REMOVAL OF ORGANIC MICRO POLLUTANTS IN BATCH EXPERIMENTS MIMICKING RIVERBANK FILTRATION

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Master Thesis report

October 2012

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ABSTRACT

The increasing occurrence of organic micro pollutants (OMPs) detected in river- and surface waters raises concerns for safe drinking water supply. Riverbank filtration (RBF) might be able to provide an effective barrier against most OMPs; however the removal mechanisms in the riverbank are not yet fully understood. The purpose of this study was to understand the main mechanism (biodegradation and/or sorption) for removal of OMPs during RBF. RBF was simulated by performing batch experiments using river water and riverbank sand from the river Lek near drinking water company Oasen in Bergambacht, the Netherlands. A distinction was made between biodegradation and sorption by comparing bottles treated with and without sodium azide, a biocide which inactivates the bioactivity. Furthermore different experimental conditions were studied; the effect of the river water matrix and the effect of dosing an additional C source (acetate) at regular intervals. The experiments were performed under oxic and anoxic conditions to mimic different redox zones in RBF.

Anoxic conditions were present, established by the reduction of nitrate. No nutrient limitation was observed under oxic and anoxic conditions. Furthermore, results showed that the dose of 0.4 g/L sodium azide was too low to completely suppress the bioactivity. This led to an underestimation of the biodegradation of OMPs in this study. Therefore the determined biodegradation only gave an indication but did not represent reality; gemfibrozil, 2,4-D, diclofenac, and diuron showed the highest biodegradation. OMP removal did not vary significantly between different experimental conditions. This may indicate that the OMP dose per mass of sand was too low in the batch bottles; meaning that not enough OMPs were available for microorganisms to be able to determine differences in removal. Furthermore, this report showed that measuring parameters such as dissolved organic carbon (DOC), bioactivity (ATP) and extracellular polymeric substances (EPS) gave a better representation of the occurring processes in the batch bottles when measured in the sand phase than in the aqueous phase. Finally, it was concluded that the amount of proteins and carbohydrates present in the biofilm in riverbank sand was directly related to the bioactivity.

CONTENTS

ABBREVIATIONS

ATP	- Adenosine triphosphate
CH	- Carbohydrates
Demi	- Demineralised water
DOC	- Dissolved organic carbon
EPS	- Extracellular polymeric substances
GAC	- Granular activated carbon
HES	- High energy sonication
HS	- Humic substances
MO	- Microorganisms
NaN_3	- Sodium azide
NF	- Nanofiltration
OMP	- Organic Micro Pollutants
RBF	- Riverbank filtration
RO	- Reverse Osmosis
QSAR model	- Quantitative structure-activity relation

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1 INTRODUCTION

Since several decades organic micro pollutants (OMPs) have been detected in surface water and groundwater near rivers. OMPs include compounds with known pharmacological actions in humans and animals, amongst others birth control and natural hormones, antibiotics, antimicrobials, pain relievers, and caffeine, but also pesticides. The occurrence of pesticides in these waters can be traced back to the pesticide use on farmers' land, where they infiltrate with rain and irrigation water into the soil to end up in the river. Furthermore the largest source of pharmaceuticals in the environment is the effluent of waste water treatment plants (WWTPs). The occurrence of pharmaceuticals can be traced back to the increasing consumption of medicines by humans. After consumption, pharmaceuticals may leave the human body non- or partly metabolised and therefore will end up in the sewer. Most pharmaceuticals will not be removed in WWTPs. Therefore treated WW effluent will discharge pharmaceuticals into the river (Onesios, 2009). In most cases, no regulatory limits are set for the discharge of these compounds from WWTPs. This leads to small but detectable (ng/L to µg/L) concentrations of pharmaceuticals and pesticides in surface water and ground water.

Although quantities of these contaminants in surface water may be low, they constitute a threat due to their constant presence (Carr, 2010, Kummerer, 2009). Effects of these compounds on human health and the environment have not been fully characterized. However toxicological studies have shown that OMPs might have an effect on aquatic life (Onesios, 2009).

A large part of the drinking water produced in the Netherlands is gained from the river Rhine and Meuse by drinking water treatment plants. Due to the aging of the population, more and more pharmaceuticals will be used in the future. This may lead to higher pharmaceutical concentrations in the river water. Some are removed with the current treatment, e.g. adsorption by activated carbon, whereas others require more complex treatment processes such as nanofiltation (NF) and/or reverse osmosis (RO). These more complex treatments require a lot of energy. Furthermore globally the fresh water availability reduces. Therefore human water use needs to be reduced and water cycles need to be closed. All these different problems contribute to the need for measures to improve the drinking water treatments in the Netherlands, to be able to secure safe and healthy drinking water in the future. Therefore further research on this topic is required.

Riverbank filtration (RBF) has shown to be able to (partly) remove many OMPs (Heberer, 2008, Hoppe-Jones, 2010), both in laboratory and field -studies. But carbamazepine, for example, has shown persistent behaviour during RBF in many studies (Carballa, 2006, Maeng, 2011, Heberer, 2007, Lim, 2008). Thus to be able to effectively remove all OMPs additional treatment is required. A solution might be combining RBF with NF and granular activated carbon treatment (GAC) to form a multi barrier treatment against OMPs. Several studies indicated that the compounds showing persistent behaviour during RBF, are removed very well with NF and/or GAC. NF mainly removes the larger polar compounds, while GAC is able to remove a-polar compounds. This new treatment concept is researched in the ESTAB (emerging substances towards an absolute barrier) project. The study described in this report is part of the ESTAB project and focuses on the first step of this threefold treatment system; the RBF process.

RBF is a natural water treatment system, that has been used for decades as potable water source especially in Eastern Europe (Benotti, 2012). During RBF surface- and/or groundwater percolates slowly through the bank of the river into the groundwater, which is subsequently abstracted by pumping wells. The infiltration of water into the soil takes weeks to multiple years, depending on the area. In Germany and Eastern Europe the retention time of the river water is usually weeks to months (Heberer, 2007), while in the Netherlands it varies from months to years. RBF was originally intended to be used for removal of organic material, bacteria, viruses and protozoa. More recent research has shown that during RBF also the OMPs are reduced or completely removed by the biological and physiological processes in the riverbank. The two main mechanisms of OMP removal have shown to be degradation by MOs and sorption onto aquifer content (Mansell, 2004, Worch et al., 2002, Maeng, 2011, Lin, 2010). Sorption will not only occur onto the sand of the riverbank but also onto biofilm present including the dead biomass in the aquifer. However to effectively use RBF as part of a multi barrier treatment against OMPs, more insight in these removal processes is required.

Literature shows that studies usually focused on only a few OMPs or one specific type. Moreover the dosed OMP concentrations were quite high; doses of OMPs in the µg/L or even the mg/L range are not

uncommon in literature, while OMPs usually only occur in the ng/L range (or very low µg/L range) in surface waters. In addition, literature has shown that the OMP concentration has influence on the removal (Maeng, 2011, Lin, 2010, Liu, 2010). Therefore in this study it was tried to represent the actual RBF operation more closely; therefore a mixture of 16 OMPs was dosed in ng/L range. This mixture of 16 OMPs was selected based on their common occurrence in the river Rhine and different physiochemical properties (charge, size and molecular weight). The results can be used as a first step to build a QSAR model (quantitative structure-activity relation). With a QSAR model it is possible to predict how specific compounds will behave in the riverbank, based on their physiochemical properties. This QSAR model is desirable because of the increase in use of pharmaceuticals (as discussed earlier). Moreover new pharmaceuticals will occur in the river water and is it unfeasible to investigate all these pharmaceuticals.

The objective of this study is to determine the main mechanism (sorption and/or biodegradation) responsible for the removal of OMPs during RBF in two different layers of the riverbank; the oxic and anoxic zone. Furthermore the removal of OMPs is determined during different experimental conditions.

Laboratory experiments were performed to mimic RBF using river water and riverbank sand from the river Lek at the RBF site of drinking water company Oasen at Bergambacht. Batch experiments were chosen due to their flexibility and ease of operation; in this way several experimental conditions could be investigated simultaneously. The experiments were executed under oxic and anoxic conditions to evaluate the different riverbank zones. In the oxic zone, oxygen is used as electron acceptor during metabolic processes by the microorganisms (MOs) present in the sand. In the anoxic zone other metabolic processes will take place due to absence of oxygen; nitrate will be reduced and thus used as an electron acceptor during these processes. During these metabolic processes OMPs are degraded, either metabolically or co-metabolically. Different studies have already found that some OMPs are better removed during anoxic conditions and others in oxic conditions (Carr, 2010, Baumgarten, 2011, Heberer, 2008, Drewes, 2001).

The OMP removal due to sorption was determined by inactivating the biomass present in the soil with sodium azide in this study. In this way removal of OMPs due to sorption can be subtracted from removal due to biodegradation (Rauch-Williams, 2010, Mansell, 2004, Lim, 2008, Maeng, 2011).

During RBF the soil is exposed to constant fresh river water flow; providing the soil with a constant flow of nutrients and organic matter present in the river water. This fresh flow cannot be mimicked with batch experiments. Therefore in this study the influence of this constant fresh inflow of nutrients and organic matter (dead and alive) in river water was determined by substituting the river water with demineralised water. Furthermore the DOC in the river water is expected to be degraded by MOs which are present in sand and river water. This could lead to a limitation of carbon source in the batch processes which does not occur during RBF, because the MOs are exposed to a constant DOC level due to the constant fresh inflow of river water. Therefore in this study to moderate the possibility of carbon source limitation, the effect of dosing acetate was investigated. Acetate is an easy degradable compound, widely used as a carbon source for MOs (Maeng, 2011).

In order to make sure the right redox conditions were maintained during the whole experiment, several parameters were measured. The water quality and nutrient supply/limitation were evaluated with parameters like oxygen, pH, ions and DOC values. The bioactivity in the water and sand phase was determined to gain knowledge about the stability and the condition of the present MOs. Furthermore more insight in the activity of the MOs in the batch was gained by analysing the biofilm. In the riverbank microorganisms form a biofilm around themselves; a protective environment containing extracellular polymeric substances (EPS). These EPS were measured in order to gain more knowledge on the bioactivity and the microbial processes, compounds such as carbohydrates and proteins which appear in the EPS of the biofilm were measured (Conrad, 2003, Flemming, 2010, Jahn, 1999).

This report consists of different chapters; Chapter 2 (Material & Methods) first gives an outline of the experiments performed and is followed by the Results & Discussion. In that chapter the results will be discussed and compared with similar research described in literature. Chapter 4 gives the conclusions and in chapter 5 recommendations for further research are given.

2 MATERIAL & METHODS

In this study batch experiments were performed to mimic RBF using river water and riverbank sand under oxic and anoxic conditions. The main objective of this study was to distinguish between OMP removal by degradation by MOs from sorption on to the sand. This was executed by comparing the removal of OMPs with and without treatment of the batches with sodium azide. Sodium azide is a biocide which inactivates the microorganisms (MOs) present in the batch bottles. This way the removal of OMPs due to biodegradation and/or sorption was estimated. Furthermore the OMP removals of different experimental conditions were compared to each other; Table 2-2 and Table 2-3 show those different conditions. First of all, OMP removal in two different redox zones was compared; the oxic and anoxic zone (resp. Table 2-2 and Table 2-3). Secondly during RBF the soil is exposed to constant fresh river water flow, which cannot be mimicked in batch experiments. To examine the influence of this, the influence of the nutrient and organic matter in river water was determined by substituting river water by demineralised (demi) water. Finally, acetate was dosed in some bottles at regular time intervals to determine the influence of an additional carbon source to replenish the easily degraded carbon in the river water. Acetate is an easily degradable carbon source which in commonly used in comparable studies. Acetate was not dosed under anoxic conditions, because it was assumed that when the river water reaches the anoxic zone all easily degradable carbon is already degraded in the oxic layer.

The trends of different variables were monitored in all bottles; therefore samples were collected from both the supernatant and the sand in glass vials (50mL) or brown bottles (250mL). From the supernatant the pH, oxygen, DOC content, OMP concentrations, bioactivity (ATP), ion concentrations, and EPS content were measured, while from the sand the DOC content and bioactivity (ATP) were determined. This chapter describes the materials and methods used in detail.

2.1 BATCH EXPERIMENTAL SETUP

Batch experiments were performed using riverbank sand (150g) in river water or demi water (350mL) in brown glass bottles (1L), spiked with OMP mix (200 ng/L per component). The bottles were kept in a dark climate room at 20°C. The experiment was performed both under oxic and anoxic conditions. Bottles under oxic conditions were covered with parafilm with small holes in it. The anoxic bottles were flushed with nitrogen gas for 4 minutes to remove all oxygen from the water. After 4 minutes an oxygen concentration below 0.1 mg/L was measured. Those bottles were closed with an oxygen tight glass cap covered with parafilm. Under both conditions different experimental setups were performed, see Table 2-2 and Table 2-3. Sodium acetate was dosed (0.5 mg/L, Merck, Germany) from day 5 on every 3 to 5 days to replenish the DOC that was degraded. Sodium azide was dosed at t=0 in a concentration of 0.4 g/L (NaN_3, Sigma-Aldrich, The Netherlands) to inactivate the MOs present. The duration of the batch experiments was 28 days (conform OECD guidelines for batch experiments), samples were taken for analysis under oxic conditions at t=0, 4, 7, 14 and 28 days and under anoxic conditions at t=0, 3, 7, 14 and 28 days. The bottles were sacrificed after taking samples.

Sodium azide is a biocide which is frequently used to suppress microbial activity in studies with abiotic experiments (Rauch-Williams, 2010, Mansell, 2004, Lim, 2008, Maeng, 2011). Sodium azide is highly toxic to cells because it significantly reduces the ATP production of cells. The mechanism responsible for this reduction is the tight binding of sodium azide to the cytochrome C at the site where oxygen usually binds. Cytochrome C is located in the inner membrane of mitochondria and is part of the respiratory chain complexes. Mitochondria can be described as the cellular power plant; in this organelle the cell energy carrier ATP is produced from its metabolite in a lower energy state ADP. Mitochondria are surrounded by two membranes; the outer membrane is semi-permeable and the inner membrane contains respiratory chain complexes. These complexes transfer electrons (gained from oxygen or another source) in steps; this is coupled to the generation of a proton gradient. This proton gradient can be used for the conversion of ATP from ADP. This protein complex accounts for 90% of the total oxygen uptake in most cells. Therefore this complex is crucial for all aerobic life (Whitford, 2005, Drewes, 2001). In this study sodium azide was used to eliminate microorganisms under both oxic and anoxic conditions. However sodium azide is only toxic for gram-negative cells; gram-positive cells have a thicker outer membrane and therefore sodium azide is not capable of penetrating through this layer.

2.1.1 River water and Riverbank sand

The river water was gained from the river Lek at the RBF site of drinking water company Oasen from the side of the river at Bergambacht (51.916665N, 4.784653E). The Lek River is branched from the Nederrijn River which is a distributary branch of the river Rhine. The riverbank sand was gained from the same location as the river water, 3 to 10 cm below the water level. A sieving test showed that the grain size of the riverbank sand used varied from 0.8 to 0.15 mm; the majority of grains (81.8%) with a diameter of 0.21 to 0.4 mm (Appendix A – Sand Data). The density of the riverbank sand was 1.56 kg/L with a porosity of 0.40; determined by comparing the mass of a 250mL cylinder filled with dry sand to a cylinder filled with wet sand (formula's shown in Appendix A – Sand Data).

2.1.2 OMPs

Sixteen different OMPs (Table 2-1, analytical grade, Sigma-Aldrich) were prepared in a 10L glass bottle containing 20 mg of each compound in 10 L tap water. This stock solution was stirred for at least 3 days at room temperature. This mix was spiked in the batch bottles in a concentration of 200 ng/L per compound. The OMPs were selected based on their common presence in water of the river Lek and varying physicochemical properties at pH 8; molecular weight, pKa, charge, and hydrophobicity (log K_{ow}/log D) (Table 2-1). The hydrophobicity log K_{ow}/log D might give an indication of the sorption potential of compounds.

2.2 Analyses

The samples were kept in glass bottles and were measured within 24 hours, except for the samples for ion chromatography, these were frozen until analysis. OMP samples were extracted on a cartridge within 1 week and frozen until analysis.

2.2.1 Measuring OMP concentrations

At several time steps, samples of 200mL in glass bottles were taken to extract the OMPs using Oasis HLB cartridges (200mL, 6cc)(Waters, USA) pre-treated with methanol (≥99.9%, Sigma-Aldrich) and demineralised water. Cartridges were frozen until analysis. The elution and analysis of the extract were performed according to the method described by Bertelkamp (2012). Furthermore the analysis parameters are described in Reungoat (2012).

2.2.2 Measuring DOC content

DOC was measured with a Shimadzu TOC analyser. All samples (20mL) were measured at room temperature after being filtered through a 0.45 µm filter (Whatman, Germany) which has been flushed twice with demineralised water. Samples were acidified by adding 1.6mL HCl (Sigma-Aldrich). High energy sonication (HES) was applied on sand samples to suspend the biomass in solution using a Branson digital sonifier (Model 250D, Boom BV Meppel). HES treatment was applied 3 times for 2 minutes on 45W to the sand samples, while replacing the supernatant with KCl solution (10mM) before each treatment. Organic carbon dissolves better in KCl solution. Furthermore after three HES treatments the recovery of sonication was less than 10%; therefore the treatment was applied three times. From all three supernatant samples 0.667mL was combined in a vial (2 mL in total) to be analysed.

2.2.3 Measuring Bioactivity

In order to determine the bioactivity in the batch bottles, cellular bound ATP (adenosine triphosphate) was measured in the supernatant and the sand. ATP is used in all cells as carrier of free energy and phosphate groups to drive many chemical reactions. One molecule of ATP contains three phosphate groups, and it is produced by ATP synthase from inorganic phosphate and adenosine diphosphate (ADP) or adenosine monophosphate (AMP). ATP plays a key role in metabolic processes in the cells and therefore can be used as a measure for living biomass (Alberts, 2002). In this study cellular bound ATP was measured using a QUENCH-GONE™ Aqueous (QGA™) test kit and a LB9509 luminometer (both Aqua tools, France). ATP is a very degradable compound and therefore the results can vary due to small variations. Therefore samples were taken and analysed in duplicates, and the ATP test kit was combined with the use of ATP standard (BioThema, Sweden) to correct for pH, temperature, aging of the ATP reagent and inhibitor effects. The ATP concentration of the supernatant (10mL) and sand (8-16g dry weight) samples were determined. Sand samples were treated with HES as described above, but the supernatant was replaced with fresh demineralised water before each treatment. From all three supernatant samples 0.667mL was combined in a vial (2 mL in total) to be analysed.

2.2.4 MEASURING IONS

Ion chromatography was used to measure nitrate, ammonium, phosphate, sulphate, magnesium, calcium and chloride concentrations from the supernatant of the batch experiment. Samples were frozen until analysis. Samples were prepared by filtering them through a 0.45 µm filter (Whatman, Germany) after flushing them twice with demineralised water. Samples were measured undiluted and after 10x dilution. Ion standards (Sigma-Aldrich) were prepared in concentration of 0.1, 1, 10 and 50 ppm.

2.2.5 MEASURING EPS

Extracellular polymeric substance (EPS) content was determined from supernatant samples of the batch experiment. EPS mainly consist of polysaccharides, proteins, nucleic acids and lipids. In order to get an indication of the EPS present, proteins and carbohydrates are often measured (Lowey, 1951, Dubois, 1956). Proteins were analysed with an UV-VIS spectrophotometer at 750 nm using a 4cm cuvette. Samples (5mL) were prepared in a plastic tube (15mL) by adding 7mL reagents D and mixing with a vortex. Reagent D was prepared by mixing reagents A, B, C in relation of 100:1:1. Reagent A, B and C were prepared in demi water; reagent A (143 mM NaOH, 270 mM Na_2CO_3), B (57 mM $CuSO_4$), C (124 mM Na_2-tatrate, $C_4H_4Na_2O_6$, or Na-K-tatrate, $C_4H_4NaKO_6$). Reagents A, B, C and E can be stored unlimited, as reagent D needs to be prepared daily. After 10 minutes of incubation at room temperature 1mL Folin-Ciocalteu phenol reagents was added. After vortexing, the samples were incubated for 45minutes at room temperature. Moreover samples were compared with a calibration curve prepared the same way using 0 – 25 mg/L BSA (Bovine serum albumin) (Lowey, 1951). Carbohydrates were analysed with an UV-VIS spectrophotometer at 487 nm using a 4cm cuvette. Samples (4mL) were prepared in a plastic tube (50mL) by adding 2mL phenol solution (5%). Then 10mL sulphuric acid (95-99%) was added. After 15minutes of cooling down, the samples were mixed with a vortex and incubated for 30 minutes at room temperature. The samples were compared with a calibration curve prepared the same way using 0 – 25 mg/L glucose (Dubois, 1956). All chemicals were of analytical standard and purchased at Sigma-Aldrich.

2.3 SODIUM AZIDE EXPERIMENT

The sodium azide experiment was performed to investigate whether the sodium azide dose (0.4 g/L NaN_3) from the experiment described above was sufficient to inactivate the bioactivity. Therefore the experiment above was repeated while comparing with a higher dose of sodium azide. Moreover two other methods were used to inactivate bioactivity according to the method of Kerr (2000); (1) dosing sodium azide with metals ($NiCl_2$ and $BaCl_2$), (2) heating the bottles. The content of the bottles from the sodium azide test is shown in

Table 2-4. The same analyses were performed on the samples. However the proteins, carbohydrates and humic substances were measured from the sand phase instead of the supernatant. To be able to measure these parameters from the sand phase, the samples were treated with a high energy sonicator (HES). This was performed as described by Onesios (2012). 20mL of demi water was added to the sand samples before HES treatment of 5min at 70W. After centrifuging (Sorvall ST16R, Sysmex, The Netherlands) for 5 minutes at 4000 rpm the supernatant was used to determine proteins and carbohydrates content as described in paragraph 2.2.5 Measuring EPS. According to Frolund (1995) humic substances interfere with the protein measurement. Therefore in addition the protein concentrations corrected for the humic substances was calculated in this study. This was done by measuring the protein concentration without interference of copper sulphate; reagent D was prepared with demi water instead of reagent B. According to Frolund (1995) when copper sulphate is omitted the colour development due to humic compounds is measured. Then the corrected protein content was calculated by using the following formula for absorbance $A_{protein} = 1.25(A_{with\ CuSO4} - A_{without\ CuSO4})$.

Table 2-1 – Components of OMP mix and their physiochemical properties

Name	MW (g mol^{-1})	pKa	Log K_{ow}	Charge at pH 8	Log D at pH 8
2,4-D	221.0	2.73	2.81	-1	-0.73
Atrazine	215.7	1.7	2.61	0	2.25
Caffeine	194.2	10.4	-0.07	0	-0.58
Carbamazepine	236.3	1; 13.9	2.45	0	2.63
Diclofenac	296.2	4.2	**4.6**	-1	1.17
Diuron	233.1	na	2.68	0	2.49
Gemfibrozil	250.3	4.43	**4.77**	-1	1.36
Ibuprofen	206.2	4.91	**3.97**	-1	0.99
Lincomycin	406.5	7.6	0.56	0	-0.64
Metolachlor	283.8	na	3.13	0	3.44
Metoprolol	267.4	9.68	1.88	1	0.18
Phenytoin	252.3	8.33	2.47	0	0.91
Roxithromycin	837.1	8.8	2.75	1	2.22
Sulfadiazine	250.3	1.8; 6.36	-0.09	0	-0.14
Tramadol	263.4	9.44	2.31	1	1.25
Trimethoprim	290.3	3.2; 7.1	0.91	-1	1.23

Table 2-2 – Experimental oxic conditions

Exp. Condition	Solid phase	Aqueous phase	C-source / biocide
River	150 g sand	350 mL river water	None
River + Acetate	150 g sand	350 mL river water	Sodium acetate (0.50 mg/L)
River + NaN_3	150 g sand	350 mL river water	Sodium azide (0.40 g/L)
Demi	150 g sand	350 mL demineralised water	None
Demi + Acetate	150 g sand	350 mL demineralised water	Sodium acetate (0.50 mg/L)
Demi + NaN_3	150 g sand	350 mL demineralised water	Sodium azide (0.40 g/L)

Table 2-3 – Experimental anoxic conditions

Exp. Condition	Solid phase	Aqueous phase	C-source / biocide
River	150 g sand	350 mL river water	None
River + NaN_3	150 g sand	350 mL river water	Sodium azide (0.40 g/L)
Demi	150 g sand	350 mL demineralised water	None
Demi + NaN_3	150 g sand	350 mL demineralised water	Sodium azide (0.40 g/L)

Table 2-4 – Experimental conditions of sodium azide experiment

Exp. Condition	Solid phase	Aqueous phase	Biocide
River	50 g sand	100 mL river water	None
High dose Azide	50 g sand	100 mL river water	Sodium azide (40 g/L)
Low dose NaN_3	50 g sand	100 mL river water	Sodium azide (0.40 g/L)
NaN_3 + Metals	50 g sand	100 mL river water	Sodium azide (5.0 g/L) + metals (8.0mM $NiCl_2$ and 3.1mM $BaCl_2$)

3 RESULTS & DISCUSSION

This chapter contains the results of the batch experiments performed under different experimental conditions in both oxic and anoxic conditions. These results are discussed and compared with literature. Moreover the results and discussion of the additional experiment will be outlined, which investigated the efficiency of dosing sodium azide as a biocide. Finally all results are summarized and combined in the last paragraph of this chapter.

3.1 WATER QUALITY PARAMETERS

3.1.1 pH AND O_2

pH and O_2 concentration were monitored during the batch experiment. Under oxic conditions the pH and oxygen concentration remained constant; there was no difference between different batches (pH was 8.0 to 8.3 and oxygen concentration was 7.2 to 8.2 mg/L). Under anoxic conditions it was not possible to measure the oxygen concentration due to equipment problems. However bacteria will use nitrate as electron acceptor when no oxygen is available; thus under anoxic conditions nitrate reduction can be observed. In paragraph 3.1.3 Ions, results of nitrate concentrations are discussed and these confirm that nitrate was reduced, concluding that anoxic conditions were obtained. The pH under anoxic conditions remained between 8.2 and 8.7.

3.1.2 DOC CONTENT

DOC was determined during the batch experiment in the supernatant (see Figure 3-1). The bottles containing river water showed a higher DOC content than the bottles containing demi water in the beginning as expected. After 14 days in the river water a small part of DOC was biodegraded, whereas in demi bottles DOC dissolved from the sand into the supernatant. This trend can be observed under both oxic and anoxic conditions. However under anoxic conditions the DOC content increases from t=14 days on. No explanation was found for this increase.

The total DOC (in mg) in the bottles was calculated from the DOC concentration in both the supernatant and the sand; the supernatant concentration was multiplied by the amount of supernatant in the bottles (350mL) and the sand concentration by the sand mass (150g). Figure 3-2 (left) shows that under oxic conditions the total DOC content varies a lot (2 to 27 mg DOC) at t=4 days, but at t=28 days the DOC levels out to around 8 mg DOC. This indicates that around 8 mg DOC per bottle was not biodegradable and therefore remained in the bottles. Furthermore the percentage of DOC originating from the supernatant was around 4 to 24% under oxic conditions and remained more or less the same in time (Figure 3-2). Thus the amount of DOC in the sand phase was much higher than in the supernatant phase. This was expected because the sand phase contains precipitated organic material from the river water as well as biofilm that contains a lot of organic material.

Figure 3-2 (right) shows that the total amount of DOC remained more or less the same during the anoxic experiment. At t=3 days the sodium azide treated river water bottles gave a high DOC value in the sand; this could be due to contamination of the sample. Figure 3-2 shows that the part of the DOC originating from the supernatant increases after t=14 days under anoxic conditions. This indicates that organic matter from the sand transfers to the supernatant phase. The ammonium and phosphate concentrations also increased in the supernatant under anoxic conditions. Interestingly, this trend seemed not to be related to the bioactivity (paragraph 3.2 Bioactivity).

Acetate was dosed every 3 to 5 days (0.5 mg/L) to keep the level of biodegradable DOC constant in the supernatant; no clear effect of dosing acetate was found, indicating that acetate was immediately degraded or incorporated in the biofilm. One exception was the bottle with demi water and acetate which showed a DOC increase at t=28d. There was no clear explanation for this.

3.1.3 IONS

Figure 3-3 shows that in the anoxic batch bottles nitrate decreased in time. This means that the conditions in these bottles were nitrate reducing; thus no oxygen was available. Moreover Figure 3-3 shows that under oxic conditions the initial nitrate concentrations in the river water were 7 to 8 mg/L. These values are comparable with the annual average Lekkanaal water data; see Table 3-1 (Stoks, 2010). Bottles containing demi water showed a much lower initial nitrate concentration and stayed low.

Under oxic conditions the nitrate concentration increased during the experiment, meaning that either microorganisms or the other chemical processes produced nitrate. Addition of acetate did not have a significant effect on the nitrate concentration. Bottles treated with sodium azide showed a very high nitrate concentration from the start and remained at more or less at the same level of 100 mg/L under both oxic and anoxic conditions (Figure 3-3). This was due to a measurement error caused by sodium azide which gives a peak at the same place as nitrate in the ion chromatograph.

Figure 3-4 (lower) shows gaps in the data for ammonium measurement. Ion chromatography was used to determine the ion concentrations, the output peaks of sodium and ammonium are very close to each other and the peak for sodium is much higher. Therefore the ammonium peak is not always detectable. This makes the results of the ammonium concentrations less reliable. Sodium was not analysed in this study. Moreover bottles treated with sodium azide resulted in higher ammonium levels under both oxic and anoxic conditions (Figure 3-4). This could also have to do with the increased sodium concentration in the bottles due to addition of sodium azide which disfigures the nitrate peaks of the ion chromatograph.

Furthermore results showed an overall decrease of ammonium under oxic conditions. This is probably caused by nitrifying bacteria which converts ammonium into nitrite, and nitrite into nitrate. This would explain the observed increase of nitrate (Figure 3-3). However this would not explain the total increase of nitrate, which is much higher (in molar) than the amount of ammonium decreased. Under anoxic conditions the ammonium concentration more or less doubled during the experiment. This can be explained by the conversion of sugar molecules and amino acids into ammonium by acidogenic bacteria. These bacteria are common in anaerobic processes. Under anaerobic conditions the nitrate concentration dropped, while no oxygen was present. Therefore it is expected that denitrification took place where nitrate transforms into nitrogen gas.

Overall the phosphate concentration increased in time by 50 to 350% for both oxic and anoxic conditions (Figure 3-4). There was no clear trend visible in the differences in increase. It should be noted that the scale is very low, thus the increase in phosphate was small. However this result was remarkable, because phosphate is an essential nutrient for microorganisms under oxic conditions, therefore a decrease in phosphate was expected.

The concentrations of chloride, sulphate and magnesium in river bottles remained more or less the same in time for both oxic and anoxic conditions (Figure 3-6). Calcium seems to be released into the supernatant under oxic conditions in time. Interestingly, this increase was not seen under anoxic conditions. The increase was probably caused by dissolution of limestone from the sand in the supernatant of the batch bottles; no explanation was found why this did not seem to happen under anoxic conditions. In demi water the components chloride, sulphate, magnesium and calcium showed a significantly lower concentration compared to river water. After 14 days the concentration of chloride and sulphate remained the same, whereas extra magnesium and calcium were released in the supernatant due to dilution from degradation of organic complexes in the biofilm. This trend was shown for both oxic and anoxic conditions. This means that under anoxic conditions no sulphate reducing conditions occurred.

All measured starting concentrations for the ions were a little lower than minimum annual Lekkanaal water values, see Table 3-1 (Stoks, 2010), except for nitrate and ammonium. The ion concentrations of the anoxic experiment were higher than the oxic ones at t=0, because the river water was taken a few weeks later from the river Lek. As the annual values of the Lekkanaal water indicate in Table 3-1, the ionic concentrations can vary in the river.

3.2 BIOACTIVITY

The bioactivity in the sand and supernatant was estimated by measuring the cells energy carrier ATP. ATP degrades very quickly and therefore this method is very sensitive. This was clearly shown by the data; the ATP concentrations varied a lot. Therefore all samples were performed in duplicates, but the data still varied.

Figure 3-5 shows the bioactivity per gram sand under oxic conditions. Overall the microbial activity shrunk with 16 to 85% in 28 days' time, due to die-off of microorganisms. This result could not be explained, because results from ion and DOC measurement showed that there was no nutrient limitation (paragraph 3.1 Water Quality Parameters).

Bottles treated with sodium azide showed a lower bioactivity. However the differences are not very large, when plotted on log-scale, which indicates that dosage of sodium azide was not sufficient to inactivate all bioactivity and/or some bacteria are resistant to sodium azide. An additional experiment was performed to test this theory. Results of this test are discussed in Paragraph 3.5 Sodium Azide and shows that the sodium azide dose was indeed too low.

The river water bottles showed a lower initial bioactivity than bottles containing demi water. This indicates that fewer microorganisms were present in the sand from the start. Another explanation can be the measurement errors due to the sensitivity of the measurement as explained in the beginning of this paragraph. This second explanation is more plausible because at t=4 and 14 days the bioactivity was at similar level as the demi water containing bottles.

Under anoxic conditions the microorganisms died off with 38 to 58% in 28 days' time; probably with the same cause as the die-off under oxic conditions, see Figure 3-5. Treating the sand with sodium azide had no effect on the bottle containing demi water. This indicates that microorganisms were able to resist the sodium azide. Gram-positive bacteria are resistant to sodium azide due to their thicker outer membrane. This could mean that the whole present population would consist of gram-positive cells, which is not plausible. However this should be further researched, for instance by studying the microbial population using DGGE (Denaturing Gradient Gel Electrophoresis). Comparing the bottles containing river water with and without sodium azide shows a clear die-off of microorganisms. However the dose was too low to suppress all bioactivity.

The ATP concentrations were also determined in the supernatant. However no clear trend was visible in this data. Moreover the concentration of microorganisms was much lower in the supernatant than in the sand. Thus more MOs are present in the sand phase of the bottle. The results for supernatant and the total bioactivity, calculated by adding the weighted data from both the supernatant and the sand, are shown in Appendix B – Bioactivity.

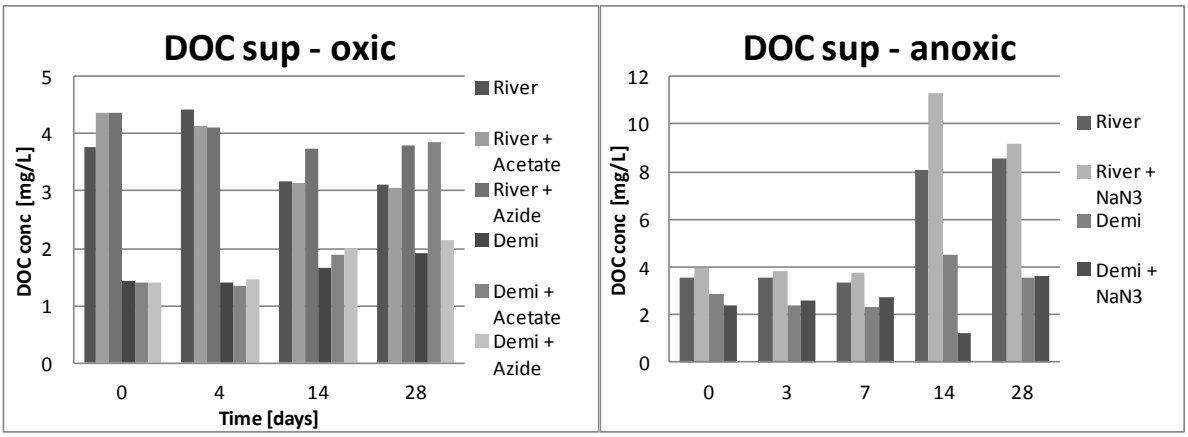

Figure 3-1 – Dissolved organic carbon (DOC) in mg/L in time measured in the supernatant of different batch bottles under oxic (left) and anoxic (right) conditions.

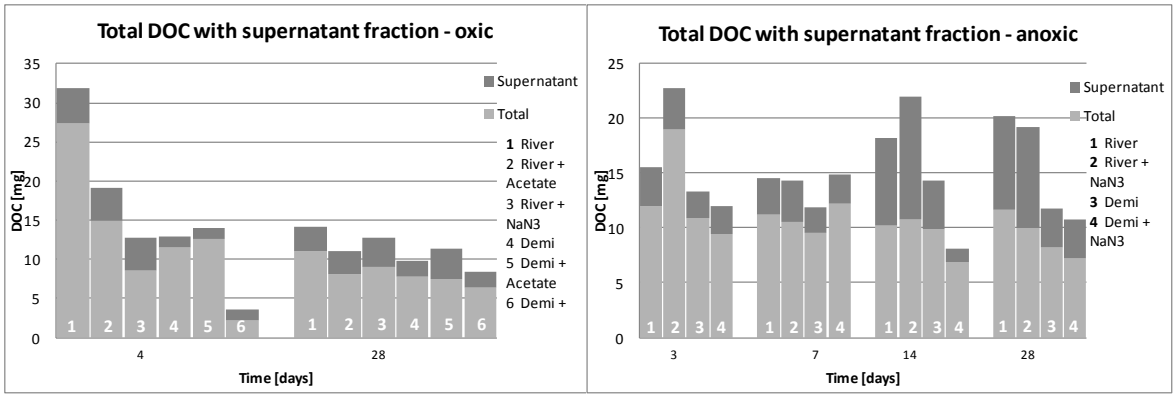

Figure 3-2 – Total DOC content (mg) in time for different batch bottles under oxic (left) and anoxic (right) conditions. The total DOC was calculated by adding up the weighted DOC concentration in supernatant and sand. The top part represents the contribution of DOC originating from the supernatant.

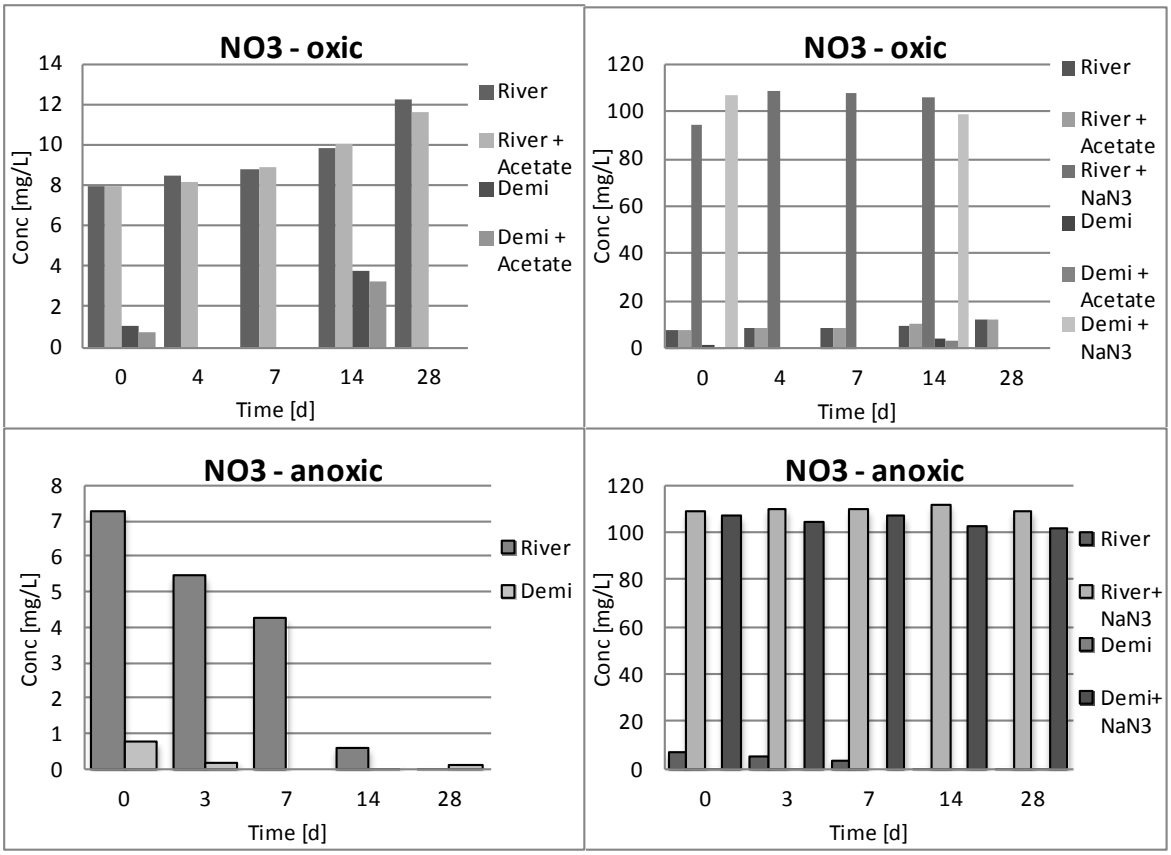

Figure 3-3 – Nitrate concentrations without (left) and with (right) sodium azide bottles shown under oxic (upper) and anoxic (lower) conditions (in mg/L).

Table 3-1 – Measured ion concentrations at t=0 for oxic and anoxic conditions, and annual Lekkanaal water values (in mg/L)(Stoks, 2010)

	Oxic t=0			Anoxic t=0		Annual Lekkanaal water		
	River	River + Acetate	River + NaN3	River	River + NaN3	Average	Min	Max
Ammonium	ND	0.20	ND	ND	ND	0.10	0.04	0.27
Calcium	32.6	40.8	31.4	42.0	41.5	70.6	60.7	91.3
Chloride	49.7	49.4	45.7	67.6	65.0	76.6	56.0	116
Magnesium	6.76	6.66	5.93	7.36	7.19	10.6	9.50	13.3
Nitrate	7.93	7.99	94.7	7.30	109	11.9	5.58	17.9
Phosphate	ND	0.10	0.10	0.12	0.09	0.45	0.30	0.70
Sulphate	42.5	43.0	41.2	52.3	50.4	57.1	45.9	72.7

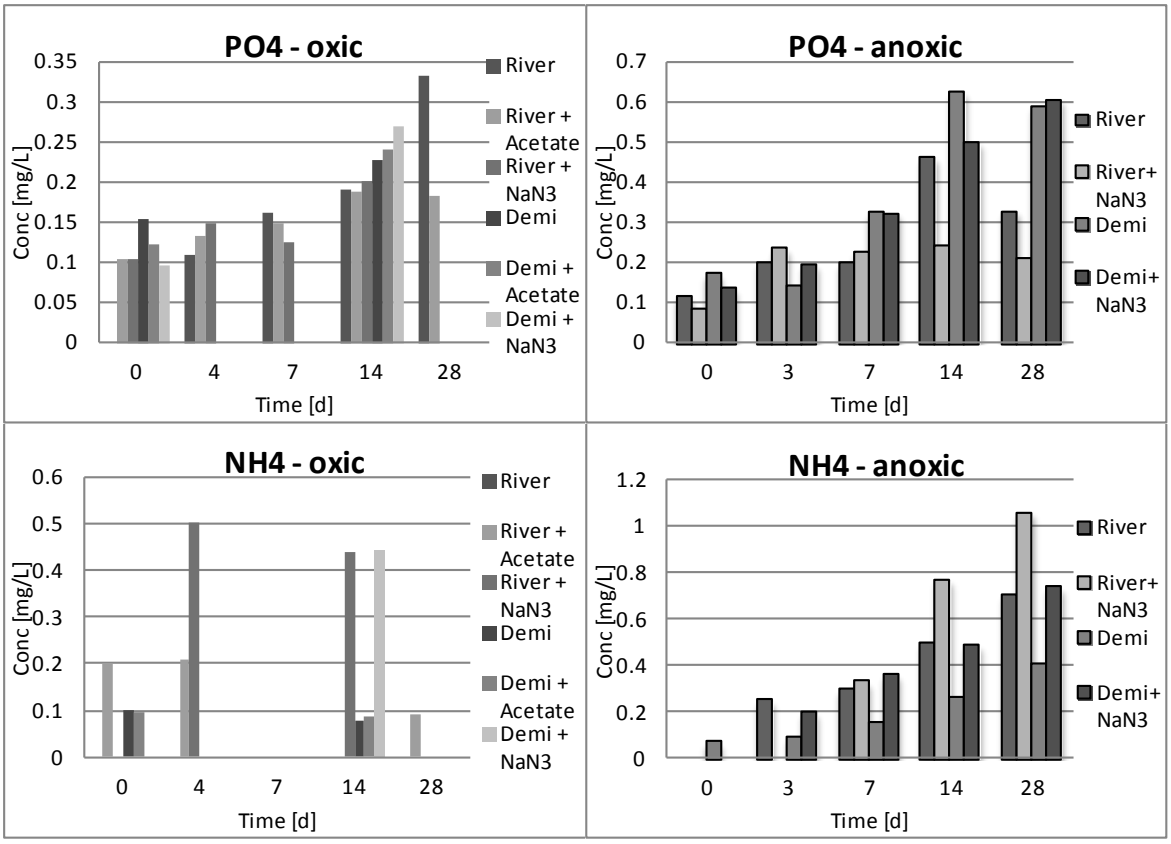

Figure 3-4 – Phosphate (upper) and ammonium (lower) concentrations under oxic (left) and anoxic (right) conditions (in mg/L). Under oxic conditions batch bottles containing demi water were only measured at t=0 and 14 days. Data of ammonium concentration is missing at some time steps.

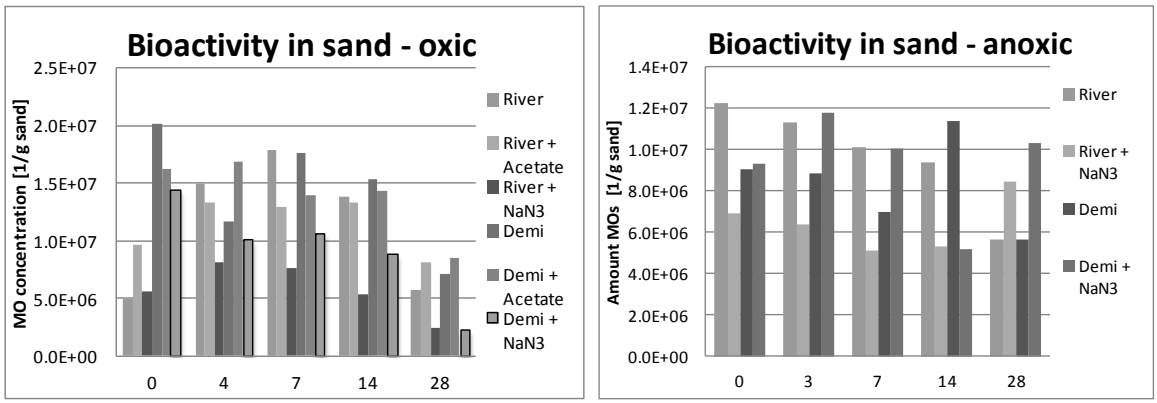

Figure 3-5 – Bioactivity measured in the sand per gram sand under oxic (left) and anoxic (right) conditions.

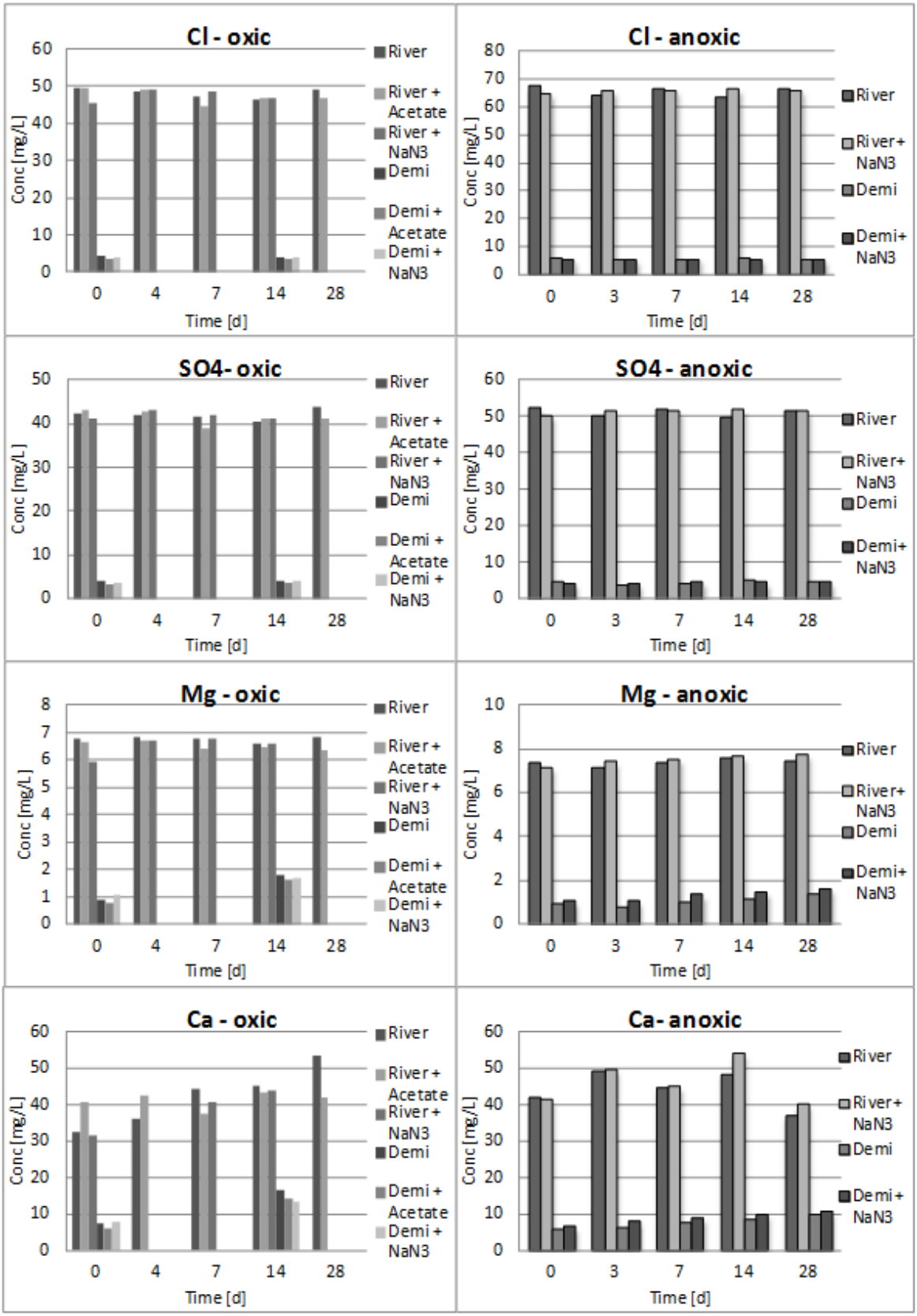

Figure 3-6 – Chloride, sulphate, magnesium and calcium concentrations under oxic (left) and anoxic (right) conditions (in mg/L). Under oxic conditions batch bottles containing demi water were only measured at t=0 and 14 days.

3.3 OMPs

3.3.1 Removal of OMPs

The removal of the selected OMPs in river water over 14 days is shown in Table 3-2 (Biodegradation explained in next paragraph); 9 of 16 compounds show a removal of more than 70% (2,4-D, caffeine, diclofenac, diuron, gemfibrozil, ibuprofen, metoprolol, roxithromycin, and trimethoprim). Table 3-3 shows an overview of removal of the selected OMPs in literature. Most removal values were obtained from (comparable) batch results, others from column, WWTP or field studies when no data from batch studies were available. The removal of a specific compound can vary strongly between different studies. Moreover the removal found in this study varied from the literature for 7 OMPs (2,4-D, diclofenac, lincomycin, metoprolol, phenytoin, sulfadiazine and trimethoprim). Caffeine, gemfibrozil, ibuprofen, roxithromycin are very well removed (>85%) during this study, this can also be confirmed by literature. In contrast carbamazepine is found to be unable to degrade or sorb during river bank filtration, this was also found in this study with a removal of 4%.

3.3.2 Different Batches

The influence of the supernatant on the removal of OMPs was determined by comparing batch bottles containing river water with batch bottles containing demi water, see Figure 3-7. Overall the removal of OMPs was relatively comparable between batches containing river water and demi water. This indicates that the water phase of the batch processes does not have much influence on the removal, and that the removal of OMPs mainly takes place in the sand. This was confirmed by a statistical test; a paired one tailed T-Test was performed on the removal of OMPs in river versus demi water batches. This gave a p-value of 0.00996, thus lower value than the threshold for a statistical difference of 5%, indicating that results from the different water phases are not statistically different.

During the experiment acetate was dosed every 3 to 5 days to maintain the amount of biodegradable DOC at a constant level, just like in the river where riverbank sand is exposed to a constant flow of fresh river water. Overall dosing of acetate resulted in a slightly better removal (less than 10%) of OMPs, see Figure 3-8. This could indicate that with more carbon available the bioactivity increased and therefore more OMPs were removed. However paragraph 3.2 Bioactivity shows no increase in bioactivity due to acetate dosing. Another possibility for the increase in OMP removal is that the removal increased because acetate was used as a metabolite and OMP degradation was co-metabolic. Thus due to the addition of acetate more OMPs could be biodegraded in the sand. However the differences between the bottles with and without dosing of acetate are too small to draw this conclusion, but were larger than the limit of detection and limit of quantification of the OMP analysing method. Moreover if this theory would be correct, difference in removal between river water and demi water is also expected, and this was not the case.

Removal of OMPs in biotic bottles was compared with bottles treated with sodium azide to distinguish sorption from biodegradation (Figure 3-8). Data of biological activity showed that the activity in the sodium azide treated batch bottles was still high. This means that the microorganisms were still active and able to biodegrade OMPs. Therefore similar removal of an OMP in the sodium azide batches compared to the biologically active batches can indicate two things; (1) removal due to sorption onto the sand or (2) the OMP is easily biodegradable also when the activity is lower. Table 3-2 shows an estimation of the absolute biodegradation, calculated by subtracting the removal of river water bottles from sodium azide treated bottles (see Figure 3-8). Gemfibrozil, 2,4-D, diclofenac, and diuron showed more than 50% removal due to biodegradation. This calculation is an underestimation of the amount of biodegradation because in the sodium azide treated bottles the bioactivity was still quite high. Therefore this determination of biodegradation is not very accurate and should be considered as a rough indication of the biodegradability of the OMPs.

Overall the different conditions (bottles containing river water versus demi water and dosage of acetate) did not seem to have a large effect on the removal of OMPs. The most plausible explanation is that the dosage of OMPs per mass of sand was too low in the batch. This means that the availability of OMPs for microorganisms is too low to determine differences in removal.

3.3.3 PHYSIOCHEMICAL PROPERTIES VS. REMOVAL

Sixteen different OMPs were selected based on their different physiochemical properties, see Table 3-2. In order to gain more insight in the removal processes of OMPs, it was tried to link these different properties to the removal of the OMPs in river water under oxic conditions (Table 3-2). No clear relation was found between the removal and the molecular weight or pKa of the selected OMPs. In general charged OMPs, both negative and positive, seem to have a higher removal than neutral compounds, except for tramadol. Better removal of positively charged components can be explained due to attachment to the negative charged biofilm present between the sand (Conrad, 2003). These OMPs will be biodegraded or remain attached/adsorb onto the biofilm. Negatively charged OMPs may also attach to the biofilm. Biofilm consist of a complex mixture of EPS. Negative OMPs might be attracted by some positive charged EPS; for instance exo-enzymes which are secreted by MOs for degradation. Moreover positively charged metal oxides are present in the soil which could attract the negatively charged compounds. The selected neutral compounds do not show a clear pattern; their removal depends on other physicochemical properties.

According to Schwarzenbach (1983) the potential of OMPs to move with infiltrating river water into groundwater can be predicted with the logarithm of the octanol/water partition coefficient, the log K_{ow} value. This value indicates the potential of compounds to sorb onto the soil. Schwarzenbach (1983) and Benotti (2012) indicate that for OMPs with a log K_{ow} values less than 3.7, there will not likely be sorption onto the riverbank soil. Table 3-2 shows that diclofenac, gemfibrozil and ibuprofen have a higher log K_{ow} values than 3.7 and thus are likely to sorb onto the soil. These OMP were all well removed. However these OMPs were not the only ones that showed a good removal. Therefore predicting the sorption with log K_{ow} is not a very good measure; it depends on more factors, this was also found by Kummerer (2009). However the log K_{ow} range used in this study was not very large, which makes it difficult to draw conclusions about the relation between log K_{ow} and the removal of OMPs.

pH-corrected solute hydrophobicity can be indicated with the log D. Log D is derived from the octanol/water coefficient log K_{ow} while taking into account the charge of the compound. According to Cunningham (2008) chemicals with a log D lower than 1 may not sorb, and those with log D higher than 3 should sorb very well onto organic matter. However in Table 3-2 no clear relation was found between these different ranges in hydrophobicity of the selected OMPs and their removal (Cunningham, 2008). However this study contained not enough compounds per range to be able to conclude that there is no relation between the OMP removal and the hydrophobicity.

Table 3-2 – Physiochemical properties of selected OMPs, the OMP removal and absolute biodegradation in percentages (removal in bottles treated with sodium azide subtracted from the removal of river water bottles, see Figure 3-8)

Compounds	MW (g mol-1)	pKa	log Kow	Charge at pH8	Log D at pH 8	Removal %	Biodegradation (%)
2,4-D	221	3	2.81	-1	-0.73	96	80
Atrazin (ATZ)	216	2	2.61	0	2.25	9	9
Caffeine (CAFF)	194	10	-0.07	0	-0.58	95	3
Carbamazepine (CBZ)	236	1; 13.9	2.5	0	2.63	4	4
Diclofenac (DCF)	296	4	4.60	-1	1.17	77	55
Diuron (DIU)	233	na	2.68	0	2.49	69	51
Gemfibrozil (GMF)	250	4	4.8	-1	1.36	98	56
Ibuprofen (IBU)	206	5	3.97	-1	0.99	99	21
Lincomycin (LNC)	407	8	0.56	0	-0.64	31	22
Metolachlor (MET)	284	na	3.13	0	3.44	39	39
Metoprolol (MTP)	267	10	1.88	1	0.18	86	39
Phenytoin (PNT)	252	8	2.47	0	0.91	9	9
Roxithromycin (ROX)	837	9	2.75	1	2.22	85	18
Sulfadiazine (SDZ)	250	1.8; 6.36	-0.1	0	-0.14	38	9
Tramadol (TML)	263	9	2.31	1	1.25	25	25
Trimethoprim (TMP)	290	3.2; 7.1	0.91	-1	1.23	97	21

Table 3-3 – Literature overview of OMP removal

Compounds	Removal		Literature
2,4-D	50%	batch	Boivin (2005)
Caffeine (CAFF)	97%	batch	Maeng (2011)
	>95%	batch; oxic	Bradley (2007)
	68+/- 10-100%	batch; anoxic	Bradley (2007)
Carbamazepine (CBZ)	0%	batch	Maeng (2011)
	0%	(M)	Carballa (2006)
	0%	(T)	Carballa (2006)
	0%	batch	Yamamoto (2009)
	0%		Lim (2008)
	0%	Field study	Heberer (2007)
Diclofenac (DCF)	94%	batch	Maeng (2011)
	20%	column	Maeng (2011)
	ND	batch (1)	Quintana (2005)
	ND	batch (2)	Quintana (2005)
	30%	batch	Yu (2006)
Gemfibrozil (GMF)	86%	batch	Maeng (2011)
	no biodegradation		Lim (2008)
	>99%	batch	Yu (2006)
	99%		Onesios (2012)
Ibuprofen (IBU)	94%	batch	Maeng (2011)
	99%		Onesios (2012)
	97-99%	batch	Buser (1999)
	ND	batch (1)	Quintana (2005)
	100%	batch (2)	Quintana (2005)
	>99%	batch	Yu (2006)
	21%	batch	Carr (2011)
Lincomycin (LNC)	69%	batch	Carucci (2006)
	0%	WWTP	Castiglioni (2006)
Metoprolol (MTP)	59%	MBR labscale	Radjenovic (2007)
Phenytoin (PNT)	50%	batch	Yu (2006)
Roxithromycin (ROX)	100%	field study; oxic	Heberer (2008)
Sulfadiazine (SDZ)	97%	WWTP, full scale	Peng (2006)
	50%	WWTP, full scale	Xu (2007)
Trimethoprim (TMP)	70%	batch (1)	Batt (2006)
	25%	batch (2)	Batt (2006)
	no biodegradation	batch	Lim (2008)
	100%	field study	Heberer (2008)

Batt (2006)	Batch: 96h incubation, 250 mg/l OMPs, inoculum from:(1)nitrifying sludge, (2)inhibited nitrifying sludge
Bendz (2005)	Batch: 96h incubation, 250 mg/l OMPs, inoculum from:(1)nitrifying sludge, (2)inhibited nitrifying sludge
Boivin (2005)	Batch 2,4-D studied in clay and loamy soils used, 10 days incubation time
Bradley (2007)	(O)oxic sediment inoculum, 3 river sediments, incubation time for caffeine was 32 d, for cotinine 72 d; (A) anoxic sediment inoculum, 3 river sediments, incubation time for caffeine was 52 d
Buser (1999)	Batch: WWTP influent inoculum and activated sludge, incubated for 8 h
Carballa (2006)	Anaerobic digester: Pilot scale, mesophilic (M) and thermophilic (T) conditions, 4–400 ug/l OMPs
Carucci (2006)	Batch: Inocula from WWTP, aerobic, 4 h incubation, 2 mg/l drugs
Carr (2011)	Batch; according to this study ibuprofen only under high-bacterial activity degradation goes fast.
Castiglioni (2006)	WWTP: 24 h time proportional, composite samples
Heberer (2007)	Field study, measurement in groudwater wells
Heberer (2008)	Field study, measurement in groudwater wells
Lim (2008)	Batch; 1,5L solution with BDOC, 1-3 µg/L OMPs; better removal when plant extracts are added
Maeng (2011)	Batch; riverwater; 30days under oxic conditions followed by 30d under anoxic conditions
Peng (2006)	WWTP: samples from 2 WWTPs; removals during act. sludge treatm. calculated
Quintana (2005)	Batch: sludge inoculum, mean removals by transformation reported, 28 d incubation; (1)20 mg l-1 OMPs as sole carbon source, (2)5 mg l-1 OMPs and 50 mg l-1 milk
Radjenovic (2007)	MBR: Lab scale, installed at WWTP, mean removals presented
Xu (2007)	WWTP: Average overall removals for 4 WWTPs, 2 WWTPs sampled as time proportional grab samples, 2 WWTPs sampled as 24 h composites
Yu (2006)	Batch: Activated sludge inoculum, 50 d incubation, 1, 10, and 50 ug l-1 OMPs
Yamamoto (2009)	Batch; eight OMPs, river water and river sediments

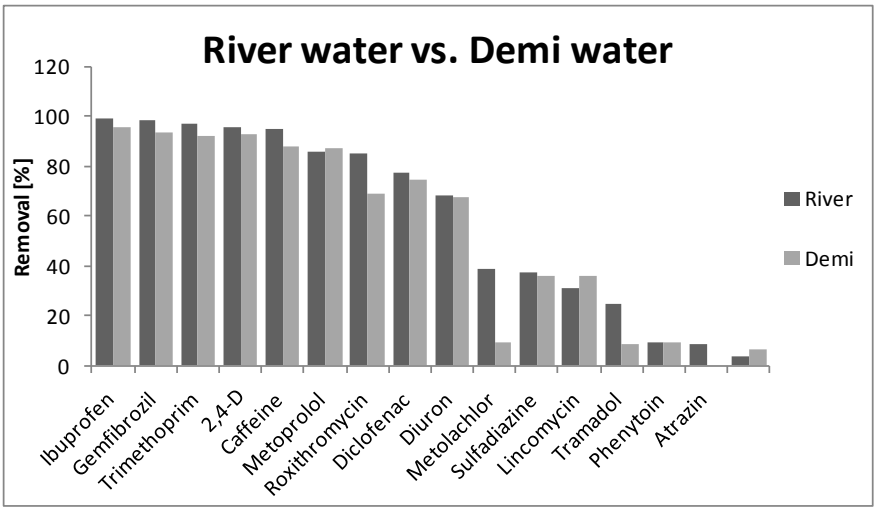

Figure 3-7 – OMP removal in percentages between t=0 and 14 days; batch bottles containing river water and demi water compared under oxic conditions.

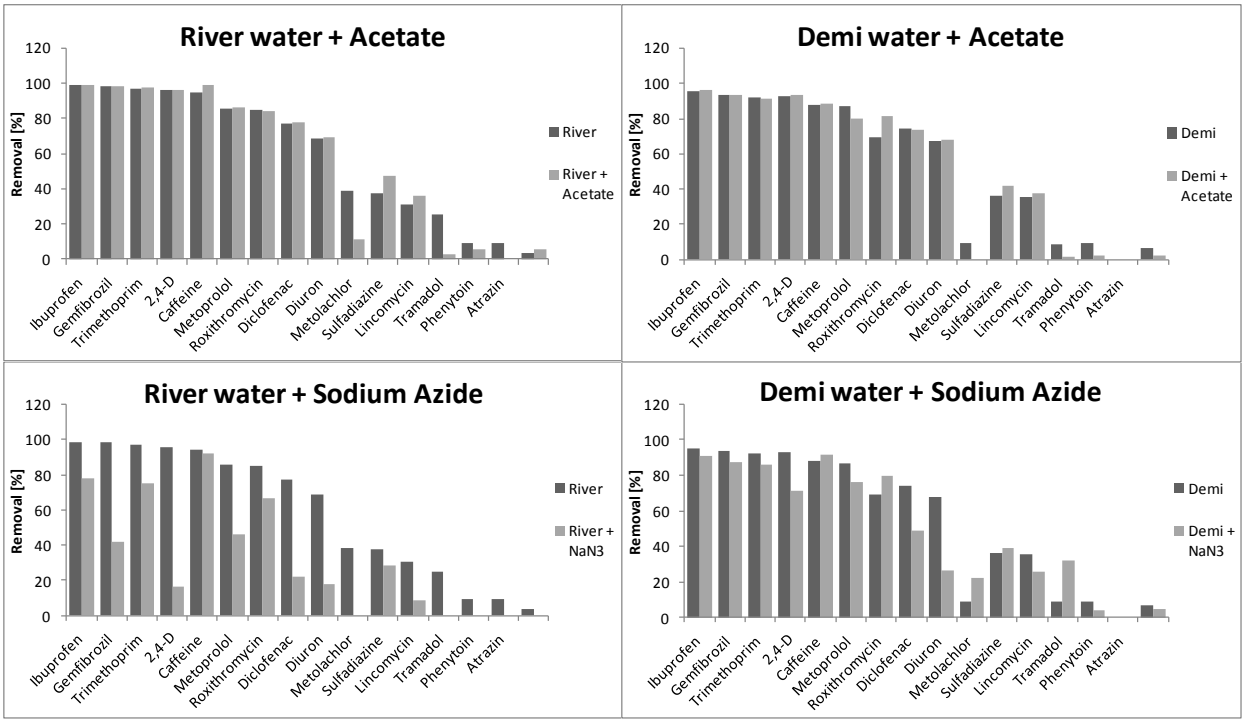

Figure 3-8 – OMP removal in percentages between t=0 and 14 days comparing different batch bottles; river (left) and demi water (right) and dosage of acetate (upper) and sodium azide (lower) under oxic conditions.

3.4 EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

In this study the change in EPS content during different experimental conditions was determined. Proteins and carbohydrates were measured and compared. This paragraphs starts with some background information about biofilm, followed by the discussion of the results of EPS measurements.

Microorganisms form a protective environment around themselves, this is called the biofilm. The biofilm increases the capacity for cohesion to surfaces; allowing cells to stick to the river sand, while water infiltrates. Biofilm contains EPS which are excreted from cells into the medium. EPS are mainly carbohydrates, proteins, nucleic acids and lipids. In most biofilms the EPS matrix accounts for 90% of the dry mass, whereas the microorganisms only account for 10% (Flemming, 2010). EPS components form a 3D polymer network, the extracellular matrix of biofilms. This matrix takes care of the stability of the biofilm and enables cells to interconnect and immobilise with the biofilm. These are a couple of the many functions biofilm carry out.

EPS acts as external digestive system. Organic and inorganic components from the water phase may be absorbed into the biofilm; these will be used as nutrient and energy source. These components are often large in size and therefore it is difficult for cells to uptake them into the cell. Therefore cells excrete extracellular enzymes which cut the nutrients in smaller pieces. Due to the matrix, the extracellular enzymes can be kept close to the cells for easy uptake of the smaller components. Moreover all components are kept within the biofilm, when cells die-off their content is released into the biofilm. All these lysed cell components are being recycled in the biofilm, used for building the matrix and as energy source. Moreover DNA can be used for horizontal gene transfer. Therefore the EPS content varies greatly between biofilms (Jahn, 1999, Conrad, 2003, Flemming, 2010).

In order to get an idea about the behaviour of the biofilm during the different batch experiments, the EPS was studied. This was done by measuring the protein and carbohydrate content of the supernatant. The protein concentration varied between bottles but did not show a trend for different experimental batch conditions (Figure 3-9). Dosing acetate or sodium azide showed no clear effect. This was not expected; the protein concentration was expected to be related to the bioactivity. Less bioactivity means fewer proteins secreted by microorganisms. In this study, treatment with sodium azide caused a lower bioactivity, but no decrease in protein concentration. However protein concentrations measured in the sand phase with a higher sodium azide dose did show this effect. These results are shown in paragraph 3.5 Sodium azide experiment. This indicates that proteins stay attached to the biofilm and are not released into the supernatant.

According to Frolund (1995) substances interfere with the analysis of proteins following the method of Lowey (1951), (Conrad, 2003, Jahn, 1999). This could be an explanation for the lack of trend between different batch bottles. The humic substances can be determined by omitting copper sulphate during analysis. Then the corrected protein content can be calculated using the formula stated in paragraph 2.3 Sodium Azide Experiment. During the sodium azide experiment the corrected protein content was calculated. However due to the correction the bottles dosed with sodium azide + metals gave negative values. This could indicate that the dosed heavy metals inferred with the absorbance spectra during measurement, but this theory should be further researched.

Two samples sprouted, under oxic conditions the bottle containing river water and acetate at t=28d and under anoxic conditions river water treated with sodium azide at t=7d. Probably this was due to a measurement mistake.

Furthermore the carbohydrates (CHs) were determined in the supernatant. Overall the carbohydrate concentrations showed first an increase but after 14 days the concentration decreased under oxic conditions. Moreover treatment with sodium azide showed a clear decrease in carbohydrates. These results were directly related to the bioactivity (paragraph 3.2 Bioactivity) (Figure 3-9). Under oxic conditions these relations were also observed.
When the bioactivity increases, more carbon source is required. In the riverbank carbon source is available in the form of chunks organic material originating from the river, like small wooden sticks, parts of dead fish, etc. To be able to bring this organic material into the cell, the organic material should be degraded into smaller pieces, carbohydrates. Cells secrete exo-enzymes to take care of this degradation. This will lead to an increase of carbohydrates in the biofilm when the bioactivity increases (Conrad, 2003, Jahn, 1999, Flemming, 2010).

The measured concentrations of proteins and carbohydrates were much lower in the supernatant than the sand (see paragraph 3.5 Sodium Azide Experiment). This indicates that most of the proteins and carbohydrates are present in the sand, and that the EPS stays attached to the biofilm on the sand. Therefore measuring proteins and carbohydrates in sand samples gives a better indication of the EPS and the biofilm present in the batch bottles.

3.5 Sodium Azide Experiment

An additional batch experiment was performed to investigate the effect of the sodium azide dose used in the previous experiment; the results of this sodium azide experiment are discussed in this paragraph. This experiment was performed because results from the previous experiment indicated that dosing 0.4 g/L sodium azide was not sufficient to significantly diminish the bioactivity in the batch. This sodium azide dose was compared with treatment with a higher dose, with heat and with the combination of sodium azide and heavy metals (8.0mM $NiCl_2$ and 3.1mM $BaCl_2$)(Table 2-4). The results of the pH, DOC (aqueous and sand phase), EPS (sand phase) and ATP (sand phase) concentration are shown in this paragraph. However the results for the heat treated batch experiment were not shown, because the method Kerr (2000) did not work out and took a lot of time.

During the sodium azide experiment the total bioactivity was analysed by measuring the ATP concentration in both aqueous and sand phase of the batch. The total bioactivity was calculated by adding the weighted data from the supernatant phase to the weighted data of the sand phase. Appendix C – Sodium Azide Experiment shows bioactivity in the supernatant and the sand separately. The bioactivity was measured before and two hours after dosing the biocide. In the bottles treated with 0.4 g/L sodium azide 18% of the microbial activity was reduced in two hours. This result was comparable with the experiments above. Moreover the activity increased already after 24h. This means that the dose was too low to effectively inactivate MOs (Figure 3-10). Dosing 40 g/L sodium azide was able to inactivate 91% of the microbial activity in 24h. Furthermore dosing of sodium azide + metals was also quite effective; removal of 77% in 24 hours. However after 16 days the microbial population increased slightly, this time step was not measured for the bottles containing low and high dose sodium azide.

Even though the high dose of sodium azide was most effective in eliminating MOs, this high dose causes the pH to increase around 8 to 9; this means that the conditions are less comparable to the natural situation (Table 3-4).Treating bottles with a low dose of sodium azide did not affect the pH and sodium azide + metals led to a small decrease in pH to 7.3.

Results from DOC measurements showed that the DOC levels remained more or less the same when bottles were treated with a low dose of sodium azide, whereas the DOC concentration increased due to treatment with the high dose of sodium azide or the combination of sodium azide with metals (Figure 3-11). This trend was observed in both supernatant and sand phase of the batch bottles. However no explanation was found for this trend, because this result was not observed in the experiments described in paragraph 3.1.2 DOC content.

Proteins and carbohydrates were measured with a different method in this experiment; taking into account the results of the experiment above. The proteins and carbohydrates were measured from the sand, because this is the place where the EPS is located, therefore it is expected that measuring the EPS in the sand should give a better representation of the EPS. The protein and carbohydrates content was approximately 10 times higher when measured in the sand (compare Figure 3-9 with Figure 3-12). Moreover the protein content was corrected for interference by humic substances during analysis; results are shown in this paragraph.

Treatment with a high dose of sodium azide and sodium azide + metals showed a decrease in protein concentration at t=2 hours (Figure 3-12). On the contrary at t=2 hours the protein concentration increased in bottles dosed with a low sodium azide concentration in time. The bottles treated with sodium azide and metals stayed quite low; 60% lower than the initial protein content before treatment with biocide. This indicates that the protein content decreased with a decrease in bioactivity. On the contrary the protein content increased in bottles treated with 0.4 g/L sodium azide at t=2 hours and even more after 24 hours. No explanation was found for this result. Figure 3-10 shows that the bioactivity was a little lower, thus this does not explain the increase in protein content.

At t=0 the carbohydrate concentration in untreated bottles was almost 60 µg/g sand (Figure 3-12). Treatment with biocide caused an immediate drop of carbohydrates; after two hours bottles treated with sodium azide showed a decrease of 61% and 10% for respectively high and low dose of NaN_3. Treatment with sodium azide and metals gave a 96% carbohydrates reduction after two hours, within 24hours this concentration stayed low. However at t=16 days the carbohydrates concentration was higher compared to t=24 hours in the bottle treated with sodium azide and metals, the other bottles were not measured at this time step. These results show that also the carbohydrate concentration in the sand is directly related to the bioactivity. This result was also found for the supernatant in paragraph 3.4 EPS.

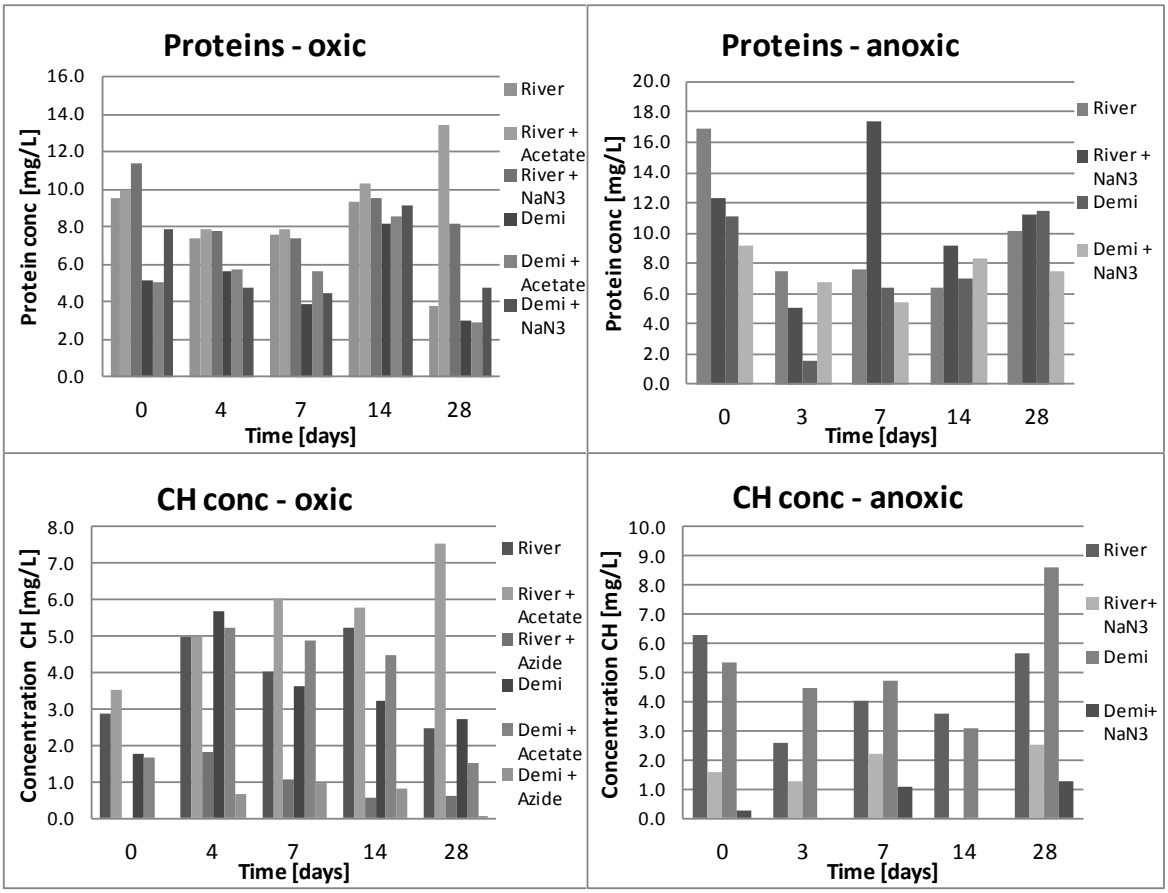

Figure 3-9 – Protein (upper) and carbohydrates (lower) in time for oxic (left) and anoxic (right) conditions.

Table 3-4 – pH of batch bottles treated with different biocide at t=0, 2 and 24h

pH					
Time	River water	High dose NaN3	Low dose NaN3	NaN3 + metals	Temp [*C]
0	8.14				20.0
2h		9.11	8.19	7.3	19.6
24h		9.01	8.33	7.36	20.0

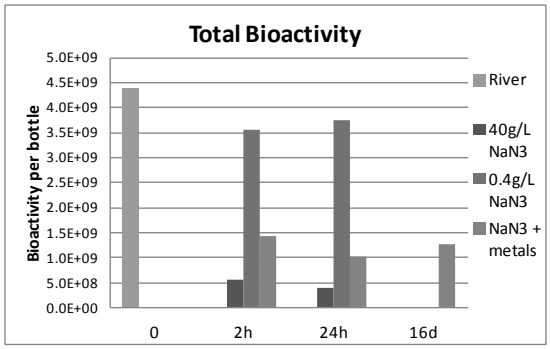

Figure 3-10 – Total bioactivity per bottle calculated by adding the weighted supernatant and weighted sand data in time.

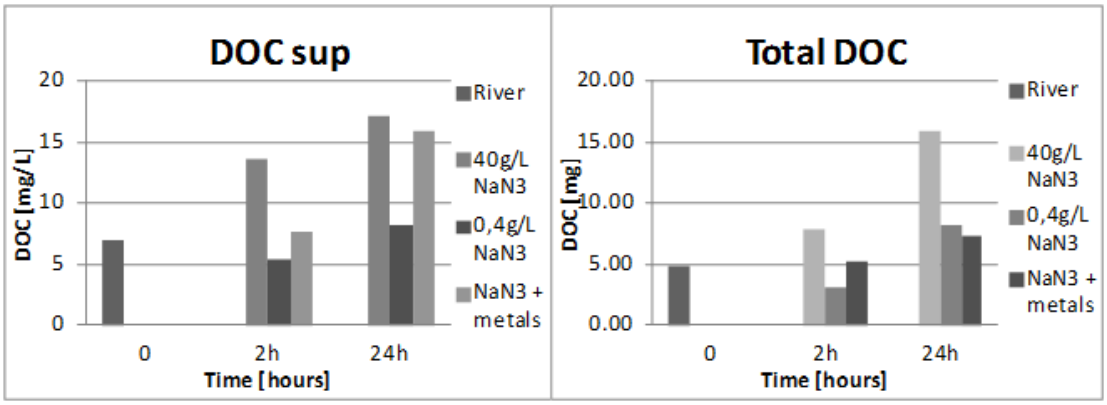

Figure 3-11 – DOC concentration in supernatant (left) and total DOC (right) which is calculated by adding the weighted DOC from the supernatant and sand.

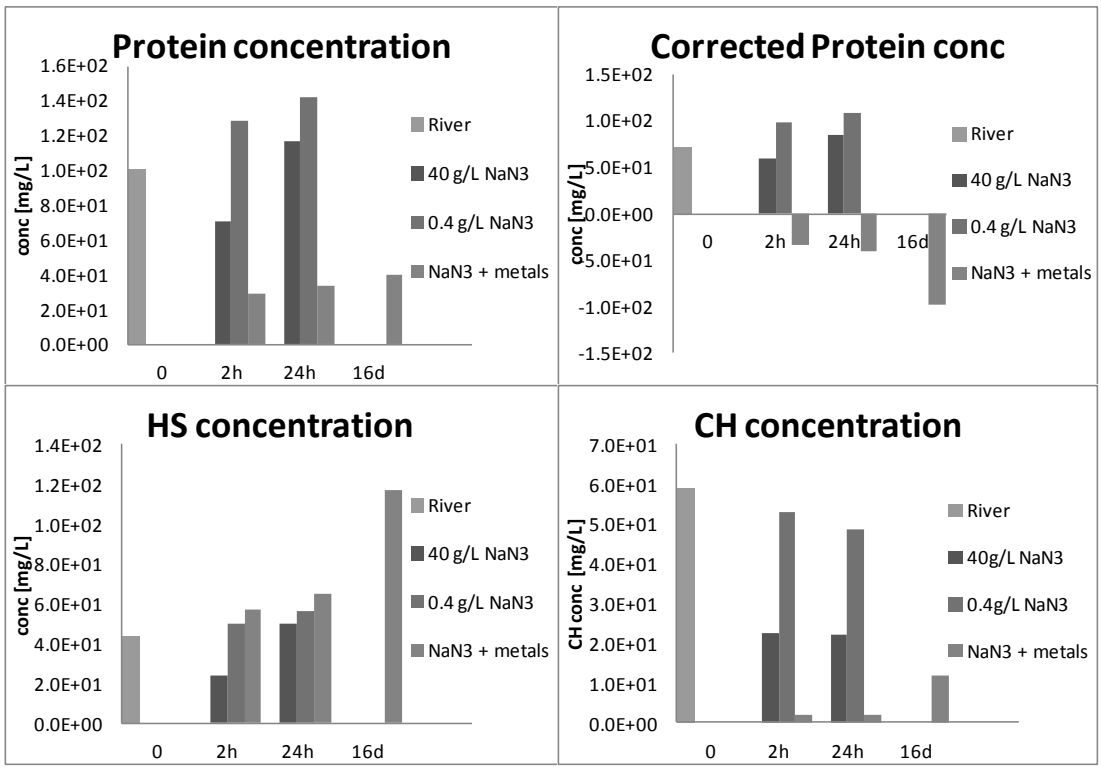

Figure 3-12 – Protein (upper), humic substances (lower, left) and carbohydrate (lower, right) concentration in time. At t=0 only the river water bottle was measured and at t=16 days only the NaN_3 + metals bottle. Corrected protein concentration (right) was calculated with humic substances using the formula in chapter 2.3 Sodium Azide Experiment.

3.6 COMBINING RESULTS

To summarize, batch experiments were performed with river water and riverbank sand under different conditions to gain knowledge on OMP removal by RBF. In this paragraph the most striking results will be discussed and results from different analyses will be combined.

During the batch experiments conditions were as close to the natural riverbank as possible; pH, DOC concentration, and nutrient and ions concentrations (Ca, Mg, NO3, NH4, SO4, Cl) started at common values for river water and remained constant during the experiment. The oxygen concentration stayed around 8 mg/L in the supernatant of the batches under oxic conditions. Under anoxic conditions nitrate was reduced, proving that a nitrate reducing zone was obtained. During oxic conditions the nitrate concentration in the supernatant increased in time. Furthermore the ammonium and phosphate concentrations increased in both oxic and anoxic batches and the DOC values remained more or less constant. Thus no nutrient limitation occurred during the experiment. However, a decrease of the nutrients phosphate, ammonium and nitrate (only under oxic conditions) was expected due to microbial activity. On the contrary an increase of those nutrients was observed, indicating that they were released into the supernatant from the degradation of organic material or the die-off of cells. The amount of DOC was much higher when determined from the sand than the supernatant; this means that the most carbon source was present in the sand.

Determining bioactivity by measuring ATP is a very sensitive method due to the high degradability of ATP; this led to varying results which are difficult to interpret. The bioactivity measured in the sand was higher than in supernatant and gave a better representation of the batch. In the oxic batches bioactivity decreased after two weeks; there was no nutrient limitation and no other explanation was found for this decrease. In the additional sodium azide experiment was found that treatment with sodium azide (0.4g/L) did not sufficiently suppress the bioactivity. Figure 3-13 shows that treatment with sodium azide (0.4mg/L) resulted in almost no log removal of bioactivity. Treatment with a higher dose (40g/L sodium azide) or treatment with the combination of sodium azide and heavy metals ($NiCl_2$ and $BaCl_2$) showed to suppress the bioactivity with approximately 0.5 to 1 log. The high dose of sodium azide caused the pH to increase from around 8 to 9; making the conditions less representative for the natural situation. Therefore using sodium azide in combination with heavy metals was found to be the best option in this study to inactivate MOs in sand.

During this study, it was tried to find the difference between biodegradation and sorption on the sand by treating the bottles with and without sodium azide to suppress the bioactivity. The sorption onto the sand was estimated by determining the OMP removal in bottles with suppressed bioactivity. However, due to the too low sodium azide dose, the bioactivity was still quite high and therefore sorption was overestimated. The biodegradation was determined by subtracting the OMP removal due to sorption from the OMP removal in untreated bottles. Therefore the results give an indication of the biodegradation but are not representative for the riverbank. Nine of sixteen OMPs showed a high removal (>70%) of which gemfibrozil, 2,4-D, diclofenac, and diuron showed more than 50% removal due to biodegradation. Literature shows that the OMP removal for different compounds can differ for different experimental conditions (Table 3-3). However most removal rates found in this study were comparable with results found in literature.

Overall the different experimental conditions did not seem to have a large effect on the removal of OMPs and the removal of most compounds was relatively high. A possible explanation is that the dosage of OMPs per mass of sand was too low in the batch. This means that the availability of OMPs for microorganisms may be too low for determining differences in removal. Furthermore the content of the water phase had no influence on removal of OMPs; a statistical t-test showed that there were no significant differences between the OMP removals in bottles containing river or demi water.

This study can possibly be used as a first step in building a QSAR model (quantitative structure-activity relation) to predict the fate of the OMPs during RBF. With this model not all OMPs need to be investigated separately. To be able to build this model relations between different physiochemical properties and their removal need to be evaluated. In this study several physiochemical properties were assessed; charge, molecular weight, pKa, log K_{ow} and log D. Charged OMPs were well removed during the experiment; negatively charged compounds may be attracted to the positively charged metal oxides in soil and positively charged compounds to the negatively charged biofilm (Flemming, 2010). No relations were

found between other physiochemical properties and the removal of OMPs. However the ranges between the different selected OMPs were not very wide.

This report shows that dosage of acetate does not seem to have a large effect on the bioactivity and the protein or carbohydrate concentration. However a small increase (around 10%) in removal of OMPs was observed due to the acetate dosing. This may indicate that acetate was used as a metabolite and OMP degradation was co-metabolic during degradation processes of OMPs, because the bioactivity did not increase. This theory needs to be researched further to be able to draw conclusions on this. However there was no difference in removal of OMPs between river water and demi water containing bottles observed, which makes this theory less likely.

The results in this study show that most activity occurs in the sand phase of RBF as compared with the supernatant. First of all the measured DOC concentrations, bioactivity and EPS concentrations were much higher in the sand. Secondly no difference was observed between bottles containing river water and demi water in DOC concentration, OMP removal and EPS concentrations. Finally the acetate dose did not seem to have an effect on all the monitored processes, except for the OMP removal results as described above. Thus this is the place were all OMP removal occurs due to both biodegradation and sorption.

Furthermore from literature can be learned that the biofilm located between the sand in the riverbank has multiple functions; enabling MOs to stick to the soil, sorption of OMPs, aggregation of bacterial cells, forming a protective barrier, providing a nutrient source, enzymatic degradation, etc (Flemming, 2010). A lot of these functions are involved or closely related to the mechanisms of OMP removal. Therefore studying the EPS matrix of the biofilm is very interesting to gain more knowledge on OMP removal mechanisms. In this study the protein and carbohydrate concentrations were studied. Results of the EPS content in both experiments showed that the carbohydrate concentration was related to the bioactivity, when measured in the sand or water phase. This was expected because with more bioactivity more carbon source is required. In the riverbank carbon source is available as chunks of organic material, like precipitated small wooden sticks, parts of dead fish, etc. To be able to bring this organic material into the cell, the organic material should be degraded into smaller pieces, carbohydrates. Cells secrete exo-enzymes to take care of this degradation. This will lead to an increase of carbohydrates and proteins in the biofilm when the bioactivity increases. However the protein concentration did not seem to change when measured in the supernatant; probably because the proteins stayed attached to the EPS matrix in the biofilm. Apparently the carbohydrates were released in the supernatant, because the carbohydrate concentration in the supernatant was also observed to be related to the bioactivity in the supernatant. According to Frolund (1995) the protein concentration should be corrected for the humic substances content, which infers with the analysis. During the sodium azide experiment this corrected protein concentration was determined. However bottles treated with sodium azide and heavy metals gave negative values. This indicates that the heavy metals absorbance spectra possibly interfere with the absorbance spectra of the protein analysis.

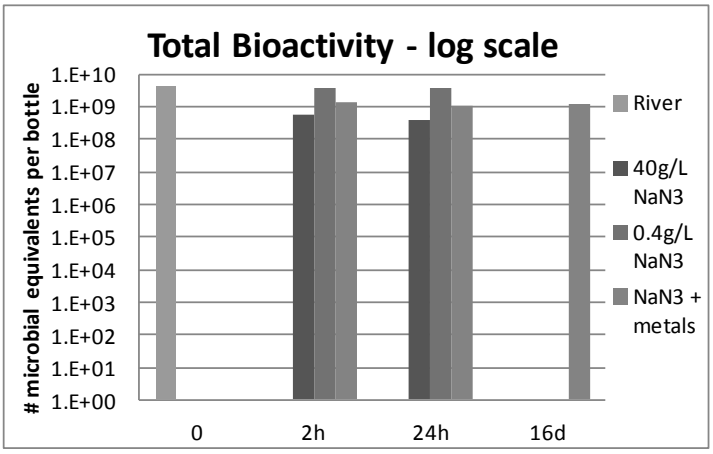

Figure 3-13 – Total bioactivity per bottle plotted on log scale in time. Results from the sodium azide experiment.

4 CONCLUSIONS

The objective of this study was to distinguish the main mechanism (biodegradation or sorption) responsible for removal of OMPs during RBF. Batch experiments using river water and riverbank sand were investigated. The bottles were treated with and without sodium azide to suppress the bioactivity and be able to make a distinction between removal due to biodegradation and/or sorption. However results showed that bioactivity was not sufficiently suppressed, caused by a too low dose of sodium azide. Therefore the estimated sorption of OMPs was overestimated, leading to an underestimation of the biodegradation of OMPs. Therefore the biodegradation calculated in this study only gave a rough indication whether biodegradation occurred or not.

An additional experiment proved that an increased sodium azide dose (40 instead of 0.4 mg/L) suppressed the bioactivity better; 0.5 to 1 log removal was observed compared to almost no log removal using the lower dose. Higher dosing of sodium azide shows an increase in pH which makes the conditions less comparable to the real situation. Furthermore the treatment with sodium azide and heavy metals ($NiCl_2$ and $BaCl_2$) showed a removal comparable with the high dose sodium azide; but did not cause an increase in pH. Therefore this treatment seems to be the most promising.

The removal of the 16 selected OMPs in river water over 14 days showed a removal of more than 70% for 9 compounds; 2,4-D, caffeine, diclofenac, diuron, gemfibrozil, ibuprofen, metoprolol, roxithromycin, and trimethoprim. The removal results were comparable with literature. Furthermore, dosing of acetate gave small increase in removal while no increase in bioactivity was observed. This may indicate that acetate was used as a metabolite and OMP degradation was co-metabolic in removal of OMPs. However the differences between the bottles with and without dosing of acetate are too small to draw this conclusion. Therefore this theory should be investigated further. No statistical difference in the removal of OMPs was obtained when river water and demi water were compared. This indicates that river water has no effect on the removal of the OMPs. Moreover different experimental conditions did not seem to have a significant impact on the removal of OMPs. This could indicate that the dose of OMPs per mass of sand was too low in the batch to be able to observe differences. This means that the availability of OMPs for microorganisms may be too low for determining differences in removal.

In order to build a QSAR (quantitative structure-activity relation) model, relations between physiochemical properties of compounds and their removal during RBF should be made. With this model the removal of different compounds can be predicted based on their physiochemical properties, this way not all compounds need to be investigated separately. This study showed that charged OMPs are removed; positively charged compounds may attach to the negatively charged biofilm and negatively charged compounds to the positive metal oxides which are present in the soil. This was the only relation found; other physiochemical properties (molecular weight, pKa, log Kow and log D) could not be related to their observed removal. However the ranges of the physiochemical properties of the different investigated OMPs were not very wide.

During the batch experiment no nutrient limitation occurred under both oxic and anoxic conditions, except for the nitrate concentration which decreased under anoxic conditions. This proved that no oxygen was present in the anoxic batch bottles. The DOC concentrations remained constant. Interestingly, ammonium, phosphate and nitrate (only under oxic conditions) increased during the experiment; these compounds were probably released into the supernatant from the degradation of organic material. Furthermore no explanation was found for the observed decrease in bioactivity after 14 days under oxic conditions, because no nutrient limitation occurred.

Measuring DOC concentrations, bioactivity and EPS concentrations (proteins and carbohydrates) were found to be much more representative in the sand phase than in the water phase of the batch. This conclusion can be made based on three different results. First the measured DOC concentrations, bioactivity and EPS concentrations were much higher in the sand. Moreover there was no difference observed between bottles containing river water and demi water in DOC concentration, OMP removal and EPS concentrations. Thirdly dosing acetate had no significant effect on the parameters analysed in this study, with exception of the OMP removal results as described above.

Finally it can be concluded that proteins and carbohydrates present in the EPS of the biofilm were directly related with the bioactivity. Furthermore, literature has shown that a lot of processes during RBF occur in

the EPS matrix of the biofilm. Therefore, to be able to gain more understanding in the processes occurring during RBF, the EPS should be investigated further.

5 RECOMMENDATIONS

This chapter will give some recommendations for further research on this topic. This research was part of ESTAB project which tries to design a multi barrier against OMPs by combining RBF, NF and GAC treatment. From this study it can be concluded that more insight is required in removal mechanisms of RBF. This should focus on the content of the different removal mechanisms, microbial population, the biofilm, and (co-)metabolic removal of OMPs. This chapter will give some recommendations for further research on this topic.

In order to get a better insight into the processes which occur in the batches, more knowledge on the microorganisms present is required. Currently not a lot of research has been done in this area, although it seems to be very important. A possible method for investigating the occurring MOs is DGGE (Denaturing Gradient Gel Electrophoresis); this method determines the microbial diversity, the dynamics of microbial community, and the functional organisation of the microorganisms (Boon, 2011, Marzorati, 2008).

In this study for each experimental conditions only one bottle was prepared to keep the experiment flexible and ease of operation. This way the design of the experiment was tested and the results gave an indication of the processes that occurred in the batch. However to be able to draw conclusions, this experiment should be repeated to confirm the trends found in this report. Some recommendations are outlined below to improve this experiment.

- First of all, to be able distinguish biodegradation from sorption; the bioactivity should be suppressed completely. This study showed that the bioactivity was better reduced with a higher dose of sodium azide or dose with sodium azide combined with heavy metals ($NiCl_2$ and $BaCl_2$). However there was still bioactivity present. Therefore another method should be investigated, for instance heating the riverbank sand. This was tried using the method of Kerr (2000); however this method did not work out due to time limitations; therefore another heating method should be tested. Other possibilities may be using radiation or microwaves to inactivate the bacteria.
- Secondly, results indicated that it was possible that the OMP dose per mass of sand (0.5ng/g) was too low to make a distinction between different experimental conditions. Other studies were able to distinguish differences between different experimental setups; they used higher doses per mass of sand, for instance Lin (2010), Liu (2010) used respectively 0.25 and 100 µg OMPs per gram sand. This means that they used a higher OMP concentration in the aqueous phase as well as a higher water/sand-ratio than the OECD guidelines for batch experiments followed in this study. It would be interesting to investigate the impact of different concentrations of OMPs, while keeping the dose as low as possible to be able to mimic the low river water concentrations and staying as close as possible to the water/sand-ratio conform the OECD guidelines.
- The results in this study indicated that acetate might be used as a metabolite and OMP degradation was co-metabolic during removal of OMPs. When this experiment will be repeated with a higher OMP dose per mass of sand, results will show if this observation can be considered as a trend to be researched further with specific (co-)metabolic experiments.
- The sand phase in batch bottles is the phase in which most processes occurred due to the higher microbial activity between the sand. Therefore further studies should focus more on the sand phase; especially when analysing the EPS, bioactivity and DOC content.
- According to Frolund (1995) the protein concentration measured using Lowey (1951) should be corrected for the humic substances. In this study, it was not possible to correct for humic substances according to Lowey (1951), because it resulted in negative values for the samples from bottles treated with sodium azide and heavy metals. It is possible that the dosing of $NiCl_2$ and $BaCl_2$ interfered with the method, this should be further investigated.
- Furthermore, results showed that measuring the bioactivity using ATP analysis is very sensitive to hinder, because ATP is fast degradable. This leads to varying data. This method was used because it is relatively simple and easy in operation. However to be able to accurately measure the bioactivity another, more reliable method should be considered. In literature often cell counts are used, measuring the amount of cells present by dying them and count them under the microscope. However it is difficult to distinguish dead cells from alive ones using this method (Magic-Knezev, 2004, Lindahl, 1995, Yu, 2010). Therefore it would be interesting to combine the ATP analysis with cells counts to be able to estimate the amount of MOs present in the batch bottles.
- The batch bottles were measured at 5 time steps; bottles were sacrificed after every measurement. However, between the different time steps a lot of change can occur. Therefore it might be interesting to perform the experiments described in this report in the same way while using a fermentor.

This way certain parameters can be monitored constantly, like oxygen, CO_2, and pH. Moreover pH can be easily adjusted. This can only be used as an additional experiment, because sand samples cannot be taken without sacrificing the fermentor content.

- Further it is recommendable to test the effect of sonication on the bacterial cells. Sonication detaches microorganisms and biofilm from the sand. During sonication the cells are disrupted, this may cause cell lysis which gives a less reliable representation of the EPS content of the biofilm in the batches. Therefore intracellular components should be measured after sonication to determine if cell lysis occurred. This can be done by measuring intracellular enzymes such as glucose-6-phosphate 1-dehydrogenase (G6PD, aka Zwf) which is involved in the metabolic pathway (Flemming, 2010). This way the amount of cell lysis that occurred can be estimated and compared between different samples.
- Finally to be able to build a QSAR model, which predicts the fate of OMPs during RBF, a larger range of physiochemical properties should be investigated. Moreover next to the properties investigated in this study (charge, molecular weight, pKa, log K_{ow} and log D) other physiochemical properties should be examined.

ACKNOWLEDGEMENTS

In this chapter I would like to sincerely thank the people that made my graduation thesis possible. First of all, I would like to thank Cheryl Bertelkamp for all her help, never ending enthusiasm and positivism. Hearing her sing in the lab made the long lab days more bearable. Furthermore I would like to thank Arne Verliefde for his positive energy, active work approach and approachability. Then, Jan Peter van der Hoek for his approachability, involvement and knowledge, which led to fruitful meetings. Not to forget Naresh Singhal for our interesting discussions, his patience and broad knowledge; this has helped me a lot during the designing of my experiments. Although I never met him, I would like to thank Julien Reungoat for the analysing OMPs with the LCMS-MS in Australia to use in this report. Finally, a special thanks to room 4.84 for keeping me motivated and sharing (very) long working days, lots of coffee and the 'graduation-thesis-experience' at the TU.

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APPENDIX A – SAND DATA

Table 0-1 – Sieving data

Diameter [mm]	Fraction [%]
0.90	-
0.80	0.01
0.63	0.05
0.56	0.18
0.40	3.83
0.30	20.8
0.21	61.0
0.18	10.0
0.15	3.47
Residue	0.63

Formula's to determine porosity and density of riverbank sand

$$Porosity\ (-) = \frac{Volume}{Mass_{sand+water} - Mass_{dry\ sand}}$$

$$Density\ of\ sand\ (\frac{g}{L}) = \frac{Mass_{dry\ sand}}{Volume}$$

Volume = volume of the cylinder
$Mass_{dry\ sand}$ = mass cylinder filled with dry sand
$Mass_{sand+water}$ = mass cylinder filled with dry sand, filled up with water

APPENDIX B – BIOACTIVITY

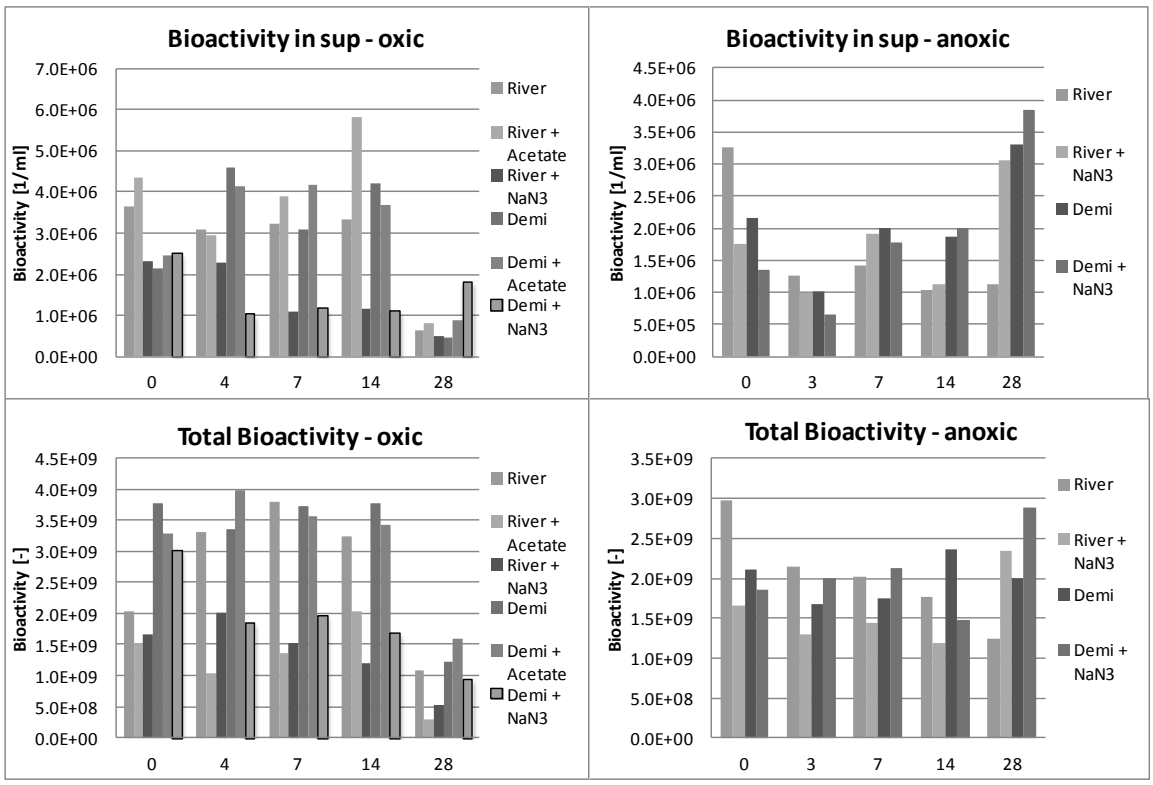

Figure 0-1– Bioactivity measured in supernatant (upper) per mL and the total bioactivity, calculated by adding the weighted data from the supernatant to the sand (lower) under oxic (left) and anoxic (right) conditions.

APPENDIX C – SODIUM AZIDE EXPERIMENT

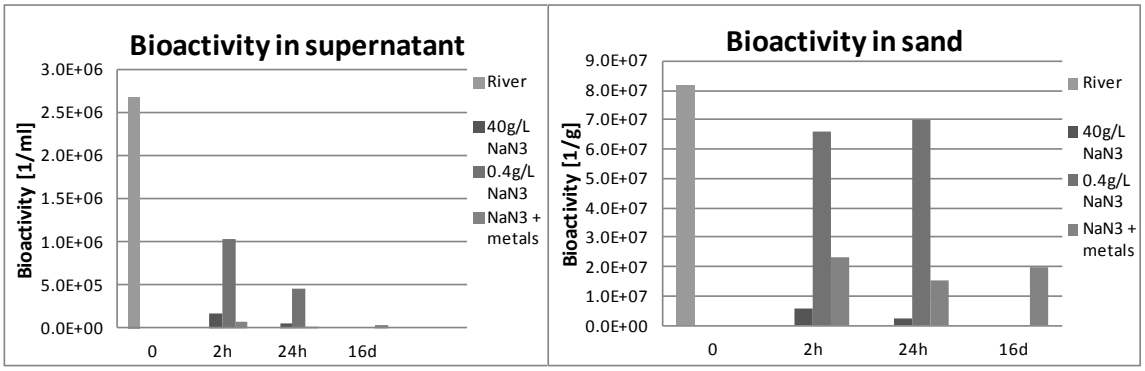

Figure 0-1– Bioactivity measured in supernatant (left) and sand (right) during oxic conditions.