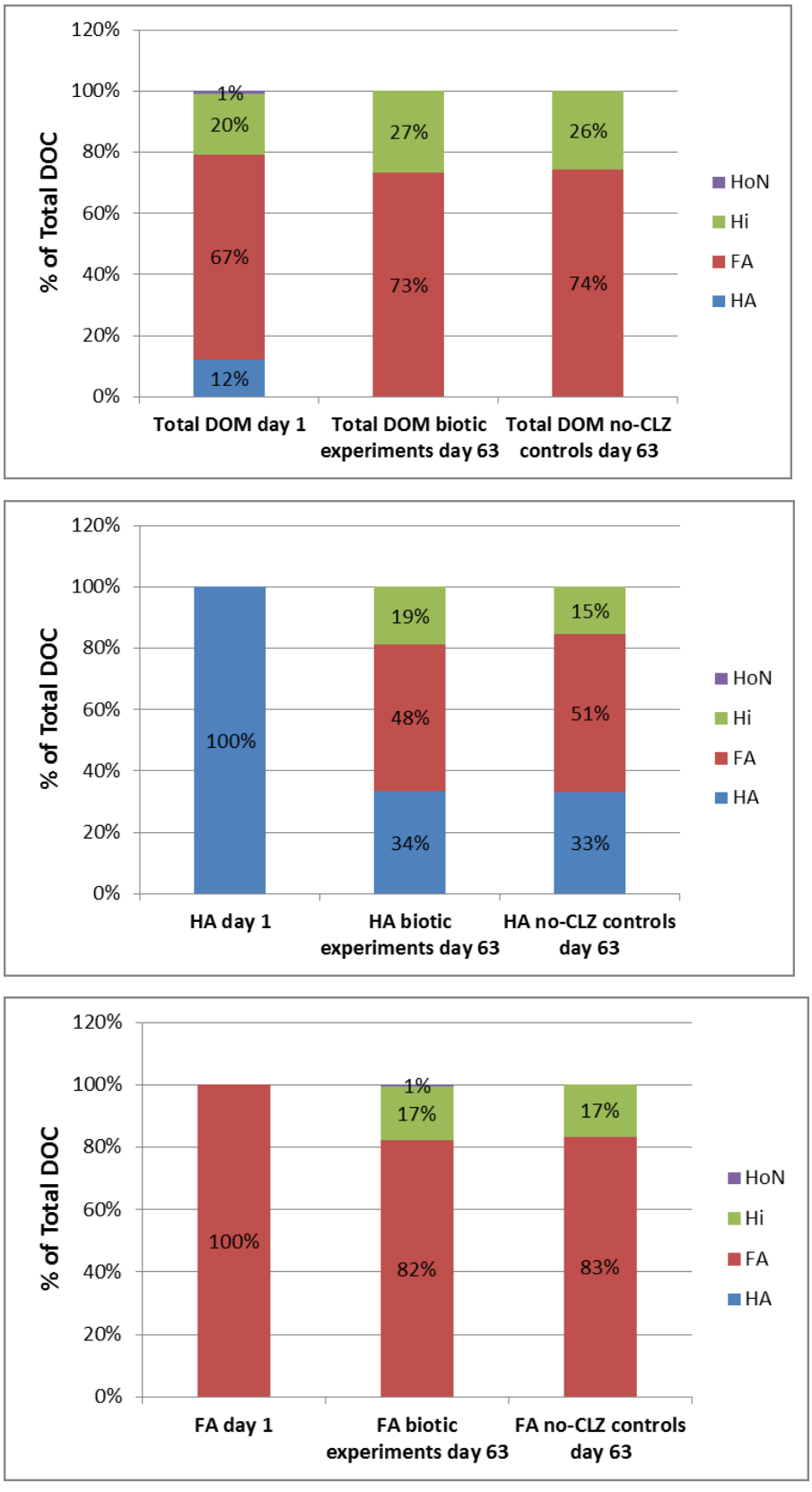


Figure 2.9. DOM fractionation in Compost experiments from start and end of experiment.

From our results it is seen that not all the types of DOM could enhance microbial degradation of Chloridazon and Chloridazon-desphenyl. Moreover, a linear relationship between microbial utilization of DOM and the Chloridazon-desphenyl biodegradation was not observed. These observations suggest that the quality of DOM available for microorganisms is the main parameter that has an influence on the microbial capacity to degrade the target compounds. As a consequence, the hypothesis according to which DOM could enhance biodegradation of Chloridazon and its products by just providing more C and energy to microorganism is refuted. Even though it is not possible to clearly identify the processes that enhance Chloridazon-desphenyl degradation, the hypothesis that considers certain types of DOM as structural analogues of Chloridazon-desphenyl and/or efficient first substrate for microbial co-metabolisms can be supported. In particular, from our results it is clear that the focus on further research should be on isolated FA and HA. In fact, these fractions showed to be more efficient in enhancing Chloridazon-desphenyl biodegradation when they are present as isolated DOM samples instead of when they are together with other DOM fractions. In fact, it is seen that in the presence of Total DOM, which differs from the other two DOM samples only for the presence of Hi, the degradation of the compound starts later than in the presence of isolated HA and FA. Therefore, it can be hypothesized that the bioavailability of relatively shorter and more readily usable C molecules contained in the Hi pool leads to their preferential utilization by microorganisms. Moreover, only after that microorganisms have degraded the readily available OC from the Hi pool, and start utilizing HA and FA, degradation of Chloridazon-desphenyl will occur.

Results show that the naturally occurring groundwater DOM studied for this research cannot enhance Chloridazon-desphenyl biodegradation. However, our results stress that microorganisms are able to efficiently remove Chloridazon and Chloridazon desphenyl in aerobic conditions and in the presence of other DOM samples, such as compost-derived HA and FA. These findings can have practical implications in in-situ bioremediation programmes. In fact, DOM samples like compost-derived dissolved HA and FA could be adopted as catalysers of biodegradation of Chloridazon and Chloridazon-desphenyl, in order to clean up water contaminated with these two target compounds. Therefore, future research shoul examine the potential for using DOM to stimulate biodegradation.

4. Conclusions

The results of this research added valuable knowledge about biodegradation of Chloridazon and its degradation products. In particular, information was gathered about biodegradation of Chloridazon and its degradation products, in the presence and absence of DOM and the structural analogue pyridine. The main results or this study were the following:

- Under aerobic conditions Chloridazon was biodegraded, both in the presence and absence of DOM and pyridine. As Chloridazon was degraded, Chloridazon-desphenyl was formed as a degradation product, while Chloridazon methyl-desphenyl was not detected during the whole experiment.
- The quality of DOM is the main parameter that influences the stimulation of biodegradation of Chloridazon-desphenyl by DOM.
- DOM samples extracted from a forest leaf-litter compost allowed complete Chloridazon-desphenyl biodegradation under aerobic conditions. In particular HA and FA degraded the compound faster than the Total DOM sample. On the other hand, the Groundwater sample of was not effective in stimulating microbial degradation of Chloridazon-desphenyl.
- Under aerobic conditions all the compost-derived DOM samples were mineralized by microorganisms, even though at relatively low rates.
- Degradation of pyridine started after a lag phase of 30 to 40 days. Pyridine, which was in a higher concentration than chloridazon, stimulated the respiration activity of the present bacteria.

Research Line 3: Understanding fate and degradation of commonly researched contaminants: MCPP,2,4-D, dichlobenil/BAM, bentazon

1. Introduction

Groundwater is an important source of freshwater on Earth, essential for drinking water production in many regions of the world. However, groundwater quality is increasingly threatened by organic micropollutants, especially pesticides. The extensive use of pesticides around the world, about two million tonnes per year (De *et al.*, 2014) combined with the persistence and mobility of pesticides results in the diffuse accumulation of pesticides in the water cycle. Pesticides can reach groundwater through run off from soil and subsequent infiltration (Stuart *et al.*, 2011). A recently published study by the Netherlands National Institute for Public Health and the Environment (RIVM) found pesticide residues at approximately 25% of the 200 drinking water production locations in the Netherlands (Swartjes *et al.*, 2016). In order to safeguard drinking water quality, it is essential to understand the environmental fate and transformation of pesticides in groundwater.

Biodegradation is viewed as the most important means for natural attenuation of pesticides in the environment. To predict and monitor the environmental fate of pesticides, it is required to have insights into the relationship among pesticides, microbial community and geochemical parameters, i.e. redox conditions and dissolved organic matter (DOM). Pesticide biodegradation is governed by microbial community (population diversity, biomass concentration, enzyme activities), substrate (chemical properties and pesticide concentration), and a range of environmental factors (pH, temperature, electron acceptors, carbon/energy sources, nutrients) (Boopathy, 2000). Thus, testing the natural potential for degradation is crucial and should focus on identifying limiting conditions in order to translate laboratory tests into field systems.

In groundwater systems, the biodegradation of pesticides is often challenged by limited carbon and nutrient sources (Egli, 2010). DOM, ubiquitously present in terrestrial and aquatic environments, therefore is believed to be an important factor determining pesticide biodegradation in such oligotrophic systems. DOM can be utilized by microorganisms since biomolecular components of DOM can provide energy and nutrients. Humic Acids (HA) and fulvic acids (FA) are currently regarded as a complex “supra-molecular” arrangement of diverse and relatively low molecular mass components, which include aromatic and aliphatic structures with associated functional groups (Leenheer et al., 2003; Sutton et al., 2005); this new view overturns the previously acknowledged refractory nature of these humic substances (Sutton et al., 2005).

However, promotion of pesticide biodegradation is not always the case. Pesticide biodegradation can be suppressed or inhibited in the presence of DOM, as DOM may be more readily degradable and more abundant than pesticides (Aislabie et al., 1995; Helbling, 2015; Willems et al., 1996). Aislabie et al. (1995) reported bacteria might less likely to degrade 2,4-D and carbofuran in the presence of alternative readily degradable carbon sources. Willems et al. (1996) found that addtion of glucose reduced the minieralizion of 2,4-D. DOM can also decrease the bioavailability of pesticides through adsorption (Dec et al., 1990; Odukkathil et al., 2013). Dec et al. suggested that the first degradation intermediate of 2,4-D, 2,4-

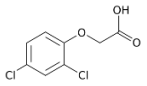
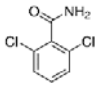
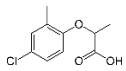
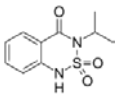
dichlorophenol, might become incorporated into humic substances, making it less susceptible to further degradation (Dec et al., 1990).

Currently, drinking water institutions have neither the necessary knowledge to predict the environmental fate and transformation of pesticides in anaerobic groundwater systems, nor available technologies to stimulate in-situ removal. Though a large body of information regarding the degradability of pesticides is available from regulatory testing for market authorization, little insights into quantitatively predict and interpret pesticide degradation in the actual field conditions are provided (Fenner *et al.*, 2013). To get a more comprehensive understanding of the environmental behaviour of pesticides in actual field conditions, this work studied pesticide biodegradation in simulated groundwater systems under different redox conditions and controlled temperature (10 °C). The influence of supplementing DOM on pesticide biodegradation was also investigated to determine if in situ biostimulation with DOM is a feasible technology. The results of this work provide insights into the interplays among pesticide biodegradation, DOM, and redox geochemistry.

Pesticides under investigation

The focus of the present study is on three different herbicides, namely 2,4-D, MCPP and bentazon, as well as on the metabolite 2,6-dichlorobenzamide (BAM) of the herbicide dichlobenil, which are frequently detected in groundwater and drinking water extraction wells in the Netherlands (Schipper et al., 2008). The concentration limit of individual pesticides and biocidal products including relevant transformation products in groundwater is at 0.1 µg l^{-1}, set by the European Commission under the EC Directive (2006/118/EC) from 2006 (EC, 2006). This quality standard also states that the sum of individual pesticides should not exceed 0.5 µg l^{-1} (EC, 2006). However, in the course of a groundwater monitoring program of pesticides in the Netherlands, over 20% of the samples exceeded the quality standard limit in phreatic groundwater and also deeper groundwater levels (>20m below soil surface) showed an exceeding of the limit in at least 7% of the samples (Schipper et al., 2008). 2,4-D, MCPP, bentazon and BAM were among these monitored substances, which were apart from 2,4-D frequently measured above the groundwater quality standard limit of 0.1 µg l^{-1}. 2,4-D exceeded the limit in one sample. A monitoring of drinking water stations in the Netherlands between 1988 and 2005 revealed that BAM, bentazon and MCPP were present in several pumping wells above the concentration limit of 0.1 µg l^{-1} (Schipper et al., 2008). A national groundwater monitoring performed in 2009 in Denmark showed similar results (Malaguerra et al., 2012). BAM detection is even considered as the main cause for the decommission of drinking water wells in Denmark (Malaguerra et al., 2012). Simonsen et al. (2006) suspect that the contamination of aquifers by the metabolite BAM is a broader problem often underestimated. Furthermore, Malaguerra et al. (2012) observed that drinking water wells showed higher susceptibility to contamination of MCPP and BAM in urban and higher susceptibility to bentazon contamination in non-urban drinking water wells (Malaguerra et al., 2012).

Table 3.1. Physico-chemical properties of the target pesticides, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,6-dichlorobenzamide (BAM), mecoprop-p (MCPP) and bentazone.

Pesticides	CAS number	Molecular structure	Molecular weight	Solubility in water mg/L	pKa[a]	Log K_{ow}[b]	Mobility[c]
2,4-dichlorophenoxyacetic acid (2,4-D)	94-75-7		221	900 (Gupta *et al.*, 2006)	2.73	2.81	Mobile
2,6-dichlorobenzamide (BAM)	2008-58-4		190	2730 (Sørensen *et al.*, 2007)	-	0.77	Mobile
Mecoprop (MCPP)	7085-19-0		214.6	620 (Heron *et al.*, 1992)	3.21	3.13	Mobile
Bentazone	25057-89-0		240.3	570 (Dousset *et al.*, 2004)	3.3	2.8	Mobile

[a] Dissociation Constants
[b] Octanol/Water Partition Coefficient
[c] Soil Organic Carbon-Water Partitioning Coefficient K_{OC}.
K_{OC}<15: Very mobile;
15<K_{OC}<75: Mobile;
75<K_{OC}<500: Moderately mobile;
50<K_{OC}<4000: Slightly mobile;
K_{OC}>4000 = Non-mobile

MCPP

First commercially produced in 1956, MCPP (mecoprop) is a selective herbicide commonly used as a control of broad-leaved weeds (more preciously: dicotyledonous plants) in cereal crops such as wheat, barley and rye but also in grasslands and under vines and fruit trees (Mackay et al., 1997). Mecoprop belongs to the group of chlorophenoxyalkanoic acids and is applied as a racemic mixture namely the two different optically active R- and S-isomers, but only the R-isomers exhibits herbicidal properties (Thrasher et al., 2004). Mecoprop acts as a synthetic auxin and inhibits hormonalgrowth (Nitschke et al., 1999; Mottier et al., 2014). Therefore, MCPP is also called an auxin agonist (Cedergreen and Streibig, 2005). After application, the leaves absorb MCPP and transport it to the roots, where it affects hormonal processes (Kirby and Sheahan, 1994). Concomitant with these hormonal growth effects, the active isomer is assumed to increase the plasticity of the cell wall as well as processes such as protein biosynthesis or ethylene production (Environmental Protection Agency, 2007). As a consequence, vascular tissue is damaged and cell division at unusually high levels take place (Environmental Protection Agency, 2007).

Due to its high water-solubility, low sorption and chemical stability in the subsurface, MCPP is susceptible to leach out of the soil column into adjacent water bodies (Fletcher et al., 2004). Since MCPP has an extremely low vapour pressure, volatilisation into the atmosphere can be neglected, see Table 3.1. Under subsurface conditions, MCPP is known to be solely degraded by microorganisms, but only limited literature about the biodegradation in subsoil and groundwater especially under anaerobic conditions is available (Thrasher et al., 2004; Buss et

al., 2006). It is suggested that microorganisms have to adapt to prevailing MCPP concentrations in order to degrade it with a significant rate by using it as a carbon or energy source (Thrasher et al., 2004).

2,4-D

2,4-dichlorophenoxyacetic acid, commonly known as 2,4-D, is a herbicide from the group of phenoxy compounds, used to control broadleaf weeds (more specifically: dicotyledonous plants) in aquatic and terrestrial environments (Vencill et al., 2002). 2,4-D's primarily mode of action is the interference with the phytohormone system of plants by mimicking the overdose effects of natural growth regulators (auxins), which are responsible for controlling plant growth and development (Grossmann, 2005). 2,4-D hereby resembles the most common auxin indoleacetic acid (Walker et al., 2012). The auxin herbicide 2,4-D was found to reveal a three-phase response in plants, which is of varying duration depending on the concentration. The three phases are stimulation along with increased production, inhibition along with growth reduction and decay along with growth inhibition (Grossmann, 2010; Michel et al., 2004). The first phase is characterized by an enormous increase in the cell wall plasticity, production of ethylene and enhancement of the biosynthesis of proteins, DNA and RNA, causing uncontrolled cell division (Vencill et al., 2002; Tu et al., 2001). The increased production of ethylene leads to a reduction of growth, which characterizes the second phase (Grossmann, 2010). The third phase is launched, if the concentration of 2,4-D is high enough, and an inhibition of cell growth can be observed (Tu et al., 2001). Above a high enough concentration, it is possible that the stimulation phase is weakly defined or even non-existent (Michel et al., 2004).

Spray and vapour drift during the application of 2,4-D can lead to phytotoxic effects on non-target organisms (Walker et al., 2012). The widely used herbicide 2,4-D is suitable for studying pesticides since it is not only similar to structures of several other compounds of interest, but also susceptible to degradation and to leaching processes (Estrella et al., 1993; Boivin et al., 2005). The relatively high water solubility, low vapour pressure as well as the low binding to soil particles result in mobility of 2,4-D in the soil and subsequently a high potential to affect groundwater. Microbial activity constitutes a major role in the breakdown of 2,4-D by using the steps of hydroxylation, cleavage of the acid sidechain, decarboxylation and ring opening (Tomlin et al., 2006). Furthermore, methylation of 2,4-D catalysed by microbial enzymes was observed (Wagner et al., 1996). Previous research found that an adaptation of the microorganisms to degrade 2,4-D is possible and even an acceleration of the microbial degradation rate after repeated application of the 2,4-D was found (Arbeli and Fuentes, 2007).

Bentazon

Bentazon is a selective contact herbicide which is applied individually as active ingredient or sometimes in combination with other active ingredients in herbicide products such as Basagran® (produced by BASF) (Huber and Otto, 1994). Bentazon is typically applied to crops, after emergence from soil, to control several dicotyledonous weeds and sedges in for instance cereals, peas, potatoes, flower bulbs, soybeans, mint, peanuts or rice (Huber and Otto, 1994). Bentazon interacts with the photosynthesis apparatus at the level of photosystem II (PSII) (Macedo et al., 2008). Strictly speaking, its target site in dicotyledonous weeds is the D1 protein in the photosystem II (Michel et al., 2004). When successfully competing with quinone B, it can block the plastoquinone binding site resulting in a decrease of the electron flow rate through the PSII electron carriers from H2O to NADP (Macedo et al., 2008). Due to the blockage of the photosynthetic electron transport, secondary effects such as the formation of reactive oxygen species, inhibition of the synthesis of lipids, proteins or RNA can emerge (Al-Mendou and Ashton, 1984; Macedo et al., 2008). Due to its low sorption affinity in soil and its relatively high solubility in water, bentazon is

suggested to have a strong potential to reach and contaminate groundwater (Das and Bagchi, 2012).

Photodegradation by UV-light and biodegradation by soil bacteria and fungi are ways to breakdown bentazon (Akbar and Lin, 2010). Similar to 2,4-D, an enhanced degradation of bentazon was found in previously exposed soils to bentazon (Arbeli and Fuentes, 2007; Wagner et al., 1996). The main biodegradation pathway is the formation of 6-OH-bentazon or 8-OH-bentazon by introducing a hydroxyl group to the 6- or 8-position of the phenyl ring of bentazon and subsequent rapid further metabolization and incorporation of the degradation products into soil organic matter (Wagner et al., 1996). Also 2-amino-n-isopropylbenzamide was found to be a metabolite of bentazon, which can be transformed by hydrolysis into anthranilic acid. However, the most found and persistent metabolite of bentazon is methylbentazon (Wagner et al., 1996). The degradation of bentazon in the upper soil layer was found to be moderate, however it seems to be persistent in aquifers (Malaguerra et al., 2012; Wagner et al., 1996).

Dichlobenil and BAM

Dichlobenil is a broad-spectrum contact herbicide, which was commercialized 1964 in the US and 1970 in Europe as component of different formulation such as Casoron® (produced by Chemtura Corporation), Norosac (trademark expired) and Prex® (produced by Syngenta) (Holtze et al., 2008). It is used at a wide range of applications sites varying from food (e.g cranberries), ornamental lawns and forest trees to industrial preservatives and aquatic sites (e.g. drainage or sewage systems) (USEPA, 1998). The target location of dichlobenil is the cellulase synthase (Michel et al., 2004). Dichlobenil inhibits the biosynthesis of cellulose leading to malformation of cell plates, accumulation of the plant polysaccharide callose and disruption of cell wall regeneration in protoplasts (Vaughn et al., 1996). Dichlobenil has a relatively high sorption affinity and a low water solubility. Therefore dichlobenil is regarded to be not highly vulnerable to leaching into groundwater but to be more immobile in soil especially in soils with a high organic matter content (Clausen et al., 2004; USEPA, 1998). Due to its relatively high vapour pressure compared to other herbicides, volatilization is regarded as an important dissipation pathway for dichlobenil, depending on soil moisture and temperature (USEPA, 1998).

Dichlobenil is not degradaed by photolysis, but hydrolysis by alkalis takes place (Holtze et al., 2007). However, biodegradation plays is more important than abiotic degradation (Holtze et al., 2007). The European Commission banned dichlobenil in 2008 due to its harmful effects and the formation of the persistent metabolite 2,6-dichlorobenzamide (BAM) (EFSA, 2010; EC, 2008a). Since then, the usage of dichlobenil in the European Union is not longer authorised. However, its metabolite BAM can still be frequently found in the environment (Schipper et al., 2008). 2,6-Dichlorobenzamide, commonly known as BAM, is the major metabolite of dichlobenil and is formed by microbial degradation (Holtze et al., 2007). If abiotic degradation processes can also lead to the formation of BAM is unknown. In contrast to its parent compound, BAM has a relatively small sorption distribution coefficient and a high water solubility, resulting in a high potential to contaminate groundwater (Clausen et al., 2004). Although BAM has no intended mode of action, it was found to induce leaf margin chlorosis after application to roots (Leach et al., 1971). Furthermore, BAM is known to influence and inhibit chlorophyll production in plants and algae (Lovecka et al., 2015; Van Leeuwen and Maas, 1985). In several studies, BAM is regarded as persistent and not vulnerable to biodegradation by soil microorganisms (Tuxen et al., 2000). However, it was found that the ability of microorganisms to degrade BAM strongly depends on the prevailing environmental conditions and the history of the investigated soil considering the antecedent treatment with plant protection products (Bjorklund et al., 2011). Furthermore, not much is known about the different metabolites produced both from dichlobenil and BAM as well as the effect of differences in the environmental conditions on the behaviour and biodegradation of

these metabolites in adjacent drinking water resources (Holtze et al., 2007; Simonsen et al., 2006). It is suggested that microorganisms need to adapt to the degradation of dichlobenil and its metabolites in order to degrade them with a significant rate (Holtze et al., 2007).

2. Materials and Methods

Chemicals and regents

DOM extracts

Two types of DOM were applied in this work. One was extracted from the Green Compost collected from a Dutch company (Van Iersel Compost, the Netherlands), with a composition of 50% screened wood, 25% grass litter, and 25% leaf litter; another type of DOM was extracted from aquifer soils natural groundwater and was provided by drinking water company (Vitens, the Netherlands). Ultra-pure water (UPW) was added to compost with a ratio 4:1 (w/w, water to soil). The mixed solution was homogenized for 2.5 hours by horizontal shaking at 120 rpm. The solution then was centrifuged at 3500 rmp for 15 min, and the supernatant was centrifuged again at 9000 rpm for 25 minutes. Finally, the supernatant solution was filtered through a 0.45 µm membrane filter (Whatman-ME 25/21 ST) using a vacuum filtration system. Prior to use, DOM solution was stored at 4 °C.

Experimental set-up

Batch Pre-batch experiments

As a proof-of-principle, liquid batch experiments were performed to study the effects of DOM on 2,4-D biodegradation under aerobic condition. Prior to experiments, aerobic enrichment cultures were prepared using sludge as inoculum. The sludge was sampled from the influent of the wastewater treatment plant in Bennekom (the Netherlands). Media preparation is as shown in Research Line 2. The effects of Green Compost DOM and Vitens DOM on 2,4-D biodegradation were tested separately by preparing batches with Green Compost DOM, Vitens DOM, and no DOM addition. Working volume of each batch bottle was 100 ml, containing 10 ml liquid enrichment cultures and 73 ml media spiked with 2,4-D (1 mg/L) and DOM (10 mg/L). Abiotic controls were performed as a reference for any abiotic degradation of 2,4-D. Nitrification processes were inhibited by adding allylthiourea (5 mg/L). The pre-batch experiments lased for 2 weeks. 2,4-D was re-spiked once at day 2, twice per day from day 4 to day 7, and three times per day from day 8 to day 14 due to the fast degradation of 2,4-D. Samples were taken at day 0, 1, 4, 7, 10, and 14. The bottles were kept in dark with a temperature of 20 °C, shaking at 120 rpm. Experiments were done in triplicate.

Simulated ground water systems

Column experiments were conducted in four continuous-fed, up-flow soil columns under different redox conditions in order to simulate groundwater systems (Figure 3.1). The cylinder glass columns were packed with real aquifer soilsmaterial collected from a drinking water production location. The aquifer soil samples contained low organic matter content, between 0.33 to 1.60%. The aquifer soils material was retained in the columns by sintered glass filters (pore size 40-100 µm). The experimental set up is located in a cabinet, and the cabin walls are covered with blue Plexiglas walls to prevent any photodegradation of pesticides.

The aquifer soils for the columns were collected at Eijbergen in the east of the Netherlands. Each column is fed media and electron acceptors to simulate the redox conditions nitrate- and sulfate- reducing and methanogenic conditions. Column 2 received no additional electron acceptor. In each media, the concentration of each pesticide (2,4-D; BAM; MCPP; and bentazone) was 1 mg/L. Column experiments lasted for 440 days, and Green Compost DOM was added to the media at day 229 to stimulate pesticide biodegradation with a concentration

of 10 mg/L. The media was placed in a fridge at 10 °C, and was continuously stirred and bubbled with nitrogen. Sulfides were added via a separate air tight bottle to ensure no release of toxic sulfides to the environment. The flow rate was around 12 ml/h. Effluent and media samples were collected for chemical analysis.

Column experiments lasted for 440 days. During the first 229 days, columns were run with only media, electron acceptors, and pesticides in order to determine the natural biodegradation capacity of the aquifer material microbial community under the different redox conditions. Thereafter Green Compost DOM was added to the media at day 229 in an attempt to stimulate pesticide biodegradation with a concentration of 10 mg/L .

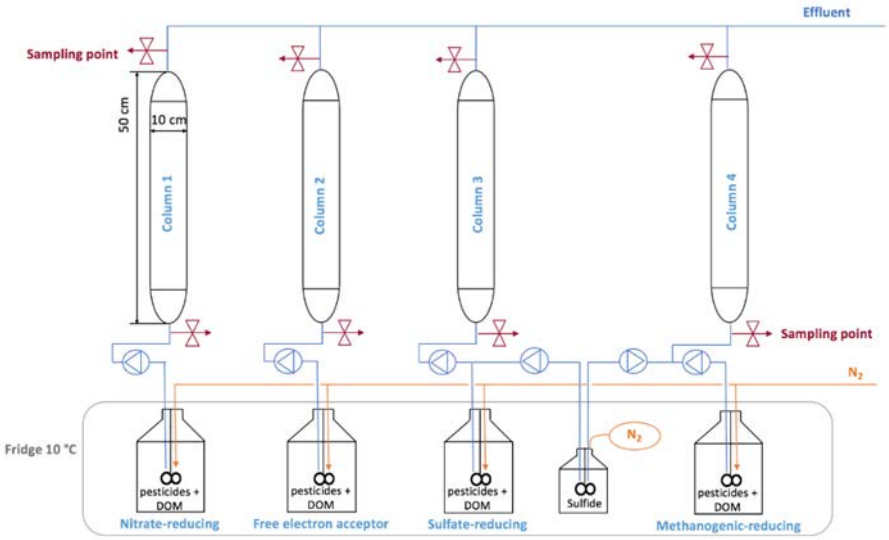


Figure 3.1. Column experimental setup simulatinged groundwater systems with different redox conditions. Column 1 was nitrate-reducing condition, column 2 was free electron acceptor condition, column 3 was sulfate-reducing, and column 4 was methanogenisisc-reducing condition. The column experiments lased for 440 days and DOM was added at day 229. Media was flushed with N2 (Column 2 was flushed with oxygen at the first 292 days).

Sampling and analysing

Pesticide analysis

Pesticide removal was calculated based on comparing the effluent concentrations per time point over the with the pesticide concentration in the media. Samples were pre-treated by centrifugation for 10 min with speed 10,000 rmp and stored at -20 °C before analysis. Pesticide concentration was measured by UPLC (ultimate 3000, Thermo, USA) with a diode array detector (DAD). UPLC is equipped with CSH phenyl-Hexyl column (3.5 mm, 300 Å,0.1 × 150 mm). The mobile phase was a mixture of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) with a flow rate at 0.3 ml/min. The sample injection volume was 50 µl. The detection results were acquired and analysed by Xcalibur software. Detection limit was 1 mg/L and the detection results of standards showed a good linearity (R^2>0.999). Samples were pre-treated by centrifugation for 10 min with speed 10,000 rmp and stored at -20 °C before analysis.

Electron acceptor analysis

Samples for nitrate and sulfate were pre-treated by centrifugation for 10 min with speed 10,000 rmp and stored at -20 °C before analysis. Nitrate and sulfate were measured by ion chromatography (Dionex ICS 2100, USA). Iron (II) was measured using Dr. Lange test kits (Hach Lange GmbH, Germany) on a Hach DR/3900 spectrophotometer. Iron (II) was

immediately analysed after sampling to prevent oxidation of Iron (II) to Iron (III). Samples were pre-treated by centrifugation for 10 min with speed 10,000 rmp and stored at -20 °C before analysis.

DOM quantification and fractionation

A rapid-batch fractionation procedure was used to describe characterize DOM before and after addition to the experimental setups and after pesticide biodegradation (van Zomeren et al., 2007). DOM was fractionated at compound-class level: humic acids (HA), fulvic acids (FA), hydrophilic acids (Hy), and hydrophobic neutrals (NoH). Samples were pre-treated by 0.45 µm filters. The starting solution of the total extracted DOM was acidified to pH 1 using 6M HCl, resulting in precipitation of HA. The acidified samples were stood incubated overnight and then were centrifuged at 3750 rpm for 20 min to separate HA from the supernatant. The precipitates (HA) were re-dissolved in a base solution of 0.1 M KOH and the concentration was determined on a Segmented Flow Analyser (SFA). DAX-8 resin (Sigma-Aldrich) was added to the supernatant composed of FA, Hy and HoN. After 1h horizontal shaking at 180 rmp, FA and HoN bound to the resin surface and the solution with Hy was collected and the concentration of Hy was analysed. Finally, 0.1M KOH were added to the DAX-8 resin to re-dissolve FA by 1h horizontal shaking at 180 rmp. This step was repeat twice in order to ensure all FA were re-dissolved in KOH solutions. NoH was the minus difference between TOC of DOM and TOC of HA, FA, and Hy . The results showed a good organic matter recovery (between 98-100.25%).

Molecular analyses

Microbial abundance and functional genes

Soil and liquid samples were taken from the top of the columns for microbial abundance and functional gene analysis. MoBio PowerSoil® DNA Isolation Kits and DNeasy® PowerSoil® Kits were used for DNA extraction. The filters, containing microbes, were cut in pieces and put in the power bead tubes. For the soil samples, 2 grams of solid were used and divided over four power bead tubes. Then DNA extraction was performed by following the protocol of the manufacturer. Quantitative polymerase chain reaction (qPCR) was used to quantify total bacterial based on the 16S rRNA gene quantification, and functional genes *tfdA* involved in the biodegradation of 2,4-D (Macur et al., 2007) was analysed by iQ SYBR Green based on Bio-Rad super mix using CFX384 Touch™ Real-Time PCR Detection System. Further, qPCR was also used to quantify other functional genes of microbes (*nirS*, *nirK*, *nosZ*, *dsrB*) related to electron acceptors to confirm the proper microbial community structure under different redox conditions. All qPCR assays were done in triplicate with a total volume of 10 µl reactions. Gene copy numbers from the results of qPCR were calculated as copies/ml sample, while for solids, the sample was calculated as copies/gram of soil. Detailed information for qPCR amplification of the functional genes can be found below.

Table 3.2. qPCR amplification of the functional genes

Functional gene	Primer sequence	Initial denaturing	Cycles	Denaturing, annealing, elongation
tfdA	(5′-GAGCACTACGCRCTGAAYTCCCG-3′) and (5′-GTCGCGTGCTCGAGAAG-3′)	10 min at 95°C	46	30 sec at 95°C 30 sec at 55°C 30 sec at gradient 56°C to 67°C 30 sec at 72°C
nirK	nirK876 (5'-ATYGGCGGVCAYGGCGA-3') and nirK1040 (5'-GCCTCGATCAGRTTGTGGTT-3')	10 min at 95°C	46	15 sec at 95°C 30 sec at 60°C 30 sec at 72°C
nirS	nirS cd3AF (5'-AACGYSAAGGARACSGG-3') nirS R3cd (5'-GASTTCGGRTGSGTCTTSAYGAA-3')	5 min at 95°C	46	15 sec at 95°C 30 sec at 56°C 30 sec at 72°C
nosZ	nosZ2F (5'-CGCRACGGCAASAAGGTSMSSGT-3') and nosZ2R (5'-CAKRTGCAKSGCRTGGCAGAA-3')	10 min at 95°C	46	30 sec at 95°C 30 sec at 60°C 30 sec at 72°C
dsrB	DSRp2060F-GC(5′-CAACATCGTYCAYACCCAGGG-3′) and DSR4R (5′-GTGTAGCAGTTACCGCA-3′)	3 min at 95°C	6	30 sec at 95°C 45 sec at 48°C 30 sec at 72°C
			40	30 sec at 95°C 45 sec at 58°C 30 sec at 82°C
Bacteria 16S rRNA	Eub341F(5-CCTACGGGAGGCAGCAG-3') and Eub534R (5'-ATTACCGCGGCTGCTGGC-3')	10 min at 95°C	40	20 sec at 95°C 30 sec at 60°C 30 sec at 72°C
Archaea 16S (787F& 1059R)	Arc787F (5'-ATTAG ATACC CSBGT AGTCC-3') and Arc1059R (5'-GCCAT GCACC WCCTC T-3')	10 min at 95°C	40	10 sec at 95°C 30 sec at 60°C

3. Results and Discussion

Batch Pre-batch experiments

Degradation of pesticides involves biotic transformation processes (mediated by microorganisms) and abiotic processes such as adsorption and chemical/photochemical reactions (Fenner et al., 2013). The results of abiotic controls suggest biotic transformation of 2,4-D was mainly involved in this pre-batch experiment (data not shown). Figure 3.2 shows the biodegradation rate of 2,4-D under aerobic condition, in relation to the DOM addition for the three experimental setups without DOM addition, with DOM GW and with DOM compost. 2,4-D degradation rate was increased with incubation time, indicating a further enrichment of 2,4-D biodegrading microorganisms. Furthermore, degradation and was promoted in the presence of DOM. Compost had a larger influence on 2,4-D degradation rate was most promoted in the presence of Green Compost DOM compared with that in the presence of than the Vitens DOM. Based on the results of this pre-batch experiment, Green Compost DOM was applied to the column systems to stimulate pesticide biodegradation under anaerobic conditions.

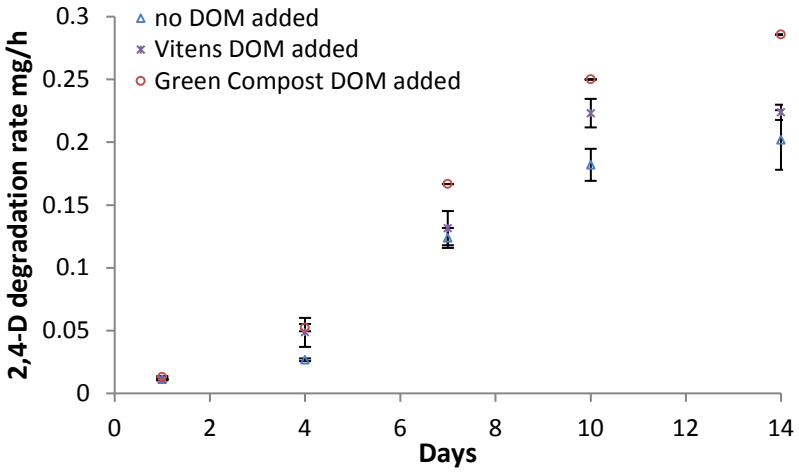


Figure 3.2 . Influence of DOM on 2,4-D biodegradation rate under aerobic condition. Green Compost DOM and Vitens DOM were applied separately, with a concentration of 10 mg/L. 2,4-D concentration was 1 mg/L. 2,4-D was re-spiked once at day 2, twice per day from day 4 to day 7, and three times per day from day 8 to day 14.

Simulated groundwater systems

Pesticide removal

Abiotic batch experiments was conducted to test the degradation of pesticides due to any physical and chemical reactions, and little changes in pesticide concentration were observed during the incubation time. Therefore , biotic transformation of pesticides was the main mean for pesticide removal in the simulated groundwater systems. Pesticide removal in column systems under different redox conditions is shown in Table 3.3. No significant reduction of BAM, bentazone, and MCPP was observed in the four columns, and the addition of Green Compost DOM had no effects on their biodegradation (DOM addition at day 229). 2,4-D had different removal patterns under different redox conditions. The biodegradation of 2,4-D under nitrating-reducing condition in Column 1 was promoted immediately after DOM addition, from 23% to 82% (average removal). In free electron acceptor Column 2, the biodegradation of 2,4-D was significantly improved by the presence of DOM with a lag phase, around 100 days. In sulfate-reducing Column 3, it seems that 2,4-D was degraded after DOM addition; however, the degradation of 2,4-D actually happened in the media instead of in the column. Batch test was performed to check the biotic and abiotic transformation of 2,4-D in sulfate-reducing media, but no degradation of 2,4-D was observed. The reasons for the elimination of 2,4-D in media are still unknown. In methanogenic-reducing condition (Column 4), DOM had no effects on 2,4-D biodegradation.

Table 3.3. Pesticide degradation data

DOM addition at day 229	2,4-D removal (%)		MCPP removal (%)		BAM removal (%)		Bentazone removal (%)		Electron acceptor consumption (%)	
	Without DOM	With DOM	Without DOM	With DOM	Without DOM	With DOM	Without DOM	With DOM	Without DOM	With DOM
Nitrate-reducing column	23.5±10.2	82.3±11.6	2.5±9.6	4.1±9.2	2.9±26.2	4.0±13.8	8.4±10.5	5.4±10.7	3.3±4.2	29.9±17.5
Electron acceptor free column	-6.3±12.6	31.1±36.3	-14.8±8.5	1.8±8.5	-11.5±23.0	-0.9±12.4	-13.3±15.5	3.4±9.3	NA	NA
Sulfate-reducing column	-2.0±4.6	44.4±37.2	-2.9±14.6	9.3±10.2	-4.7±7.6	15.0±14.5	-4.4±10.5	6.5±10.0	4.1±14.4	2.8±4.5
Methanogenic-reducing column	9.8±15.6	14.7±9.5	-3.5±8.9	9.3±6.2	-5.9±7.9	12.6±8.7	-7.4±10.9	12.0±7.6	NA	NA

Electron acceptor consumption

Consumption of electron acceptors was observed in nitrate-reducing conditions, and the consumption pattern was in consistent with 2,4-D removal (Table 3.3). The consumption of nitrate increased from 3.3% to 29.9% after DOM addition. Though no electron acceptors were added to Column 2, 2,4-D was degraded. It was hypothesized that there would be iron (III) naturally present in the aquifer soils, thus resulting in iron-reducing conditions. Therefore, iron (II), the reduced form of iron (III), in the effluents was tested. However, no iron (II) was found. No reduction of sulfate was observed, which was reasonable since the degradation of 2,4-D was did not occurred in the column. CH4 was not analysed in this work because no biodegradation of pesticides was observed in methanogenic column.

Changes in composition of DOM fractions

Since no significant reduction of pesticides removal was found in Column 3 and Column 4, DOM was not analyzed in applied in these two columns was not analysed. Figure 3.3 shows the consumption and composition change of DOM in nitrate-reducing column and free electron acceptor column. DOM consumption was observed. In the no electron acceptor addition column, total DOM was reduced from 11.7 mg C/L to 9.0 mg C/L. An decrease in the concentrations of HA, FA, and Hy was observed, and FA content was the main contributor. Conversely, the concentration of HoN increased, which could be mainly resulted from the shift of FA to HoN during pesticide biodegradation processes.

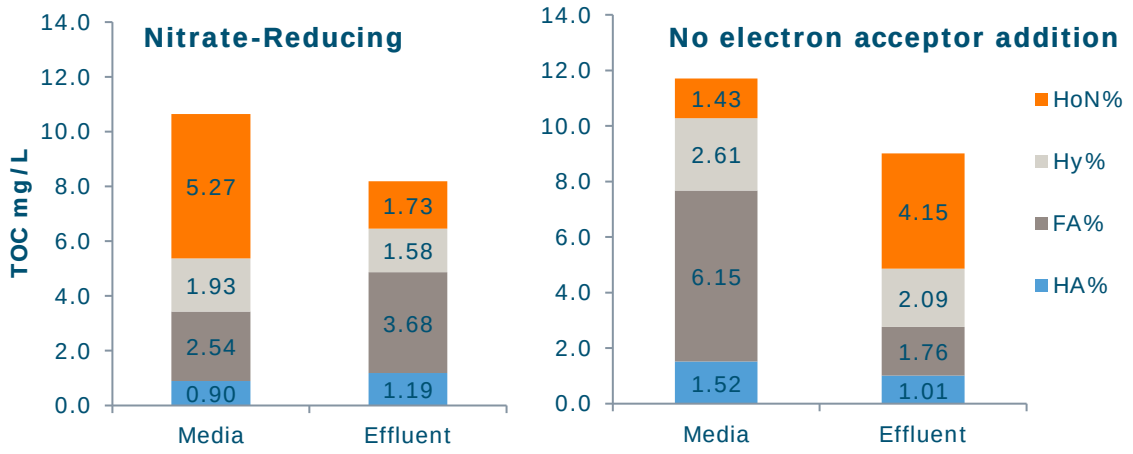


Figure 3.3. Changes in DOM fractions before and after pesticide biodegradation under nitrate-reducing condition and free electron acceptor condition. Media sample was taken at day 405, and effluent sample was taken at day 412, 7 days after media refresh. Humic acids (HA), fulvic acids (FA), hydrophilic acids (Hy), and hydrophobic neutrals (NoH) were fractionated from total Green Compost DOM.

Quantitative PCR

QPCR was used to analyze the influence of DOC addition on the microbial community size and population composition (Figure 3.4). Total bacteria and total archaea analyses indicated no significant growth of the population on the soil matrix due to DOC amendment. This is notable, considering that significant DOC and electron acceptor consumption were observed in, for example, the nitrate-reducing column. Analysis of the liquid effluent population size indicates a slight increase in total bacteria and total archaea in the liquid phase. Liquid phase samples increase by about one order of magnitude in total bacteria and total archaea following DOC amendment. This results indicates biomass wash-off from the columns, which seems to suggest that DOC stimulates biomass growth, which is subsequently washed off the columns.

Quantification of functional genes for nitrate-reduction (*nirS, nosZ,* and *nirK*) indicated very limited enrichment of nitrate-reducing-specific microbial communities. Before DOC addition, no significant enrichment of nitrate-reducing functional genes was observed, and DOC addition did not further select for nitrate-reducing bacteria. In contrast, there was some enrichment of the *dsrB* gene for sulfate reduction in soil samples from the sulfate-reducing column as compared to other columns. Further enrichment of *dsrB* was observed in the sulfate-reducing soil samples following DOC addition. Finally, no clear enrichment of the *tfdA* genes was observed following DOC addition.

Figure 3.4. qPCR results for Total bacteria; Total archaea; *nirS, nosZ, and nirK* (involved in denitrification); *dsrB* (involved in sulfate-reduction); and *tfdA* (aerobic 2,4-D biodegradation gene). Results are given for triplicate assays of DNA extracted from soil and from liquid for each column. Samples were collected before DOC addition (Day 210) and after DOC addition (Days 275 and Day 405).

Effects of DOM on pesticide biodegradation

Pre-batch experiments

In the pre-batch experiments, 2,4-D biodegradation under aerobic condition was promoted in the presence of DOM. In oligotrophic conditions, DOM can act as a carbon and energy source in addition to pesticides to support microbial growth (Bowen *et al.*, 2009; Wiedemeier, 1999), or act as a limiting substrate for co-metabolic biodegradation of pesticides (Dalton *et al.*, 1982; Marschner *et al.*, 2003; Wiedemeier, 1999). Research has shown that biodegradation of pesticides can be stimulated by artificially adding carbon substrates (Harris, 1967; Horvath, 1973; McCormick *et al.*, 1966; Roeth *et al.*, 1969; Semprini, 1997). More recent research also showed the addition of DOM promote the removal of emerging trace organic chemicals in managed aquifer recharge systems (Hoppe-Jones *et al.*, 2012; Maeng *et al.*, 2011; Rauch-Williams *et al.*, 2010). It is highly possible that DOM served as a supplemental carbon and energy sources, instead of primary substrate, to accelerate 2,4-D biodegradation in this case.

The pre-batch experiments also found that different types of DOM affected pesticide biodegradation differently. Green Compost DOM was found to have the most positive effects compared to Groundwater DOM. DOM structure, composition, and biodegradability are highly variable and depend on its source (Leenheer *et al.*, 2003). DOM extracted from groundwater systems is supposed to be more recalcitrant to biodegradation than Green Compost DOM, since the more easily metabolized components have already been utilized before entering groundwater systems.

Simulated groundwater systems

In our simulated groundwater systems (before DOM addition), little biodegradation of the selected four pesticides (2,4-D, MCPP, BAM, and bentazone) was observed. This confirms that pesticides have been observed to be persistent in groundwater systems (Fenner *et al.*, 2013). The observation that no biodegradation was observed prior to DOM addition, indicates 1) natural attenuation is most likely very limited in the field; and 2) conditions optimized in column studies such as nutrient addition, temperature, and sufficient pesticide concentration are not limiting factors. Rather, even when these conditions are optimized, degradation is not observed, indicating that other factors are limiting. One candidate is DOM. To stimulate the biodegradation of pesticides, Green Compost DOM was added to the columns at day 229 because it was found to stimulate 2,4-D biodegradation more than Vitens DOM in the pre-experiment.

In nitrate-reducing column, 2,4-D biodegradation was enhanced significantly and nitrate was consumed after DOM addition. It is highly possible that DOM served as a primary substrate controlling pesticide biodegradation in anaerobic conditions. This is different from what we found in aerobic condition (DOM acted as a secondary substrates). The stimulated 2,4-D biodegradation could be due to enhanced enzyme activity. As mentioned above, DOM can serve as structural analogue. As a result, 2,4-D could be attacked by the enzymes which were produced by microorganisms to degrade DOM.

It is very interesting to notice that 2,4-D biodegradation was stimulated in column without electron acceptor addition after DOM addition. The immediate stimulation fo 2,4-D degradation by DOM indicates that the proper microbial community was present, but that DOM was limiting biodegradation. The role of DOM in this column is unclear. It was hypothesized that iron naturally present in aquifer material could be an electron acceptor; however, no iron(II) was observed. It could be hypothesised that DOM acted as electron acceptors in this case. Several studies have proved that humic substances (HA, FA) can act as terminal electron acceptors in anaerobic microbial oxidation of organic compounds (Benz *et al.*, 1998; Lovley *et al.*, 1996; Scott *et al.*, 1998). Quinone moieties as well as other redox-active functional groups in DOM play an important role in the microbial reduction of DOM (Aeschbacher *et al.*, 2009; Aeschbacher *et al.*, 2011; Lovley *et al.*, 1996; Newman *et al.*, 2000; Nurmi *et al.*, 2002; Scott *et al.*, 1998). Research found that the presence of humic

substances that contained quinone moieties enhanced the anaerbic biodegradaiton of benzene in incubation of iron (III)-reducing sediments, becasue humic susbstances could act as electron shuttles (Coates *et al.*, 1998; Lovley, 2000; Lovley *et al.*, 1996). Klüpfel *et al*. have reported that humic susbstances were biologically reduced in growth meida contianing the eletron donor lactate, and the reduced humic substances could be re-oxidized in the presence of oxygen (Klüpfel *et al.*, 2014).

In the free electron acceptor column, the changes in DOM fractions before and after pesticide biodegradation were observed. The interactions between aquifer soils and added DOM might result in changes in DOM fraction. However, the columns have been running around 210 days, and the interactions between aquifer soils and DOM solutions have already reached equilibrium when the changes in DOM fractions were analysed (samples were taken at day 412, 183 days after DOM addition). Therefore, it is reasonable to conclude that the changes in DOM fractions were mainly due to biotic transformation. This suggests that FA of Green Compost DOM might contain quinone moieties or other redox-active functional groups, and act as electron acceptors in 2,4-D biodegradation.

4. Conclusions

This research investigated the transformation of a list of priority compounds under different redox conditions. Furthermore, the role of DOM in stimulating biodegradation was investigated. Overall, results indicate that:

- No natural biodegradation was observed prior to DOM amendment, indicating that the availability of electron acceptors, nutrients, proper temperature, or high pesticide concentrations was not limiting biodegradation.
- Following DOM addition, 2.4-D biodegradation was observed, indicating that the availability of electron donor was to some extent limiting biodgradation.
- No significant growth of the microbial community in the solid phase was observed, while total bacteria and archaea in the liquid phase increased following DOM addition, indicating the wash off may limit the population size.
- No significant enrichment of nitrate-reducing bacteria was observed while some enrichment of sulfate-reducing bacteria was measured.
- MCPP, BAM, and Bentazone remained recalcitrant to biodegradation.

References

Abbasian, F., Lockington, R., Palanisami, T., Ramadass, K., Megharaj, M., Naidu, R. (2016). Microbial diversity and hydrocarbon degrading gene capacity of a crude oil field soil as determined by metagenomics analysis. Biotechnology progress.

Aeschbacher, M., Sander, M., Schwarzenbach, R. P. (2009). Novel electrochemical approach to assess the redox properties of humic substances. Environmental science & technology, 44(1), 87-93.

Aeschbacher, M., Vergari, D., Schwarzenbach, R. P., Sander, M. (2011). Electrochemical analysis of proton and electron transfer equilibria of the reducible moieties in humic acids. Environmental science & technology, 45(19), 8385-8394.

Aislabie, J., Lloyd-Jones, G. (1995). A review of bacterial-degradation of pesticides. Soil Research, 33(6), 925-942.

Aislabie, J., Davison, A. D., Boul, H. L., Franzmann, P. D., Jardine, D. R. Karuso, P. (1999) Isolation of Terrabacter sp strain DDE-1, which metabolizes 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene when induced with biphenyl. Applied and Environmental Microbiology, 65(12), pp. 5607-5611.

Aleksieva, Z., Ivanova, D., Godjevargova, T., Atanasov, B. (2002). Degradation of some phenol derivatives by Trichosporon cutaneum R57. Process Biochemistry, 37(11), 1215-1219.

Amon, R. M., Benner, R. (1996). Bacterial utilization of different size classes of dissolved organic matter. Limnology and Oceanography, 41(1), 41-51.

Baelum, J., Prestat, E., David, M. M., Strobel, B. W., Jacobsen, C. S. (2012). Modeling of phenoxy acid herbicide mineralization and growth of microbial degraders in 15 soils monitored by quantitative real-time PCR of the functional tfdA gene. Appl Environ Microbiol, 78(15), 5305-5312.

BCPC (2011) The e-pesticide manual: a world compendium, Alton: BCPC [online], available: http://library.wur.nl/WebQuery/clc?q=%22The+pesticide+manual%22

Benz, M., Schink, B., Brune, A. (1998). Humic acid reduction by Propionibacterium freudenreichii and other fermenting bacteria. Applied and environmental microbiology, 64(11), 4507-4512.

Boesten, J., Van der Linden, A. (1991). Modeling the influence of sorption and transformation on pesticide leaching and persistence. Journal of Environmental Quality, 20(2), 425-435.

Bowen, S. R., Gregorich, E. G., Hopkins, D. W. (2009). Biochemical properties and biodegradation of dissolved organic matter from soils. Biology and fertility of soils, 45(7), 733-742.

Brunner, W., Sutherland, F. H. Focht, D. D. (1985) Enhanced Biodegradation of Polychlorinated Biphenyls in Soil by Analog Enrichment and Bacterial Inoculation. Journal of Environmental Quality, 14(3), pp. 324-328.

Buttiglieri, G., Peschka, M., Frömel, T., Müller, J., Malpei, F., Seel, P., Knepper, T. P. (2009). Environmental occurrence and degradation of the herbicide< i> n</i>-chloridazon. Water research, 43(11), 2865-2873.

Cabral, M., Viegas, C., Teixeira, M., Sa-Correia, I. (2003) Toxicity of chlorinated phenoxyacetic acid herbicides in the experimental eukaryotic model saccharomyces cerevisiae: role of ph and of growth phase and size of the yeast cell population. Chemosphere, 51(1),47-54.

Campanaro, S., Treu, L., Kougias, P. G., Francisci, D., Valle, G., Angelidaki, I. (2016). Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnology for biofuels, 9(1), 26.

Campbell, B. J., Yu, L., Heidelberg, J. F., Kirchman, D. L. (2011). Activity of abundant and rare bacteria in a coastal ocean. Proceedings of the National Academy of Sciences, 108(31), 12776-12781.

Carvalho, M. F., Ferreira, M. I. M., Moreira, I. S., Castro, P. M., Janssen, D. B. (2006). Degradation of fluorobenzene by Rhizobiales strain F11 via ortho cleavage of 4-fluorocatechol and catechol. Appl Environ Microbiol, 72(11), 7413-7417.

Chen, Y., Senesi, N. Schnitzer, M. (1977) Information provided on humic substances by E4/E6

ratios. Soil Sci. Soc. Am. J., 41, 352–358.

Coates, J. D., Ellis, D. J., Blunt-Harris, E. L., Gaw, C. V., Roden, E. E., Lovley, D. R. (1998). Recovery of humic-reducing bacteria from a diversity of environments. Applied and environmental microbiology, 64(4), 1504-1509.

Coelho, M. R. R., De Vos, M., Carneiro, N. P., Marriel, I. E., Paiva, E., Seldin, L. (2008). Diversity of nifH gene pools in the rhizosphere of two cultivars of sorghum (Sorghum bicolor) treated with contrasting levels of nitrogen fertilizer. FEMS microbiology letters, 279(1), 15-22.

Dalton, H., Stirling, D., Quayle, J. (1982). Co-metabolism [and discussion]. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 297(1088), 481-496.

De, A., Bose, R., Kumar, A., Mozumdar, S. (2014). Targeted delivery of pesticides using biodegradable polymeric nanoparticles: Springer.

de Lipthay, J. R., Aamand, J., Barkay, T. (2002). Expression of tfdA genes in aquatic microbial communities during acclimation to 2, 4-dichlorophenoxyacetic acid. FEMS microbiology ecology, 40(3), 205-214.

de Lipthay, J. R., Srensen, S. R., Aamand, J. (2007). Effect of herbicide concentration and organic and inorganic nutrient amendment on the mineralization of mecoprop, 2, 4-d and 2, 4, 5-t in soil and aquifer samples. Environmental Pollution, 148(1),83-93.

Dec, J., Shuttleworth, K., Bollag, J.-M. (1990). Microbial release of 2, 4-dichlorophenol bound to humic acid or incorporated during humification. Journal of environmental quality, 19(3), 546-551.

Dousset, S., Babut, M., Andreux, F., Schiavon, M. (2004). Alachlor and bentazone losses from subsurface drainage of two soils. Journal of environmental quality, 33(1), 294-301.

EC. Directive 2006/118/ec of the european parliament and of the council on the protection of groundwater against pollution and deterioration. EUR-Lex: European Union Law: http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32006L0118&from=DE, 2006. European Commission (EC).

European Food Safety Authority (EFSA) Scientific Report (2007) 108, 1-82, Conclusion on the peer review of chloridazon.

Egli, T. (2010). How to live at very low substrate concentration. water research, 44(17), 4826-4837.

Embree, M., Nagarajan, H., Movahedi, N., Chitsaz, H., Zengler, K. (2014). Single-cell genome and metatranscriptome sequencing reveal metabolic interactions of an alkane-degrading methanogenic community. The ISME journal, 8(4), 757-767.

Fenner, K., Canonica, S., Wackett, L. P., Elsner, M. (2013). Evaluating pesticide degradation in the environment: blind spots and emerging opportunities. Science, 341(6147), 752-758.

Fetzner, S. (1998) Bacterial degradation of pyridine, indole, quinoline and their derivatives under different redox conditions. Applied Microbiology and Biotechnology, 49, pp. 237-250.

Garrity, G. M., Holt, J. G., Spieck, E., Bock, E., Johnson, D. B., Spring, S., Maki, J. S. (2001). Phylum BVIII. Nitrospirae phy. nov Bergey's Manual of Systematic Bacteriology (pp. 451-464): Springer.

Gondar D., Lopez R., Fiol S., Antelo J.M., Arce F. (2005) Characterization and acid–base properties of fulvic and humic acids isolated from two horizons of an ombrotrophic peat bog, Geoderma 126 367–374

Gupta, V. K., Ali, I., Saini, V. K. (2006). Adsorption of 2, 4-D and carbofuran pesticides using fertilizer and steel industry wastes. Journal of Colloid and Interface Science, 299(2), 556-563.

Harris, C. I. (1967). DECOMPOSITION IN SOIL Fate of 2-chloro-s-triazine herbicides in soil. Journal of Agricultural and Food Chemistry, 15(1), 157-162.

Helbling, D. E. (2015). Bioremediation of pesticide-contaminated water resources: the challenge of low concentrations. Current opinion in biotechnology, 33, 142-148.

Heron, G., Christensen, T. H. (1992). Degradation of the herbicide mecoprop in an aerobic aquifer determined by laboratory batch studies. Chemosphere, 24(5), 547-557.

Hertfordshire, U. o. (2014a) Pesticide Properties DataBase: Chloridazon, Available: http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/141.htm

Hertfordshire, U. o. (2014b) Pesticide Properties Database: Chloridazon-desphenyl, Available: http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1104.htm

Hertfordshire, U. o. (2014c) Pesticide Properties Database: Chloridazon-methyl-desphenyl, Available: http://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1105.htm

Hogan, D., Buckley, D., Nakatsu, C., Schmidt, T., Hausinger, R. (1997). Distribution of the tfdA gene in soil bacteria that do not degrade 2, 4-dichlorophenoxyacetic acid (2, 4-D). Microbial ecology, 34(2), 90-96.

Holtze, M. S., Sorensen, S. R., Sorensen, J., Aamand, J. (2008). Microbial degradation of the benzonitrile herbicides dichlobenil, bromoxynil and ioxynil in soil and subsurface environments{insights into degradation pathways, persistent metabolites and involved degrader organisms. Environmental pollution, 154(2), 155-168.

Hoppe-Jones, C., Dickenson, E. R., Drewes, J. E. (2012). The role of microbial adaptation and biodegradable dissolved organic carbon on the attenuation of trace organic chemicals during groundwater recharge. Science of the Total Environment, 437, 137-144.

Horvath, R. (1973). Enhancement of co-metabolism of chlorobenzoates by the co-substrate enrichment technique. Applied microbiology, 25(6), 961-963.

Itoh, K., Tashiro, Y., Uobe, K., Kamagata, Y., Suyama, K., Yamamoto, H. (2004). Root nodule Bradyrhizobium spp. harbor tfdAα and cadA, homologous with genes encoding 2, 4-dichlorophenoxyacetic acid-degrading proteins. Applied and environmental microbiology, 70(4), 2110-2118.

Junghans, M., Backhaus, T., Faust, M., Scholze, M., Grimme, L. (2006). Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. Aquatic Toxicology, 76(2),93-110.

Kalbitz, K., Schmerwitz, J., Schwesig, D., Matzner, E. (2003). Biodegradation of soil-derived dissolved organic matter as related to its properties. Geoderma, 113(3), 273-291.

Kim, M. H., Hao, O. J. (1999). Cometabolic degradation of chlorophenols by Acinetobacter species. water research, 33(2), 562-574.

Kim, S.-J., Park, S.-J., Jung, M.-Y., Kim, J.-G., Madsen, E. L., Rhee, S.-K. (2014). An uncultivated nitrate-reducing member of the genus Herminiimonas degrades toluene. Applied and environmental microbiology, 80(10), 3233-3243.

Kitagawa, W., Takami, S., Miyauchi, K., Masai, E., Kamagata, Y., Tiedje, J. M., Fukuda, M. (2002). Novel 2, 4-dichlorophenoxyacetic acid degradation genes from oligotrophic Bradyrhizobium sp. strain HW13 isolated from a pristine environment. Journal of bacteriology, 184(2), 509-518.

Klüpfel, L., Piepenbrock, A., Kappler, A., Sander, M. (2014). Humic substances as fully regenerable electron acceptors in recurrently anoxic environments. Nature Geoscience, 7(3), 195.

Kolpin, D. W., Thurman, E. M., Linhart, S. (1998). The environmental occurrence of herbicides: the importance of degradates in ground water. Archives of Environmental Contamination and Toxicology, 35(3),385-390.

Kutvonen, H., Rajala, P., Carpén, L., Bomberg, M. (2015). Nitrate and ammonia as nitrogen sources for deep subsurface microorganisms. Frontiers in microbiology, 6.

Leenheer, J., Croué, J. (2003). Aquatic organic matter: understanding the unknown structures is key to better treatment of drinking water. Environ. Sci. Technol, 1, 19.

Li, J., Cai, W. Cai, J. (2009) The characteristics and mechanisms of pyridine biodegradation by Streptomyces sp. Journal of Hazardous Materials, 165(1–3), pp. 950-954.

Lin, Q., Donghui, W. Jianlong, W. (2010) Biodegradation of pyridine by Paracoccus sp. KT-5 immobilized on bamboo-based activated carbon. Bioresource Technology, 101(14), pp. 5229-5234.

Lindeboom, R., Fermoso, F., Weijma, J., Zagt, K., Van Lier, J. (2011). Autogenerative high pressure digestion: anaerobic digestion and biogas upgrading in a single step reactor system. Water Science and Technology, 64(3), 647-653.

Lingens, F., Blecher, R., Blecher, H., Blobel, F., Eberspächer, J., Fröhner, C., Layh, G. (1985). Phenylobacterium immobile gen. nov., sp. nov., a gram-negative bacterium that degrades the herbicide chloridazon. International journal of systematic bacteriology, 35(1), 26-39.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Gawlik, B. M. (2010). Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. Water Research, 44(14), 4115-4126.

Lovley, D. R. (2000). Anaerobic benzene degradation. Biodegradation, 11(2-3), 107-116.

Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J., Woodward, J. C. (1996). Humic substances as electron acceptors for microbial respiration. Nature, 382(6590), 445.

Macur, R. E., Wheeler, J. T., Burr, M. D., Inskeep, W. P. (2007). Impacts of 2, 4-D application on soil microbial community structure and on populations associated with 2, 4-D degradation. Microbiological research, 162(1), 37-45.

Maeng, S. K., Sharma, S. K., Lekkerkerker-Teunissen, K., Amy, G. L. (2011). Occurrence and fate of bulk organic matter and pharmaceutically active compounds in managed aquifer recharge: a review. water research, 45(10), 3015-3033.

Malaguerra, F., Albrechtsen, H.-J., Thorling, L., Binning, P. J. (2012). Pesticides in water supply wells in zealand, denmark: A statistical analysis. Science of The Total Environment, 414, 433-444.

Marschner, B., Kalbitz, K. (2003). Controls of bioavailability and biodegradability of dissolved organic matter in soils. Geoderma, 113(3), 211-235.

McCormick, L. L., Hiltbold, A. (1966). Microbiological decomposition of atrazine and diuron in soil. Weeds, 77-82.

Monard, C., Martin-Laurent, F., Devers-Lamrani, M., Lima, O., Vandenkoornhuyse, P., Binet, F. (2010). atz gene expressions during atrazine degradation in the soil drilosphere. Mol Ecol, 19(4), 749-759.

Monard, C., Martin-Laurent, F., Lima, O., Devers-Lamrani, M., Binet, F. (2013). Estimating the biodegradation of pesticide in soils by monitoring pesticide-degrading gene expression. Biodegradation, 24(2), 203-213.

Müller, A. L., Kjeldsen, K. U., Rattei, T., Pester, M., Loy, A. (2015). Phylogenetic and environmental diversity of DsrAB-type dissimilatory (bi) sulfite reductases. The ISME journal, 9(5), 1152.

Navarrete, C. M., Olmedo, J. G., Valsero, J. D., Gomez, J. G., Espinar, J. L., De la Orden Gomez, J. (2008). Groundwater protection in mediterranean countries after the european water framework directive. Environmental geology, 54(3), 537-549.

Newman, D. K., Kolter, R. (2000). A role for excreted quinones in extracellular electron transfer. Nature, 405(6782), 94.

Nurmi, J. T., Tratnyek, P. G. (2002). Electrochemical properties of natural organic matter (NOM), fractions of NOM, and model biogeochemical electron shuttles. Environmental science & technology, 36(4), 617-624.

Odukkathil, G., Vasudevan, N. (2013). Toxicity and bioremediation of pesticides in agricultural soil. Reviews in Environmental Science and Bio/Technology, 12(4), 421-444.

PAN (2014) Pesticides Database: Chloridazon, Available: http://www.pesticideinfo.org/Detail_Chemical.jsp?Rec_Id=PC34290#

Perdue, E. M., J. D. Ritchie. 5.10: Dissolved organic matter in freshwaters. Eds. K. K. Turekian, and H. D. Holland. Amsterdam, Netherlands: Elsevier, 2003.

Piccolo, A. (2001). The supramolecular structure of humic substances. Soil science, 166(11), 810-832.

Pimentel, D. (1995). Amounts of pesticides reaching target pests: environmental impacts and ethics. Journal of Agricultural and environmental Ethics, 8(1), 17-29.

Plugge, C. M., Zhang, W., Scholten, J. C., Stams, A. J. (2011). Metabolic flexibility of sulfate-reducing bacteria. The microbial sulfur cycle, 127.

Podosokorskaya, O. A., Kadnikov, V. V., Gavrilov, S. N., Mardanov, A. V., Merkel, A. Y., Karnachuk, O. V., Kublanov, I. V. (2013). Characterization of Melioribacter roseus gen. nov., sp. nov., a novel facultatively anaerobic thermophilic cellulolytic bacterium from the class Ignavibacteria, and a proposal of a novel bacterial phylum Ignavibacteriae. Environ Microbiol, 15(6), 1759-1771.

Ramiro-Garcia J, Hermes GDA, Giatsis C, Spikema, D., Zoetendaal, E.G., Schaap, P., Smidt, H. (20016). NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. F1000Research 5, 1791.

Ratledge, C. (1994) Biochemistry of Microbial Degradation, Dordrecht/Boston/London: Kluwer Academic Publishers.

Rauch-Williams, T., Hoppe-Jones, C., Drewes, J. (2010). The role of organic matter in the removal of emerging trace organic chemicals during managed aquifer recharge. water research, 44(2), 449-460.

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N. N., Anderson, I. J., Cheng, J.-F., Gies, E. A. (2013). Insights into the phylogeny and coding potential of microbial dark matter. Nature, 499(7459), 431-437.

Rodríguez-Cruz, M. S., Bælum, J., Shaw, L. J., Sørensen, S. R., Shi, S., Aspray, T., Bending, G. D. (2010). Biodegradation of the herbicide mecoprop-p with soil depth and its relationship with class III tfdA genes. Soil Biology and Biochemistry, 42(1), 32-39.

Roeth, F. W., Lavy, T., Burnside, O. (1969). Atrazine degradation in two soil profiles. Weed Science, 202-205.

Sagarkar, S., Mukherjee, S., Nousiainen, A., Bjorklof, K., Purohit, H. J., Jorgensen, K. S., Kapley, A. (2013). Monitoring bioremediation of atrazine in soil microcosms using molecular tools. Environ Pollut, 172, 108-115.

Schulze-Makuch, D., Haque, S., de Sousa Antonio, M. R., Ali, D., Hosein, R., Song, Y. C., Guinan, E. (2011). Microbial life in a liquid asphalt desert. Astrobiology, 11(3), 241-258.

Schipper, P., Vissers, M., Linden, A. v. d. (2008). Pesticides in groundwater and drinking water wells: overview of the situation in the netherlands. Water Science and Technology, 57(8), 1277-1286.

Scott, D. T., McKnight, D. M., Blunt-Harris, E. L., Kolesar, S. E., Lovley, D. R. (1998). Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environmental science & technology, 32(19), 2984-2989.

Semprini, L. (1997). Strategies for the aerobic co-metabolism of chlorinated solvents. Current opinion in biotechnology, 8(3), 296-308.

Simonsen, A., Holtze, M. S., Sorensen, S. R., Sorensen, S. J., Aamand, J. (2006). Mineralisation of 2, 6-dichlorobenzamide (bam) in dichlobenil-exposed soils and isolation of a bam-mineralising aminobacter sp. Environmental Pollution, 144(1), 289-295.

Sørensen, S. R., Holtze, M. S., Simonsen, A., Aamand, J. (2007). Degradation and mineralization of nanomolar concentrations of the herbicide dichlobenil and its persistent metabolite 2, 6-dichlorobenzamide by Aminobacter spp. isolated from dichlobenil-treated soils. Applied and environmental microbiology, 73(2), 399-406.

Straathof, A. L., Chincarini, R., Comans, R. N., Hoffland, E. (2014). Dynamics of soil dissolved organic carbon pools reveal both hydrophobic and hydrophilic compounds sustain microbial respiration. Soil Biology and Biochemistry.

Stuart, M., Manamsa, K., Talbot, J., Crane, E. (2011). Emerging contaminants in groundwater.

Sun, L., Perdue, E. M., Meyer, J. L., Weis, J. (1997). Use of elemental composition to predict bioavailability of dissolved organic matter in a Georgia river. Limnology and Oceanography, 42(4), 714-721.

Suthersan, S. S. (1996). Remediation engineering: design concepts. CRC Press.

Sutton, R., Sposito, G. (2005). Molecular structure in soil humic substances: the new view. Environmental Science & Technology, 39(23), 9009-9015.

Swartjes, F., van der Linden, A., van der Aa, N. (2016). Bestrijdingsmiddelen in grondwater bij drinkwaterwinningen: huidige belasting en mogelijke maatregelen. RIVM rapport 2016-0083.

Thrasher, J., Morgan, P., Buss, S. Attenuation of mecoprop in the subsurface. Environmental Agency: https://www.gov.uk/government/publications/attenuation-of-mecoprop-in-the-subsurface, 2004.

Throbäck, I. N., Enwall, K., Jarvis, Å., Hallin, S. (2004). Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS microbiology ecology, 49(3), 401-417.

van Zomeren, A., Comans, R. N. (2007). Measurement of humic and fulvic acid concentrations and dissolution properties by a rapid batch procedure. Environmental science & technology, 41(19), 6755-6761.

Vewin, 2014 Available on : http://www.vewin.nl/probleemstoffen

Weishaar, J. L., Aiken, G. R., Bergamaschi, B. A., Fram, M. S., Fujii, R., Mopper, K. (2003). Evaluation of specific ultraviolet absorbance as an indicator of the chemical composition and reactivity of dissolved organic carbon. Environmental Science & Technology, 37(20), 4702-4708.

Wiedemeier, T. H. (1999). Natural attenuation of fuels and chlorinated solvents in the subsurface: John Wiley & Sons.

Willems, H. P., Lewis, K., Dyson, J., Lewis, F. (1996). Mineralization of 2, 4-D and atrazine in the unsaturated zone of a sandy loam soil. Soil Biology and Biochemistry, 28(8), 989-996.

You, I. S. Bartha, R. (1982) STIMULATION OF 3,4-DICHLOROANILINE MINERALIZATION BY ANILINE. Applied and Environmental Microbiology, 44(3), pp. 678-681.

Zhang, L., Zhang, C., Hu, C., Liu, H., Qu, J. (2015). Denitrification of groundwater using a sulfur-oxidizing autotrophic denitrifying anaerobic fluidized-bed MBR: performance and bacterial community structure. Applied microbiology and biotechnology, 99(6), 2815-2827.