NTNU – Trondheim
Norwegian University of
Science and Technology

Removal of the Micropollutants DEET and DEP in biological Grey Water Treatment and the Effect of DEP on microbiological Processes

Eva-Linde Geiling

Civil and Environmental Engineering (2 year)
Submission date: June 2015
Supervisor: Stein Wold Østerhus, IVM
Co-supervisor: Frank Persson, Chalmers University of Technology
Viggo Bjerkelund, IVM

Norwegian University of Science and Technology
Department of Hydraulic and Environmental Engineering

Abstract

The insect repellent N,N-diethyl-m-toluamide (DEET) and the plasticizer Diethyl Phthalate (DEP) are commonly used in externally applied personal care products. Washed off the skin, they are found in grey water. If the grey water is reused for high body contact applications in order to lower the potable water demand, the removal efficiency for these micropollutants (MP) during treatment is crucial. The presented Master's thesis quantifies the removal of DEET and DEP during biological grey water treatment. It evaluates the effect of DEP on microbial processes and nutrient removal.

Beakers spiked with 2.5, 5, 10 µg/L DEET and 10, 50, 100 µg/L DEP were aerated for 3.5h and 6h. Residual MP concentrations were measured with a high performance liquid chromatography (HPLC). Therefore, samples had to be purified and up-concentrated 200 times (DEP) and 500 times (DEET) with a solid phase extraction (SPE).
Biological phosphorous removal, nitrification and de-nitrification under the influence of 0 and 100 µg/L DEP were quantified. Oxygen uptake rates and metabolic activity under the influence of 0 to 10 000 µg/L DEP were measured. TOC removal under the influence of both MP was determined in all set-ups. Selected samples were analyzed with size exclusion chromatography (LC-OCD).

At minimum, 11.7% (13.4 %) DEET, i.e. 0.12 µg/gSS (0.17 µg/gSS) is removed during 3.5h (6h) aeration. DEET does not adsorb onto sludge.
At minimum, 86.9% (100%) DEP, i.e. 2.2µg/gSS (2.3 µg/gSS), is removed during 3.5h (6h) aeration. 6.6% DEP, i.e. 2 µg/gSS, adsorb onto sludge.
None of the compounds evaporates during biological treatment.
Biological phosphorous and nitrogen removal are not significantly influenced by 100 µg/L DEP. In contrast, TOC removal is severely hampered by the presence of >50 µg/L DEP. Depending on retention time, 100 µg/L DEP decreases the TOC removal efficiency by up to 47.7%. The residual TOC in the spiked beaker is up to 39 mg/L higher than in the blank. The increased residual TOC consists of low molecular weight substances (<< 350 g/mol).

DEP concentrations between 100 µg/L and 10 000 µg/L trend to increase the oxygen uptake rate. This suggests that DEP poses oxidative stress on cells. In this case, the increased residual TOC would indicate cell damage. However, elevated TOC levels can also be caused by overproduction of extracellular polymeric substances as a successful defense against the environmental stress factor DEP. In this case, the effect of DEP on microbes in the activated sludge has to be considered less harmful.

Acknowledgments

If not stated differently, the persons are associated with the NTNU Trondheim.

Thanks to

Viggo A. Bjerkelund for the project idea, advice and help with experimental set-ups, developing HPLC methods, and input for chapter 3; Professor Stein W. Østerhus for supervising experimental plans and improving them, and giving scientific advice; Professor Frank Persson (Chalmers, Gothenburg) for feedback on the thesis draft, valuable literature suggestions and input for chapter 4.8; Trine Margrete Hårberg Ness for support with lab instruments and measurements. Special thanks for her concern about my safety while handling mercury and for organizing an extensive risk evaluation for this; Gøril Thorvaldsen for TOC measurements, ATP measurements, and general support in the laboratory; Peiyao Zhang for running the pilot plant and providing sludge and grey water; Professor Thomas Meyn for carrying out the LC-OCD measurements and for helping with their evaluation; Professor Paige Novak (University of Minnesota) for advice and input for chapter 4.5. Aleksandra Migowska for drawing the aeration unit.

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Abbreviations

ACN	ACETONITRIL
BB	BUILDING BLOCKS
BP	BIOPOLYMERS
DEET	N,N-DIALKYLAMIDE
DEP	DIETHYL PHTHALATE
EBNR	ENHANCED BIOLOGICAL NUTRIENT REMOVAL
EBPR	ENHANCED BIOLOGICAL PHOSPHOROUS REMOVAL
EPS	EXTRACELLULAR POLYMERIC SUBSTANCES
GW	GREY WATER
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
IFAS	INTEGRATED FIXED-FILM ACTIVATED SLUDGE (PROCESS)
LC-OCD	LIQUID CHROMATOGRAPHY - ORGANIC CARBON DETECTOR
MBBR	MOVING BED BIOFILM REACOTR
MBR	MEMBRANE BIO REACTOR
MP	MICROPOLLUTANT
MQ	MILLIQ (ULTRAPURE WATER TYPE 1)
OUR	OXYGEN UPTAKE [mgO2/h]
PAO	PHOSPHOROUS ACCUMULATING BACTERIA
PHB	POLY-B-BUTYRATE
ROS	RADICAL OXYGEN SPECIES
SEC	SIZE EXCLUSION CHROMATOGRAPHY
SMP	SOLUBLE MICROBIAL PRODUCTS
SOUR	SPECIFIC OXYGEN UPTAKE [$mgO_2/gSS/h$]
SPE	SOLID PHASE EXTRACTION
SRT	SOLID RETENTION TIME [h]
SS	SUSPENDED SOLIDS [g/L]
STD	STANDARD DEVIATION
TOC	TOTAL ORGANIC CARBON [here mg/L]
VFA	VOLATILE FATTY ACIDS
VSS	VOLATILE SUSPENDED SOLIDS [g/L]

1 Introduction

1.1 Background – introduction problem

Population growth and urbanization lead to an increasing water demand in urban areas that necessitates the exploitation of new fresh water sources. Even in water ample regions this development is detrimental, since it demands a costly upgrade of supply infrastructure and causes environmental damage. (Friedler and Hadari 2006) In more arid regions, climate change might exacerbate water stress, making fresh water availability a major social and economic challenge (Kharraz et al. 2012). Hence, the lowering of the potable water demand in order to prevent a depletion of fresh water resources is of increasing interest, in both humid and arid countries. One measure to lower domestic water consumption could be the separation and reuse of different wastewater fractions. (Ottoson and Stenström 2003)

1.1.1 Grey water (GW) – its potentials, treatment, reuse

One possible resource for reuse of domestic wastewater is grey water (including water from bath tubes, washing machines, showers, hand-washing basins and kitchen basins). Firstly, because it constitutes 60-80% of domestic waste water (Hocaoglu et al. 2013) and secondly, because it contains – compared to a combined domestic waste water including black water - low concentration of suspended solids, nitrogen, total carbon and pathogens (Atasoy et al. 2007).

There are established methods for GW treatment: it is commonly treated by sand filtration and disinfection, constructed wetlands, membrane technologies (pore sizes ranging from microfiltration to reverse osmosis), physical-chemically (adsorption or ozonation) and biologically. Each GW treatment has its' strengths and weaknesses: for instance, ozonation is reported to be cost-efficient to remove organic pollutants, but might lead to toxic by-products. (Liu et al. 2010, Hernández Leal et al. 2012) Studies show that moving bed and membrane bioreactors are a promising technology for GW: the effluent reaches the quality requirement for reuse in terms of standard parameters such as phosphorus, nitrogen, BOD, COD, turbidity and coliforms (Merz et al. 2007, Jabornig and Favero 2013).

Treated in this way, GW can then be reused for toilet flushing, which reduces the fresh water of the dwelling by up to 30%. It can also be reused for irrigation, where it reduces the potable water demand by 40-60%. (Al-Hamaiedeh and Bino 2010) Apart from this, grey water

treatment might also reduce the release of household derived pollutants into the environment – but only, if they are removed by the treatment.

1.1.2 Micropollutants in GW treatment

Can household chemicals be removed from GW with commonly used treatment technologies? Established methods for GW treatment achieve good nutrient removal efficiencies. But apart from nutrients, household chemicals also contain micropollutants (MPs) (Ottoson and Stenström 2003, Friedler and Hadari 2006). These MPs are synthetic substances that originate for instance from personal care products and cosmetics and thus can be found in washing wastewater. They might be harmful for human health (Fuhrman 2012). Furthermore, they could be - unlike nutrients - resistant towards the established treatment technologies. Research is still needed on the GW treatment efficiency for those compounds. (Donner et al. 2010)

If GW should later be used for high body contact applications – as it is the case in this project - general quality requirements are higher than for irrigational purposes. A reuse of grey water for high-body contact purposes also means, that grey water is recycled, since water is not only used twice (e.g. first in the shower, afterwards for irrigation), but multiple times. This is crucial, since MPs can be up-concentrated during recirculation, if they are not removed in the treatment. Hence, for the assessment of grey water treatment, it is of vital importance to quantify MP removal.

1.2 Aims and Objectives

The aim of this master thesis is to research biological removal of the micropollutants DEET and DEP from grey water. Furthermore, the effect of DEP on the biological activity such as nutrient removal, microbial growth and respiration should be studied.

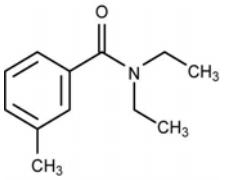
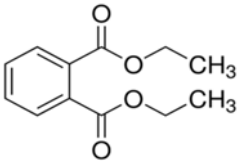
- Overall removal of the micropollutants from biological batch reactors should be measured.
- In order to characterize biological removal of DEET and DEP, the contribution of abiotic removal (adsorption, evaporation) to overall removal should be quantified.
- The compounds' influence on the bacteria's performance should be characterized by measuring nitrogen-, phosphorous- and TOC-removal as well as oxygen uptake. Cell growth under the influence of DEP should be estimated, if procurable.
- The data generated in the foregoing specialization project suggests, TOC removal is inhibited by DEP. This should be verified or falsified.

- If procurable, part of the experiments should be conducted under conditions similar to those in the pilot plant (sequence of anaerobic, anoxic, and aerobic).
- The theoretical part of the thesis should provide for justifying the choice of compounds, doses and treatment, i.e. it should present usage, properties and adverse effects of DEET and DEP. It also should introduce all relevant biotic and abiotic removal mechanisms. The generated data should be evaluated in the context of the reviewed literature.

2 Theory

In the following chapter, first MPs are introduced and then the relevant physical and chemical properties of two example compounds are presented (see Table 1). Their occurrence and adverse effects on the environment and humans are described in section 2.2 and 2.3. In section 2.4, general degradation mechanisms (abiotic, biotic) as well as sorption mechanisms for any pollutant are explained. Kinetic laws that describe removal are introduced in 2.5. In 2.6, nutrient removal mechanisms by microorganisms are described, since these mechanisms might be influenced by the MP. Specific degradation and sorption behavior of the two model compounds are elaborated in sections 2.7 and 2.8.

Table 1 - physical and chemical properties of DEET and DEP
(Jun Sekizawa 2003, Jackson 2008, Thomsen, Rasmussen and Carlsen 1999, Sudakin and Osimitz 2010, Roháč et al. 2004, GSI 2013, Hyland et al. 2012, NLM 2001)

	DEET	DEP
		
Partition coefficient (log K_{OW})	2.02	2.51
Molecular weight	191.3 g/mol	222.2 g/mol
Solubility in water at 25° C	>1000 mg/L	1080 mg/L
Sorption (log K_{OC})	1.97 - 2.97	2.65
Vapor pressure (Pa) at 25°	0.75	0.099

2.1 Micropollutants (MP)

MPs can be measured in trace concentrations ranging from ng/l to µg/l in the aquatic environment. Their ecotoxicological effect is not fully researched yet (Hollender 2009). Some categories of MPs, such as pharmaceuticals and steroid hormones, can be found in black water (the fecal stream of domestic wastewater), whereas grey water contains the categories industrial chemicals (e.g. plasticizers, fire retardants), pesticides (herbicides, insecticides), personal care products (e.g. fragrances, disinfectants, insect repellents) and surfactants. (Luo et al. 2014) Their effect on humans are not fully elucidated (Fuhrman 2012).

2.2 Properties N,N-diethyl-m-toluamide DEET

2.2.1 DEET - Source, fate and occurrence in the aquatic environment

DEET is an odorless, colorless insect repellent in the family of N,N-dialkylamide. It is formulated in various ways such as liquids, pressurized liquids and aerosols, gels, sticks and

lotions. It can be applied to textiles, skin or headgear (Jackson 2008). More than one third of the American population uses it every year, the worldwide consumption is estimated to exceed 200 000 000 application per year (Barnard 2000). The concentration of DEET in those products ranges from 4% – 100%. DEET can penetrate the skin, however, less than 20% of DEET content of a product is absorbed while applied (Stinecipher and Shah 1997, Costanzo et al. 2007). Accordingly, a major pathway of DEET towards the aquatic environment is the waste water effluent after washing-off of the products. Around the world, the detected concentration of DEET in aqueous samples ranges from 4 to 3000 ng/l. In surface waters in the USA, Australia, Germany and the Netherlands (140 samples analyzed) concentrations range from 19 to 97 ng/L (Costanzo et al. 2007). A study evaluating the effluent of 90 wastewater treatment plants in 17 European countries concludes DEET can be detected in 100% of the treatment plants' effluent. The average concentration is 678 ng/L. (Loos et al. 2013)

However, in grey water the percentage of washed-off insect repellent creams can be higher than in municipal wastewater. Hence, for the presented study, concentration in the range of µg (and not ng) are chosen.

In Norwegian sea water, DEET was detected in concentrations ranging from 0.4 to 13 ng/L (Weigel et al. 2004). Tran, Hu and Urase (2013a) report furthermore the detection of DEET in groundwater and conclude that it is persistent during wastewater treatment. This is supported by Yang et al. (2011), who found DEET frequently in the effluent of an advanced treatment plant for wastewater reclamation.

This last property makes DEET interesting as an example compound for a study. Apart from that, it is also considered as a possible barrier against malaria (Mark Rowland 2004). Since water scarcity can trigger an increase in malaria vector breeding (WHO 2007), the demand for grey water reuse might correlate with a demand for DEET. At the same time, DEET might up-concentrate in biological grey water treatment– depending on the bacteria's capacity to degrade it. Its ubiquitous use in the form products that are easily washed off, its persistency during treatment and the correlation between a demand for DEET and a demand for grey water recycling make DEET it an important compound and motivate its choice as a model MP for this study.

2.2.2 DEET - Adverse effects

DEET is found to be nontoxic to small mammals, but slightly toxic to birds, fish and freshwater invertebrates with LC_{50} values of 71.3 - 76 mg/l for these organisms. However, DEET is likely to sorb to sediments where it affects sediment-based species in the long term. This chronic exposure is not accounted for with acute toxicity test (Costanzo et al. 2007).

Toxicological effects on humans depend on the exposure pathway. Reported symptoms like irritation where linked to ocular and skin exposure. Gastrointestinal symptoms included nausea and vomiting. The relation between DEET exposure and neurological symptoms is unproven. (Katz, Miller and Hebert 2008) (Osimitz et al. 2010).

Though DEET appears to be harmless, a definitive toxicity of DEET taking into account chronic exposure, bioaccumulation and synergetic toxicity remains undetermined (Brausch and Rand 2011, Costanzo et al. 2007).

This might be because until 1998, the U.S. EPA considered DEET to be an indoor compound that is not even emitted to the aquatic environment. And since “ecological risk assessments are not conducted for pesticides with exclusively indoor use patterns” (U.S.EPA 1998), little environmental toxicity data is available. DEET is, however, not an indoor pesticide but part of outdoor equipment and also emitted with wastewater. Thus, it is ubiquitous in the aquatic environment (Costanzo et al. 2007, Loos et al. 2013).

2.3 Properties Diethyl phthalate DEP

2.3.1 DEP - Source, fate and occurrence in the aquatic environment

DEP is a low molecular phthalic ester and is applied in cosmetics and personal care products. It is used to prolong the duration of a perfume scent by inhabiting evaporation or as a methanol denaturant (Abdel daiem et al. 2012) It is applied as a plasticizer and in medical treatment tubing, for oils, tablets and in salts for bathing, for hair spray, nail polish and skin lotions (Abdel daiem et al. 2012, Jun Sekizawa 2003). Since there are no covalent bonds between the plasticizer and the PVC-matrix of the plastic, DEP can leach into the environment. Approximately 1% of the phthalate ester content of plastic materials leaches in direct contact with water or other liquids. (Wu et al. 2015, Gómez-Hens and Aguilar-Caballos 2003). DEP was found in surface waters at concentrations ranging from <1 to 10 µg/L and in drinking-water at concentrations ranging from 0.01 to 1.0 µg/L (Jun Sekizawa 2003). DEP concentrations in

the biological step of municipal sewage plants range from approx. 1 to 50 µg/L (Dargnat et al. 2009, Vogelsang et al. 2006)

Its behavior in the environment is closely linked to solubility, partitioning (K_{OW}, octanol/water partitioning) and sorption (K_{OC}, soil organic carbon/water partitioning) (Thomsen et al. 1999). DEP was chosen as an example compound because it is dermally applied and water soluble. That makes it relevant for grey water. Furthermore, it might be up-concentrated in the course of grey water recycling, which could exacerbate its adverse effects.

2.3.2 DEP - Adverse effects

DEP has a low acute toxicity with LC_{50} of 1-30 g/kg bodyweight, but dose related chronic damage of the thyroid gland, kidney and liver are reported (Abdel daiem et al. 2012). Studies also show a cumulative, dose additive effect with other toxic substances (Kapanen et al. 2007). The overall hazard posed to human health is object to debates (Abdel daiem et al. 2012): some studies describe only slight primary dermal irritation or eye irritation after exposure to DEP. They stress that no carcinogenic, teratogenic or other endpoint can be identified. (Api 2001) Other researchers conclude, DEP causes abnormalities in the reproductive system of female vertebrates and has a toxic effect on the male reproduction system (Kumar et al. 2014). Carcinogenicity remains questionable (Abdel daiem et al. 2012).

The U.S. EPA thus established an ambient water quality criterion for DEP of 350 mg/l [sic]. The oral daily reference dose for humans was set to 0.75 mg/kg bodyweight. This dose is expected to be without risk for cancer or other toxic effects (Api 2001).

2.4 Mechanisms for pollutant removal

The decrease of a micropollutant's concentration in wastewater is a combination of abiotic and biotic processes. The processes shown in the graphic are elaborated in the paragraphs following thereafter.

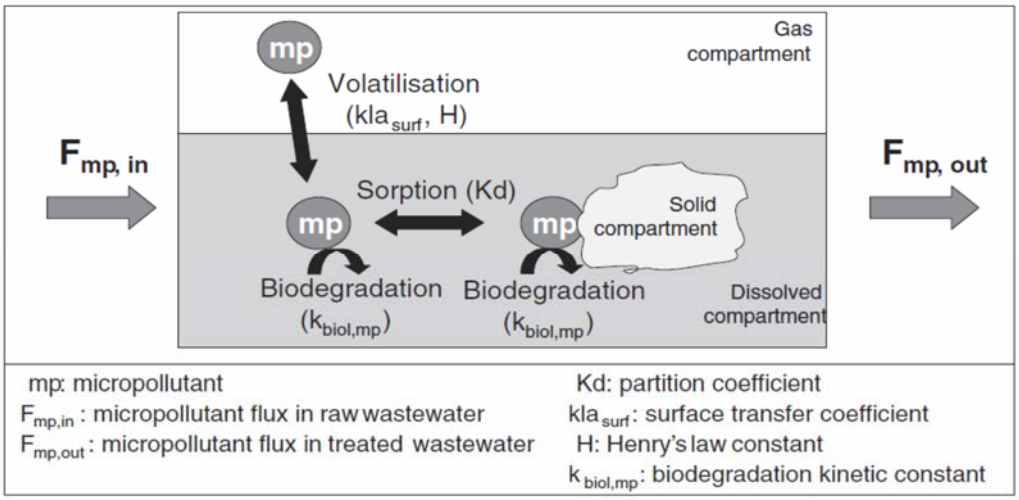


Figure 1 - Fate of micropollutant (mp) in a biological reactor.
The MP can volatilize (i.e. enter the gas phase) or sorb onto solids. It can also be metabolized by microorganisms (bacteria or fungi) in the sludge. Note: the less important abiotic degradation processes hydrolysis and oxidation are omitted. (Pomiès et al. 2013).

2.4.1 Degradation in general

Degradation can be defined as a decrease of concentration of a compound because of a nonreversible change of its chemical structure. A partial change of the contaminant's structure is called transformation. Degradation includes biotic processes, where the compounds are mineralized under the release of carbon dioxide (enzyme catalyzed) and abiotic degradation, such as photolysis or hydrolysis (water, chemical or UV-light as catalyzer). The compounds can also attach to solid substances (sorption), which is not termed degradation, since it does not necessarily include a change of chemical structure. (Felsot 2005) Biosorption describes the binding of pollutants to functional groups on the outside of the cell wall of microorganism (i.e. a binding to the organic matrix). It also plays a major role in the removal of MPs during biological treatment, especially for MBRs (Rattier et al. 2014). It depends on the partition coefficient and solubility, so also on the pH and on hydrophobicity. (Vijayaraghavan and Yun 2008, Tadkaew et al. 2011)

Abiotic processes are relevant pathways of DEP's and DEET's fate in the aquatic environment (Calza et al. 2011, Peng, Feng and Li 2013).

2.4.2 Biological degradation

Organic compounds can be transformed by many organisms, but microorganisms are the most important actors in transforming anthropogenic MPs (Schwarzenbach, Gschwend and Imboden 2005c, Tran et al. 2013b).

In order to degrade recalcitrant MPs, appropriate microorganisms (MO) have to be present and have to acclimatize. Besides, long-term exposure to the substance might be needed to trigger and sustain the synthesis of enzymes needed for the degradation. (Schwarzenbach, Gschwend and Imboden 2005a) There are three main ways, how microorganisms make use of a substance in their metabolism and thus lower its concentration. 1) *Assimilative metabolism*: the compound is a growth substance (carbon or nutrient source) for the microorganism; 2) *dissimilative metabolism*: the organic MP serves as an electron donor or acceptor; and 3) *co-metabolism:* the MP is degraded in the presence of a growth substrate (primary substrate), but is not itself a part of the MO's metabolism. That means there is no specific enzyme in charge with breaking down the MP. The MP is then a non-growth secondary substrate, i.e. it is not beneficial for the MO's cell growth and a primary substrate is obligatory. (Tchobanoglous, Burton and Stensel 2003, Rittmann 1992) This pathway is the major degradation mechanism for organic MPs in wastewater treatment (Fernandez-Fontaina et al. 2014, Sathyamoorthy, Chandran and Ramsburg 2013, Tran et al. 2013b).

How the microorganisms use the pollutant metabolically depends on the reduction potential of the oxidation or reduction of the pollutant compared to the reduction potential of other available electron acceptors (or donors, respectively). I.e. if a good electron donor (e.g. glucose) is available, but no good electron acceptor (e.g. oxygen or nitrate), the pollutant is likely to serve as an electron acceptor. If no good electron donor, but an excellent electron acceptor is available, the pollutant will probably serve as an electron donor. (Madigan 2015a, Rittmann 1992) Figure 2 illustrates how the pathways are combined and explains the steps of biotransformation.

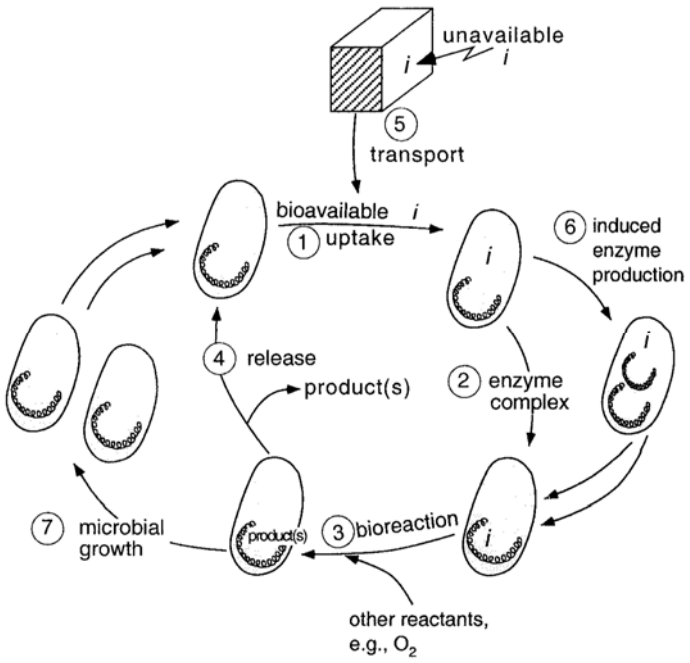


Figure 2 - sequence of events in biological degradation.
i in this case would be a micropollutant mp.
1: Uptake of chemical i by bacterial cell. 2: Binding of i to enzyme. 3: Reaction of enzyme-i-complex, production of transformation products. 4: Release of transformation pro-ducts. Processes 5-7 depict other influence factors of the transformation rate. 5: availability of i for the bact-erium.
6: Enhancement of enzyme pro-duction in the bacterium. 7: Growth of the total microbial production and thus increase of biotransformation rate. (Schwarzenbach et al. 2005a)

The process is influenced (and thus limited) by the delivery of the compound to the MO's metabolic apparatus, the presence of enzymes and their ability to catalyze the initial breakdown of the compound (Michaelis-Menten kinetics, 2.5.2) and the growth of the microbial population (Monod kinetics). In engineered systems, the latter is dependent on the solid retention time (SRT); hence with a high SRT, higher degradation rates can be observed (Fernandez-Fontaina et al. 2012). Nevertheless, not only the concentration of microorganism, but also their access to the substrate is crucial; the substrate delivery is influenced by the diffusivity, by its hydrophobicity, and by mass transport. Mass transport to a cell can be impaired, when cells are buried under each other in a biofilm. The water-biota partitioning (i.e. polarity and hydrophobicity) determines whether the compound can passively diffuse through the lipid-rich cell membrane. That means non-polar substances can be taken up into the cell interior, even if the MO does not have a system associated to the membrane to actively pick up the compound. (Schwarzenbach et al. 2005a)

The compound structure (i.e. the length of the side chains, their complexity and functional groups) impact the biodegradability of a compound: linear compounds with short side chains, unsaturated aliphatic compounds or compounds with electron donating groups are easily biodegradable. Branched and long side chains, a saturated or polycyclic structure and halogen, sulfate or electron-withdrawing functional groups make a compound recalcitrant towards

degradation. (Luo et al. 2014) However, a definitive prediction on biodegradability based on structural properties cannot be given (Rattier et al. 2014).

Also other factors, such as conditions in the reactor influence the degradation; e.g nitrifying conditions have been shown to increase the biodegradation of MP in different systems (fixed bed reactor and activated sludge process) (Luo et al. 2014).

2.4.3 Microbial growth

No matter how fast and efficiently microbes metabolize either the MP or another substrate, the degradation of these depends on how many microbes are in the system. The pace of growth is linked to the stage of growth. Microbial populations grow exponentially after a lag phase. In the exponential phase, growth rates in which the biomass duplicates are highest and cells are in their healthiest state. In a batch reactor, exponential growth cannot be maintained, but is limited by substrate depletion or waste product accumulation, which lead to a stationary phase. In this stage, the growth rate is zero and the populations size is constant. The population size declines in the death phase. (Madigan 2015b)

2.4.4 Substrate interaction and inhibition

Another substrate (e.g. TOC) can influence or be obligatory for the degradation of a pollutant (see "co-metabolism", 2.4.2), but also the MP can influence the degradation of other substrates. Substrates influence the production of enzymes that are needed for the substrate's utilization in different ways:

1. Enzyme repression/inhibition: Here, the transcription of a gene synthesising an enzyme is repressed, because of the presence of a substance. If for instance an enzyme is needed to synthesize a certain amino acid, but this amino acid is already sufficiently present in a bacterial cell. In order not to produce that enzyme, the amino acid represses the transcription of the gene that codes for the enzyme by blocking the RNA polymerase. (Madigan 2015a) The co-metabolite (e.g. an MP) can also repress the expression of an enzyme needed to utilize the primary substrate. (Pablo B. Saéz 1993)
2. Enzyme induction: The production of an enzyme is a response to a signal, i.e. the presence of a certain substrate. In the presence of that substrate, the gene for the synthesis of a necessary enzyme is transcribed. If for instance lactose is present, the

gene for the enzyme needed to degrade lactose is expressed. That assures that catabolic enzymes are only produced when they are needed. (Pablo B. Saéz 1993, Madigan 2015a) In the case of co-metabolism, however, also the primary substrate A can induce the gene repression for an enzyme catabolising substrate B, if the substrates A and B are structurally similar. (Pablo B. Saéz 1993)

3. Competitive inhibition: If an enzyme suitable to metabolize a substrate already exists, a competitive inhibitor can bind to the active site of the enzyme, preventing the substrate to bind. The substrate then cannot be utilized. Competitive inhibition can also be allosteric, i.e. the inhibitor binds to an allosteric site, which still prevents the substrate to bind to the active site. (Blat 2010)
4. Non-competitive inhibition: The inhibitor does not prevent the substrate from binding to the enzyme, but it reduces the enzyme activity. However, non-competitive inhibitors can bind to the active site as well (in case of enzymes using exosites, multiple substrates or for two-step-binding inhibitors). (Blat 2010) The differences between competitive and non-competitive inhibition can also be illustrated with the influence they have on enzyme kinetics (further elaborated in 2.5.2).

2.4.5 Sorption

During wastewater treatment, the concentration of trace organic compounds can also be lowered in the liquid phase by sorption of the compound onto activated sludge, i.e. biosorption and adsorption on suspended solids (Hyland et al. 2012, Stevens-Garmon et al. 2011, Luo et al. 2014).

Sorption describes the interaction between a compound in solution (“solute”, „sorptive“ or „sorbate“) and a solid phase („sorbent“). The interaction between sorptive and sorbent can be categorized: 1) physical interaction, i.e. dipole interaction that can be amplified by hydrophobicity, 2) chemical interaction involving covalent and hydrogen bonds and 3) electrostatic interactions involving ion-ion and ion-dipole forces. The latter electrostatic forces might be relevant for sorption onto activated sludge, since the surface of the microorganisms is negatively charged. However, no charge state (positive, negative) shows considerably higher sorption on activated sludge than another, since electrostatic interaction is not the solemn interaction mechanisms for charged compounds. (Hyland et al. 2012).

The intensity of interaction between the sorptive and the sorbent depends on the physic-chemical properties of the sorbent (grain size distribution, specific surface area, pH, fraction of organic matter and mineral matter, cation exchange capacity) as well as on properties of the sorptive (polarity and thus octanol-water partitioning, solubility, other features of chemical structure) (Site 2000).

The sorption of organic contaminants is often described with the solid-water distribution coefficient K_p, valid for systems in equilibrium:

$$K_p = \frac{[mp]_{solidphase}}{[mp]_{solution}} \left[\frac{L}{kg_{SS}} \right] \quad (0.1)$$

With [mp] being the concentration of micropollutant.

K_p is also sometimes called K_D. Sorption can also be described with the partition coefficient K_{OC} on sediments:

$$K_{OC} = \frac{K_P}{carboncontent_{sorbent}} \left[\frac{L}{kg_{SS}} \right] \quad (0.2)$$

K_{OW} values are also used to estimate the sorption of a contaminant, since for neutral compounds there are empirical linear correlations between log K_{OC} and log K_{OW} (Stevens-Garmon et al. 2011). This is due to the fact that soil organic matter has a similar function for the organic contaminant like octanol in octanol-water-partitioning (Keeley 1990). It has been shown that this is also true for the sorption of personal care products on activated sludge; the higher the K_{OW}, the more hydrophobic are the substances and the more likely is their partitioning into organic matter and hence onto activated sludge (Hyland et al. 2012). Thus with K_{OC} values from literature and a prediction of the carbon content in the sludge, the amount of pollutant adsorbed onto the sludge could be estimated. This, however disregards the mechanisms that govern the sportive behavior (Keeley 1990) . Neutral compounds with a high K_{ow} can also sorb onto mineral colloids in the activated sludge. In this case, the sludge's carbon content is not relevant (Hyland et al. 2012).

Isotherms describe the relationship between the concentration of a compound in solution [µg/L] and the amount, which is adsorbed onto the slid phase [µg/g]. One empirical model for isotherms, which will be applied later, is the Freundlich model:

$$q = k \cdot C^n \tag{0.3}$$

Here,

q is the equilibrium load of MP on the sorbent, e.g. in [µg/gSS],

n is the Freundlich exponent [-]

k is the Freundlich coefficient with a dimension depending on the Freundlich exponent: $\left[\frac{\mu g}{mg} . \left(\frac{\mu g}{L}\right)^{-n}\right]$

(Heinrich Sontheimer 1980)

2.5 Kinetics

If MPs should be degraded by bacteria, it is interesting to know, how long the bacteria needs to be in contact with the MP, how old the bacteria should be, how much other substrate and nutrients they need and how fast they consume those. Thus, the next paragraph presents laws that govern the pace of biological reactions.

2.5.1 Degradation kinetics

Biological degradation mechanisms of pollutants in heterogeneous matrices are more complex than chemical reactions with a distinct number of educts and products. Yet, kinetic laws can help to describe an empirically observed decrease of concentration in a batch experiment. One has to bear in mind that the kinetic laws do not reveal reaction mechanisms. Furthermore, they are based on empirically found rate constants. They also can hardly be linked to structural properties of the compound.

The progress of a chemical reaction can be described with the rate law. It is a mathematical function linking the turnover rate of a compound (*i)* to the concentration of all species involved in the reaction. The following equation describes the rate in a general way:

$$\frac{d[mp]}{dt} = -k_{biol,mp}[mp]^i[B]^b[C]^c \quad (0.4)$$

Whereas

$\frac{d[mp]}{dt}$	describes the turnover (or disappearing) rate of a micropollutant (mp),
B, C	other wastewater components needed for degradation (e.g. substrate or nutrients for the bacteria)
exponents *i, b, c*	indicate the rate of reaction with respect to substrate components
$k_{biol, mp}$	is the n^{th} order rate constant for biological micropollutant degradation .

The total order n of the reaction is given by the sum of the exponents n = i + b + c.

A first-order-rate law describes the turnover rate of a substance, if this turnover rate is proportional to the substance' current concentration :

$$\frac{d[mp]}{dt} = -k_{biol,mp}[mp] \quad (0.5)$$

$K_{biol, mp}$	is the first-order rate constant [T^{-1}].

(0.5) is a differential equation and can be solved by integrating from $[mp]=[mp]_0$ at $t=t_0$ to $[mp]=[mp]_t$ at time t. Hence the concentration of compound mp can be described at any time with

$$[mp]=[mp]_0 \cdot e\text{^}(-k_{biol,mp}\, t) \quad (0.6)$$

Equation (0.6) implies that plotting the ratio $\frac{[mp]}{[mp]_0}$ on a logarithmic scale yields a straight line through the origin with slope $-k_{biol,mp}$. Thus, k can be determined by linear regression. (Schwarzenbach, Gschwend and Imboden 2005b)

2.5.2 Enzyme kinetics –Michaelis-Menten

Enzymes catalyse reactions (i.e. enhance reaction rates) so that they are compatible with the bacteria's need. (J. M. Berg 2002) Metabolic reactions such as the degradation of nutrients or MPs (termed "substrate" in the following) could be possible examples for such reactions. The substrate (S) forms a complex (ES) with the enzyme (E). This complex either dissembles again or forms a product (P).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E + P \qquad (0.7)$$

Where

E	is the enzyme,
ES	is the enzyme-substrate complex,
P	is the reaction product,
k	are rate constants (k_{-1}, k_{-2}: dissociation and k_1, k_2: formation).

To simplify the kinetics of the enzyme reaction with a metabolized substrate, one can assume that the concentration of a reactant slowly decreases while the concentration of a product slowly increases and the concentration of an intermediate (enzyme-substrate-complex) is constant. The latter assumes that the formation rate of ES equals the dissociation rate. The rate of catalysis V_i describes a reaction velocity and is dependent on the substrate concentration. It can be expressed by using the initial reaction equation (0.7) and simplify with the above named assumption:

$$V_i = V_{\max} \frac{[S]}{[S] + K_M} \qquad (0.8)$$

Where

V_i	is the rate of catalysis at a certain substrate concentration,
[S]	is the substrate concentration,
V_{max}	is the maximal rate, when all the reactive sites of an enzyme are saturated with substrate,

K_M is the Michaelis constant $K_M = \frac{(k_{-1} + k_2)}{k_1}$, a constant describing the substrate-enzyme interaction (J. M. Berg 2002)

Figure 3 provides an illustration of this function (Vi=f([S])) as well as for the influence of inhibitors on the rate of catalysis (compare to 2.4.4).

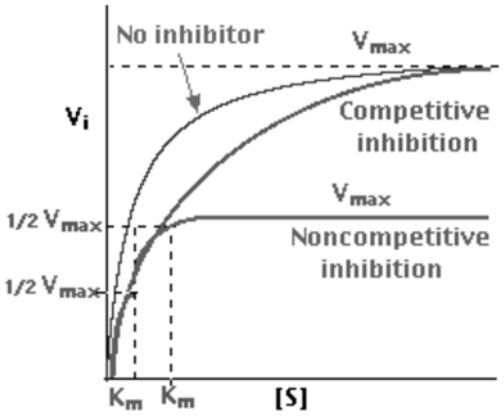


Figure 3 - enzyme kinetics;
The higher the substrate concentration [S], the higher the rate of enzyme catalysis V_i. K_M corresponds to the substrate concentration, where half of the maximal catalysis rate is reached. Competitive inhibitors increase substrate K_M, but do not affect V_{max}. Non-competitive inhibitors decrease V_{max}, but do not affect K_M, i.e. they lower the enzyme activity independently from substrate concentration. Picture from Blat (2010).

If a compound (e.g. a MP) is degraded by non-growing biomass or biomass growing on another substrate (e.g. TOC), Michaelis-Menten is applied. (Brandt 2002)

2.5.3 Substrate kinetics – Monod

Monod kinetics describe the growth of bacteria as a function of substrate concentration. It relates the specific substrate consumption U to the substrate concentration [S] in the following way (Persson 2014):

$$U = U_{\max} \frac{[S]}{[S] + K_S} \tag{0.9}$$

Where

U_{max} is the maximal specific substrate consumption

K_S is the half saturation concentration (constant)

Since microbial growth is related to enzymatic reactions, the equation of Monod kinetics is based on the Michaelis-Menten-Model. Accordingly, the plot of the function looks very similar to the curve in Figure 3, with U corresponding to V and K_S corresponding to K_M. Monod can be understood as a chain of enzymatic reactions with the rate of enzymatic catalysis as a limiting

step. However, the two models are based on different assumptions: Monod assumes that a single substrate is the limiting factor of growth and only one species of bacteria is growing with one singular specific yield. (Brandt 2002)

2.6 Nutrient removal by bacteria

Since the thesis should evaluate the influence of DEP and DEET on bacterial processes, it is important to understand bacterial activities without the influence of the MPs.

2.6.1 Nitrification

Nitrification is performed by autotrophic bacteria and takes two steps; first ammonium is oxidized to nitrite by *Nitrosomonas* (see (0.10)). Thereafter, nitrite is converted to nitrate (see (0.11)) by a group of bacteria known as *Nitrobacter*. (Also other groups of bacteria are capable to nitrify, but their metabolism does not differ significantly from *Nitrosomonas* and *Nitrobacter*.) Most nitrifying bacteria are autotrophic, i.e. they need CO_2 as a carbon source. (M. Henze 2002)

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + H_2O + 2H^+ \tag{0.10}$$

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \tag{0.11}$$

Ammonium oxidation yields a low energy gain, thus nitrifiers are slow growers. Accordingly, in an attached growth process they live on the carriers, since a biofilm allows slow and more specialized bacteria to grow at their pace and undisturbed. (Østerhus 2015) (This is also visible in an experiment in Appendix F, p.95.)

Nitrifying bacteria are affected by certain parameters:

- pH: since the oxidation of 1 mole NH_4^+ consumes 2 moles of HCO_3, alkalinity is affected by the first step of nitrification. In addition, a NH_3 and H_2N can inhibit nitrification. The presence of these species (NH_3/NH_4^+ and HNO_2/NO_2^-) is also pH dependent. Accordingly, a low pH hampers nitrification. Optimal pH lies in the range of 8-9.
- Substrate concentration: growth of nitrifiers depends on how much of their carbon and nutrient source is available. This can be described with Monod kinetics (see (0.9)).
- Oxygen concentration: nitrifiers are more sensitive towards low O_2 concentrations than heterotroph bacteria. This dependency can also be described with Monod kinetics.

- Temperature: nitrifiers are disturbed by sudden changes in temperature. So even if the maximal specific growth rate is achieved at around 25-30^{0}C, the increase in growth rate is not as high as expected, if this temperature is set abruptly. Nitrification is inhibited at over 35^{0}C.
- Inhibiting substances: nitrifiers are not more sensitive towards inhibiting substances such as (heavy) metals than other micro-organisms.

(M. Henze 2002)

2.6.2 Denitrification

Denitrification is a form of anaerobic (bacterial) respiration, in which NO_3^- is used as an electron acceptor. NO_3^- is then reduced to NO_2, NO, N_2O and finally to N_2. N_2 is released into the environment. Figure 4 displays the stepwise reduction of NO_3^- and the necessary organisms and enzymes. (Madigan 2015d)

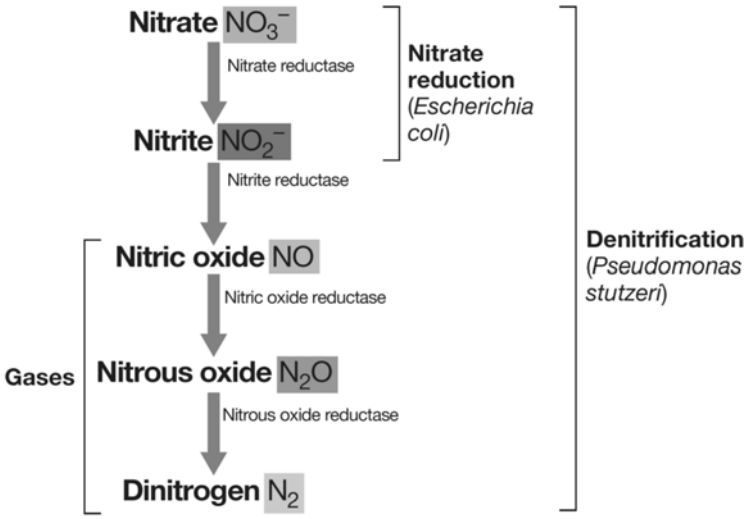


Figure 4 - steps of denitrification
The reduction from NO_3^- to N_2 requires several steps and key enzymes (reductases) as catalyzers.
Some organisms are only capable of the first step. Also other proteobacteria and archaea can perform denitrification.
The intermediate products NO_2^-, NO and N_2O are toxic and undesirable (M. Henze 2002)
Picture from (Madigan 2015d).

Most denitrifiers are chemoorganotrophs that use organic carbon as their carbon source and electron donor. But inorganic materials are also used as energy sources (Henze 2008). Denitrifiers are facultative aerobes, i.e. they prefer O_2 as an e^--acceptor, if it is present. In this case they will not perform nitrate/nitrite reductase (i.e. denitrification will not work) but respire on oxygen. (Madigan 2015c). Compared to aerobic heterotrophic conversion, the yield of denitrification [kg biomass/kg organic matter] is small. The following parameters influence denitrification:

- pH: Denitrifiaction increases alkalinity; for every mole nitrate transformed, one equivalent alkalinity is produced. Optimal pH is between 7 and 9. A low pH influences the end product and leads to increasing formation of nitric oxides.
- Energy source: e.g. methanol yields a high, organic wastewater compounds a medium and endogenous sources a low denitrification rate.
- Oxygen inhibits denitrification.
- The rate increases with increasing temperature according to Arrhenius' law. (Dawson and Murphy 1972) Thermophilic denitrifiers (50-60 °C) have been observed.

(M. Henze 2002)

2.6.3 Enhanced biological P-removal (EBPR)

EBPR is based on the enrichment of bacteria stems (PAO = phosphate accumulating organism) that are able to take up and assimilate inorganic polyphosphate (de-Bashan and Bashan 2004). During anaerobic conditions these bacteria release phosphate, during aerobic conditions they take up more phosphate than they released – thus, EBPR requires alternating incubation conditions, e.g. by a sequence of reactors. (Wilén 2014a).

In the carbon-rich anaerobic incubation, easily biodegradable carbon (e.g. acetate or other volatile fatty acids = VFA) is converted to poly-hydroxy alkanoates, for instance poly-β-butyrate (PHB) with the help of glycogen. PHB is stored in the cell. The required energy for this comes partly from the hydrolysis of intracellular polyphosphate (poly-P) to soluble phosphate ions (PO_4^{3-}). PO_4^{3-} is then released. (Smolders et al. 1994)

In the aerobic phase, energy from PHA degradation to CO_2 is used to take up phosphate. Together with magnesium, potassium and other positively charged ions, the bacteria builds up poly-P as well as cell mass. Poly-P contains high energy bonds and thus helps to store excess energy from the heterotrophic oxidation of COD/BOD. This so-called "luxury up-take" results in a higher uptake during the aerobic phase compared to what was released in the anaerobic phase. (Moore 2010)

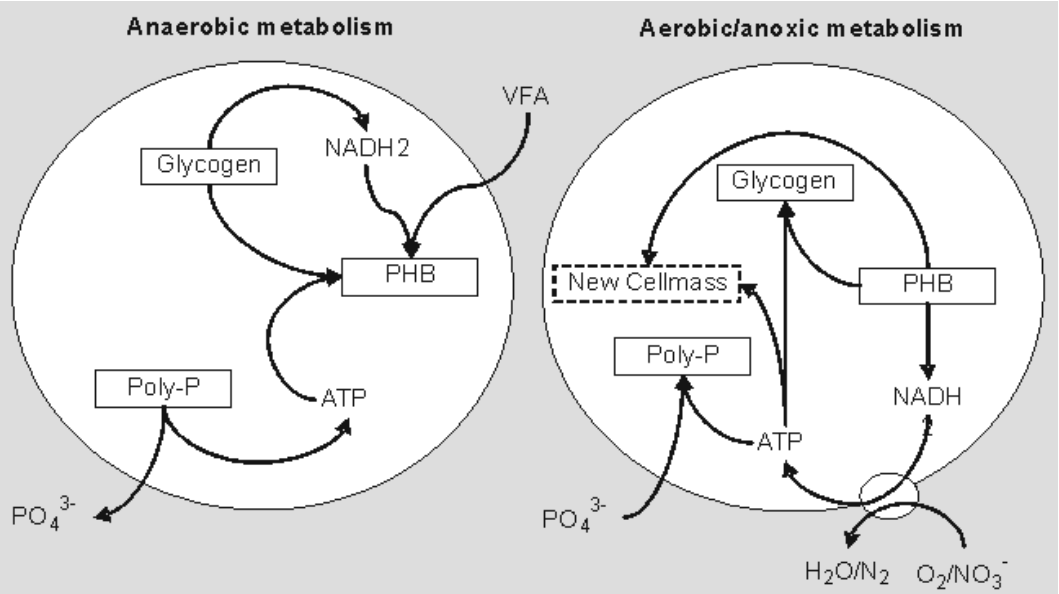


Figure 5 - PAO metabolism under anaerobic and anoxic/aerobic conditions.
In anaerobic conditions, VFA is converted to PHB. The degradation of poly-P to PO_4^{3-} delivers energy. PO_4^{3-} is released. In aerobic conditions, stored PHB is metabolized with the help of O_2 and the e^--transport chain. Cell mass is built up. Energy is stored by the up-take of PO_4^{3-} and its conversion into high energy poly-P. (Moore 2010) (picture based on Smolders et al. 1994)

2.7 Mechanisms for DEET removal

Up to here, the theoretical background tackles removal of compounds in general and introduces general microbial activities and their governing laws. The following section, in contrast, deals specifically with degradation pathways and removal of DEET.

2.7.1 Abiotic degradation

DEET is stable to hydrolysis at environmental pH (Winter 2005).

It undergoes photooxidation and forms various transformation products in natural aquatic environments. The reported half-life (MQ water, 20°, UV radiation) ranges from 6 to 13 minutes. (Benitez et al. 2013) DEET is either degraded by direct or by indirect photolysis. In indirect photolysis, other natural species absorb radiation and form radicals which transform DEET. Indirect photolysis can also trigger a number of reactions: dealkylation, mono- and poly-hydroxylation, oxidation of the hydroxyl groups and cleavage of the alkyl chains. It can also lead to a combination of biotic and abiotic degradation involving organic matter, nitrite and nitrate ions, H_2O_2 and iron species. (Calza et al. 2011)

2.7.2 Biotic degradation

DEET can be degraded by an aerobic mixed culture of for instance *Pseudomonas putida* (but also other organisms) via enzymatic dealkylation and enzymatic oxidation. Figure 6 shows the detailed degradation pathway. DEET serves the bacteria as a carbon source. (Ellis, Roe and Wackett 2006) *Arthrobacter* and *Pseudomonas* can utilize DEET as a nitrogen source (Kagle et al. 2009). Not only bacteria, but also fungi can degrade DEET: the compound can serve as an electron donor for the fungal oxireductase enzyme laccase. Adding this enzyme and redox mediators, the DEET concentration in wastewater could be halved. (Tran et al. 2013a). The degradation of DEET is not influenced by the presence of nitrifying bacteria (Rattier et al. 2014). Though these studies suggest a metabolic capacity of DEET degradation, in engineered environments it remains unclear, how DEET is actually degraded. (Kagle et al. 2009)

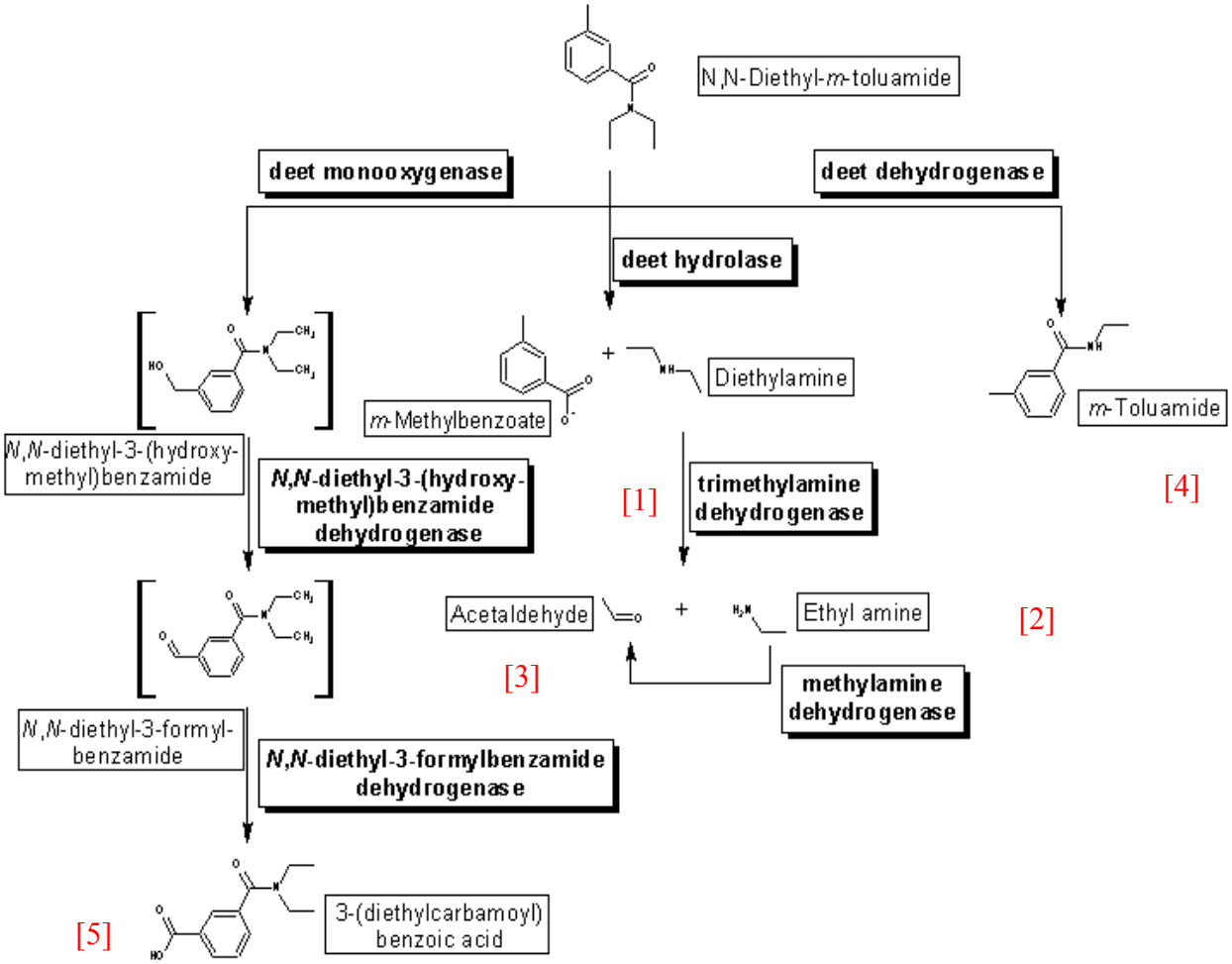


Figure 6 - biotic degradation: microbial degradation pathway DEET;
Enzyme catalyzed hydrolysis of the amide bond produces 3-methylbenzoate and diethylamine (1), 3-methylbenzoate is further metabolized through the *meta* cleavage pathway into ethylamine (2), which is then hydrolyzed to acetaldehyde before it enters the central metabolism (3). The enzymatic removal of the alkyl group is shown on the right side (4), the oxidation on the left (5). (Huebert 2014)

2.7.3 Microbial degradation vs. sorption

DEET also sorbs on solids in the sludge (Hyland et al. 2012). How much sorption could be expected is dependent on the suspended solid concentration in the reactor and on the amount of carbon in the sludge. It can then be estimated using eq. 0.4 and 0.5 (section 2.4, page 8). Log K_P values found for DEET in activated sludge range from 30 to 100 [l/kg_{SS}]. (Stevens-Garmon et al. 2011)

2.8 Mechanisms for DEP removal

This section describes specific removal mechanisms of DEP. A table with selected studies about removal efficiencies (biotic and abiotic) is given at the end of this chapter.

2.8.1 Abiotic degradation

DEP is susceptible to *hydrolysis*, forming an acid and an alcohol. It undergoes a hydrolytic step producing first a mono- ester and an alcohol moiety and afterwards a phthalic acid and a second alcohol. (Huang et al. 2013) The hydrolysis rate of DEP, however, is negligible and results in an estimated aquatic half-life of 8.8 years. (Stales et al. 1997)

Aqueous *photolysis* of DEP occurs by absorption of UV radiation in the region of 200-400nm. Energy high UV waves can either be directly absorbed by the DEP and break covalent bonds or UV radiation is absorbed by water, where it forms radicals that then react with the phthalate ester. However, aqueous photooxidation is slow (estimated half-life range from 2.4 to 12 years) and thus not considered a major pathway in the aquatic environment. In the atmosphere, in contrast, photodegradation plays a vital role. (Stales et al. 1997)

DEP might also *volatilize* in an aerated sludge basin due to its high vapor pressure. (Dargnat et al. 2009)

2.8.2 Biotic degradation

Microbes from diverse habitats including gram-positive, gram-negative bacteria as well as actinomycetes are capable of degrading DEP under aerobic and anaerobic conditions. DEP serves the microbes as a source of energy or a carbon source. (Cartwright et al. 2000) There are two different possible degradation pathways:

The degradation of DEP (aerobic as well as anaerobic) can start with the formation of a monoester and alcohol. Under aerobic conditions the monoester is enzymatically degraded to phthalic acid and from there by mono decarboxylation to procatechuate (Mohan et al. 2006). Procatechuate is then transformed by a ring cleavage either in pyruvate and oxaloacetate or into acetyl CoA and succinate (C. Vamsee-Krishna 2008) (see Figure 7, steps 2. and 3.). The latter can be used for microbial anabolism. (Stales et al. 1997) Following secondary biodegradation can then result in mineralization by a number of pathways. (Cartwright et al. 2000) Under anaerobic conditions, phthalic acid is formed under consumption of ATP and CoA and is transformed by removal of double bonds into acetate. (Stales et al. 1997)

In soil co-contaminated with methanol, Cartwright et al. (2000) suggest a second biodegradation pathway: A sequential hydrolysis of the C-O bonds is followed by transesterification (Figure 7 step 1a), which finally forms the toxic metabolites ethyl methyl phthalate and mono methyl phthalate. Amir et al. (2005) report this pathway as a major degradation sequence for DEP during the composting of activated sludge. They also suggest de-esterification (step 1a) as an alternative route. From phtalic acid, this pathway could also lead to formation of protocatechuic acid and a ring cleavage (steps 2. and 3. Figure 7) (Mohan et al. 2006, Amir et al. 2005).

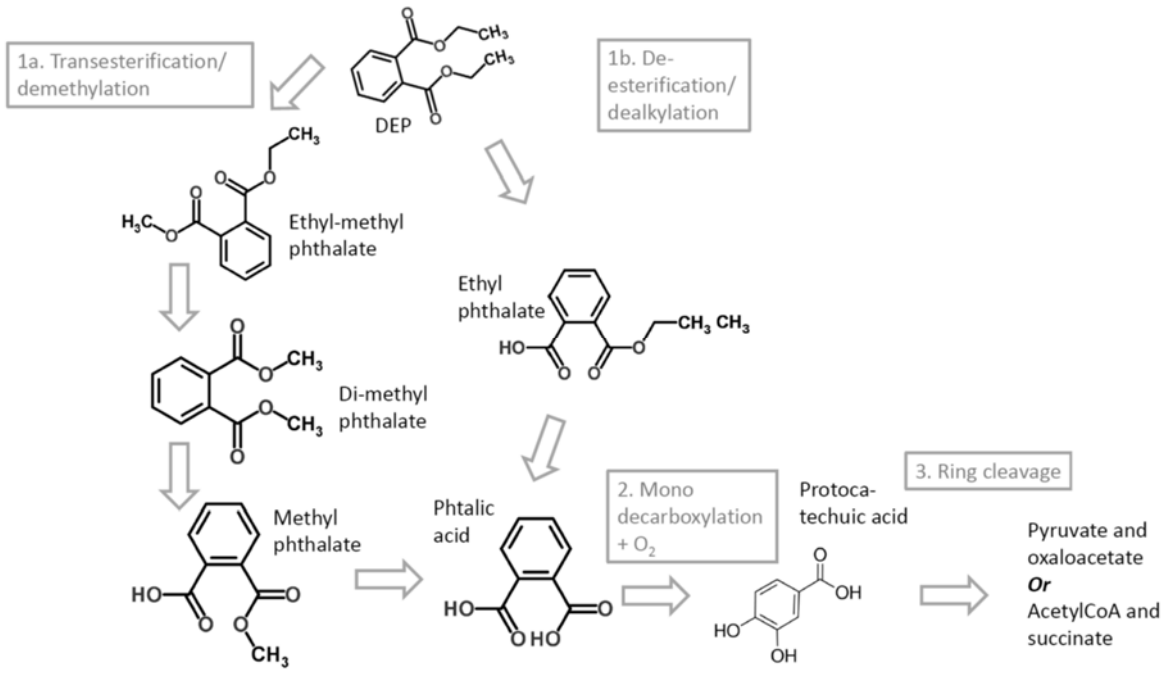


Figure 7 - One possible degradation pathway DEP
It starts with an alkyl side chain reaction, which leads to the formation of phtalic acid and finally to a cleavage of the aromatic ring. Steps 1a and 1b are suggested among other by Amir et al. 2005, steps 2 and 3 by Mohan et al 2006, Stales et al 1997, Cartwright et al. 2000.

Generally, biological growth that uses DEP as a singular substrate, is suboptimal and results in a low cell density, a slow biodegradation rate and overall limited biodegradation. Adding another carbon and energy source in form of glucose, succinate or citrate increases the biodegradation of DEP about ten times. Biodegradation of DEP can be enhanced furthermore with the use of another organic compound like yeast as an auxiliary nutrient and mineral source. This leads to co-metabolism and high cell growth. (Navacharoen and Vangnai 2011)

Measurements of removal efficiencies in wastewater treatment plants (WWTP) are summarized in Table 1.

Table 2 - removal efficiencies for DEP

Abiotic			
Applied technology	**removal efficiency**	**Observed mechanism**	**Kinetics**
UV/H_2O_2 UV: 133.9 µW/cm^2 (60min), H_2O_2 : 20 mg/L (Xu et al. 2007)	98.6 % Initial conc.: 1mg/L	Photolytic degradation: cracking of aliphatic chain followed by opening of aromatic ring by OH• radicals	Pseudo-first order kinetics, k linearly connected to UV and H_2O_2 concentrations
O_3/activated carbon (AC) coupling (de Oliveira et al. 2011a, de Oliveira et al. 2011b)	Complete removal (mineralization) Initial conc.: 0.2g/L	Degradation by radical reaction promoted by deprotonated acid groups on AC surface	Pseudo-first order, rate constants strongly dependent on pH
Biotic			
WWTP: Primary treatment, biological treatment including nitrification (Dargnat et al. 2009)	Primary clarifier 58.9%, aeration basin 34.3%, Initial conc.: 1.6 – 25 µg/L	Sorption on suspended matter, biodegradation	
Comparison between chemical, mechanical and biological WWTP (Vogelsang et al. 2006)	>80% - 90% Initial conc.: 2.8-4.9 µg/L	Biological degradation. no removal in chemical/mechanical treatment plant	

2.8.3 Microbial degradation vs. sorption

Fang and Zheng (2004) evaluated, whether the removal of DEP during wastewater treatment was due to biological degradation or sorption to either the biomass or the extracellular polymeric substances (EPS). EPS is a product of cell excretion, lysis or external organic matter. It contributes with 80% to the mass of activated sludge. It is not only a protective layer around the cells against environmental influences, but can also serve as a carbon source, when substrate

concentration is low. (Wilén 2014b, Yu Tian 2006) Fang and Zheng (2004) conclude that activated sludge and EPS are strong adsorbents for DEP due to hydrophobic interactions. The adsorption can be modeled by Freundlich and Langmuir isotherms. This implies adsorption is dependent on the initial concentration, but a maximal adsorption capacity for sludge (0.73 mg DEP/g sludge) and EPS (14.3 mg DEP/g EPS) could be found. Also other studies show that DEP is adsorbed by sediments particles such as clay (Wu et al. 2015).

3 Materials and Methods

3.1 Chemicals

DEP (99% purity) was provided by Alfa Aesar (U.S.). Two standards (1 000 000µg/L in methanol and 100 000 µg/L in MilliQ) were created in November 2014 and used until April 2015. After that, both standards were renewed.

DEET (97% purity) was provided by Aldrich Chemistry (Germany). Two standards (1 000 000 µg/L in methanol and 10 000 µg/L in MilliQ) were made in November 2014 and used throughout the experiments (until May 2015).

For both chemicals, standards were kept in transparent, white flasks in the fridge. Plastic lids were avoided, if possible.

3.2 Sludge

The sludge was taken from a pilot scale integrated fixed-film activated sludge system (IFAS) for enhanced biological nutrient removal (EBNR) from grey water. It is operated as a cascade of membrane bio-reactors (MBR). Originally, the activated sludge in the IFAS-EBNR_MBR came from an enhanced activated sludge plant with biological P-removal in Helsingborg, Sweden (Öresundsverket).

In order to characterize the sludge better, the operation of the pilot plant is described in this section. More details are provided with the help of a flow scheme in 0, p.83.

A mixture of 1L grey water concentrate and 199L tap water enters the pilot plant in a anaerobic reactor with a flow rate of Q_{in}=3.8 L/h. The anaerobic reactor is followed by an anoxic and an aerobic tank. The return flow rate (anoxic to anaerobic) was 4.8 L/h. The nitrate return (aerobic to anoxic) was 19.8 L/h. All three tanks were filled with K1 medium. The sludge age in the system is 20 days. The excess sludge flow rate is 0.09 L/h (i.e. 4.5L/48h cycle).

Table 3 - conditions in the three tanks of the pilot plant

	Anaerobic	Anoxic	Aerobic
Volumes [L]	12.74	6.83	25.38
HRT [h]	1.48	0.36	1.08
DO [mg/L]	0.004±0.002	0.007±0.003	5.4±0.5
SS [gSS/L]	~3	~5	~6
Total carrier surface [m^2]	-	1.75	4.9
Processes	P-release TOC removal	Denitrification/ P-release/ TOC removal	Nitrification/ P-uptake

3.3 Grey water

The synthetic grey water contains commercial personal care products, household products, potassium monohydrogen phosphate, proteins from meat, urea, full milk, acetate and hydrochloric acid. The personal care products and the household products contain more than 200 different organic compounds. Among these compounds are also biozides and tensides. The chemical parameters are listed in Table 4.

Table 4 - chemical properties raw grey water

pH	**7.6**	**EC [µS/cm]**	**338**
Alk [meq/L]	2.0	SS [mg/L]	62
Turbidity [NTU]	80	VSS [mg/L]	31
COD [mg/L]	517	FCOD [mg/L]	400
BOD_5 [mg/L]	264	TOT-P [mg/L]	5.67
TOT-N [mg/L]	20	NH_4-N [mg/L]	1.38
NO_3-N [mg/L]	< 0.32	Non-ion. Sur. [mg/L]	3.78
Anion. Sur. [mg/L]	65	**COD : N : P**	**100 : 3.5 : 1.1**

Note for NO_3-N and NH_4-N that for some phases of the experiment nitrate and ammonia was added to the shown concentration.

The toxicity of the grey water on aquatic organisms was tested in the framework of another project with a whole effluent toxicity (WET) test, which measures the overall impact of a wastewater effluent on organisms. If the concentration of grey water exceeds 12.5% in an effluent, the bacteria *vibrio fisheri* exposed to that effluent reduces 80% of its luminescence.

3.4 Set-up batch experiments

The first parts of this section (3.4.1 and 3.4.3) are dedicated to experiments in which the removal of MP from the beakers is examined. The other parts (3.4.4 and 3.4.5) describe experiments carried out to determine the effect of DEP on microbial activity.

3.4.1 Aeration

For the batch experiments, 6 beakers have been filled with 1/3 raw grey water and 2/3 aerobic sludge. They were spiked with DEET and DEP and varying combinations:

1. beaker 1: blank
2. beaker 2: 10 µg/L DEP and 2.5 µg/L DEET
3. beaker 3: 10 µg/L DEP and 10 µg/L DEET
4. beaker 4: 50 µg/L DEP and 5 µg/L DEET
5. beaker 5: 100 µg/L DEP and 2.5 µg/L DEET
6. beaker 6: 100 µg/L DEP and 10 µg/L DEET

The beakers were aerated by ceramic diffusors. The air flow coming directly from the compressor was controlled with a reducer at the aeration unit and rotameters in front of every beaker (see Figure 8) In this way, the air inflow into every beaker was kept constant at 200 L/h. The air concentration in the beakers was measured throughout the experiments (ranging from 6-8 mg/L), so was the pH (ranging from 8.2 to 9.1). The beakers were spiked and put on aeration with a time laps. In this way, the sludge could be collected right before the start of the aeration. The aeration was kept on for 3.5 hours (1st batch experiment) and for 6 hours (2nd batch experiment). These times were selected accordingly to the hydraulic retention time in the treatment system the experiments should be used for. Another reason for the time chosen was that 3.5 h are relevant for removal of organic compounds; 6 h are expected to be sufficient for nitrification.

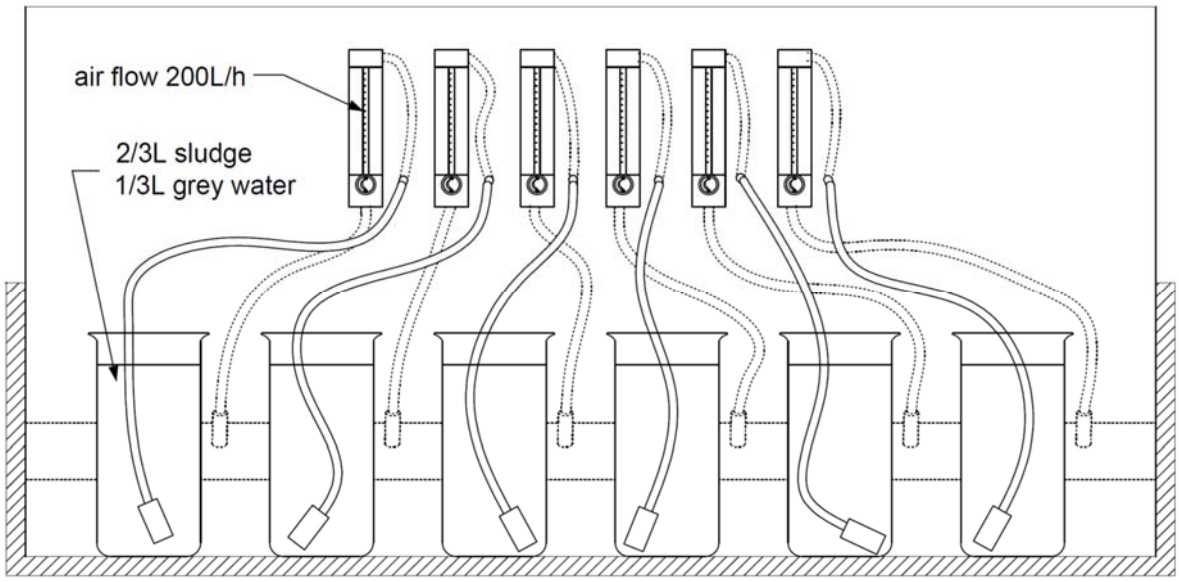


Figure 8 - sketch aeration unit
The rotameters are fixed onto a Plexiglas pane. Behind the Plexiglas, the global air supply is visible (dotted lines). Six different combinations of concentrations DEET (2.5µg/L, 5µg/L, 10µg/L) and DEP (10µg/L, 50µg/L, 100µg/L) were spiked. The beakers were switched on with a time lapse, which allowed for sample preparation after the end of the experiment (drawing by courtesy of Aleksandra Migowska).

After 3.5 h (6 h respectively) the content of the beaker was collected in 4 x 250mL centrifuge tubes and centrifuged with 13 000 rpm for 3 minutes. The supernatant was then filtered through a glass filter (GF/C with 0.7 µm pore size) and collected. Samples for TOC, SS and VSS measurements were taken. TOC samples were acidified with 2 drops H_3PO_4 and analysed after maximal four days of storage (4 °C). They were measured with a TOC-fusion system "Teledyne" (provided by "Tekmar"). The 600 mL of the filtered supernatant was and stored in the fridge for 14h, until the SPE was carried out. Details of the sample preparation are provided in 3.5, p. 34.

3.4.2 Evaporation

6 beakers filled with MilliQ were spiked with the following combination of concentrations:

1. beaker 1&2: 10µg/L DEP and 2.5 µg/L DEET
2. beaker 3&4: 50 µg/L DEP and 5 µg/L DEET
3. beaker 5&6: 100 µg/L DEP and 10 µg/L DEET

The beakers were aerated with a flow rate of 200 L/h for 6 hours. A 600mL sample was taken from each beaker and up-concentrated with the SPE. (Centrifuging and filtering was unnecessary.) HPLC analysis was performed with the up-concentrated samples.

3.4.3 Adsorption

In order to determine the amount of MP removed by adsorption, three different experiments were conducted; in one set-up, activated sludge was spiked with MP and immediately afterwards centrifuged (1). In two other set-ups, activated sludge was inhibited (thermally and with a biocide) (2).

Immediate adsorption (1): For a first estimate of adsorption of the MP onto sludge, 6 volumetric flasks (50mL) were spiked with 5000µg/L DEET and 20 000 µg/L DEP. Two blank samples contained only activated sludge. Right after spiking, the flasks' content was centrifuged and analysed with the HPLC (see Sample analysis). Up-concentration with the SPE was not necessary, due to the high MP concentration.

Inactivation (2): It has been shown that thermal inactivation techniques alter sludge adsorption capacities and rheological properties. Thus, they are not suitable to ultimately determine the role of sorption for the removal of MP from mixed liquor. (Hamon, Villain and Marrot 2014) Accordingly, the set-up and results of the thermal inactivation are presented in Appendix C, p. 88. Based on Hamon et al. (2014), mercury chloride ($HgCl_2$) and the procedure described in the following has been used to inactivate bacteria chemically: The sludge was exposed to 30 mg/gSS $HgCl_2$, i.e. in this case 120 mg/L for 2/3L of sludge. (The SS content of the aerobic sludge from the pilot is around 6 mg/L.) $HgCl_2$ was dosed 2h before mixing with 1/3L grey water, because of the necessary reaction time of the biocide. The grey water was spiked with 100 µg/L DEP and 10 µg/L DEET. The high concentrations and the high retention time were chosen in order to quantify the maximal adsorption per gSS, so that a minimum biodegradation can be quantified. In order to prevent toxic aerosols, the beakers were not aerated, but put on a shaker in closed bottles for 6h. Since no degradation processes were expected to take place, O_2 was not necessary and hence this variation (shaking instead of aeration) in the set-up was valid. Three replicates were produced.

The following paragraph motivates the choice of the inactivation method: The methods are compared in Table 5. Here it can be seen that mercury is not completely surpressing microbial activity. Microbial activity was determined by measuring ATP with a cell visibility assay (BacTiter-Glo by "Promega"). The inactivation by pasteurization is higher than from mercury. However, according to the staff engineer and Oexle, Gnaiger and Weiss (1999), ATP results are influenced by the iron background in the water used to perform the analysis. Furthermore,

high ATP results might also be due to a long storage time (up to 3 days at 4°C). Apart from this, after pasteurization, the viscosity of the sample was visibly higher, resulting in problems for decantation and suggests that the cells were lysing. That means pasteurization alternates also the adsorption capacity of the sludge used in this study, coherently to what Hamon et al. (2014) showed. Hence, the biocide $HgCl_2$ was used for inactivation, although the ATP levels measured after pasteurization were lower than after the biocide dosage.

Table 5 - comparison of activation methods
The standard deviations (STD) for no inactivation and the pasteurization are derived from the different results depending on different dilutions (i.e. method inherent STD). The STD for the $HgCl_2$ inactivation is calculated based on the ATP result of 6 different beakers. (Also, the ATP results of each beaker varies depending on the dilution, but these variations are minor compared to the differences in results from different beakers.)

method	Cellular ATPafter inactivation [nmol/L]	STD of cellular ATP
No inactivation	4176	606
Pasteurization	3.661	2.6
30 mg/gSS $HgCl_2$	174	101

3.4.4 Oxygen uptake rate (OUR) experiments combined with ATP measurement

In order to estimate the impact of DEP on the bacterial activity, OUR (aside of TOC removal) was measured. Two experiments were conducted; in the first one, 0, 100, 10 000 µg/L DEP were spiked into 0.67L aerobic sludge and 0.33L grey water. The beakers were aerated. OUR was measured five seconds and five minutes after dosing grey water and DEP, and after the beakers had been aerated for 30 minutes.

The OUR measurement itself has been conducted in compliance with the ISO standard 8192 for inhibition of oxygen consumption (ISO 2010); 150mL of mixed liquor was poured into an Erlenmeyer flask. A dissolved oxygen meter provided by WTW (model Oxi 3310) was submerged. The orifice of the flasks around the electrode was sealed with parafilm. The meter had been logged in advance to measure and record dissolved oxygen concentrations in the flasks in 5 seconds intervals. To calculate the OUR [mg/L/h], the values in the range of 7 mg/L and 2 mg/L and their respective times were used, as suggested in the ISO 8192. This corresponded to the linear sections on the plots.

The specific oxygen uptake rate (SOUR) has been calculated by dividing OUR by the SS content [g/L] of the beaker. This is in accordance with the US. EPA Method 1683 (U.S.EPA 2001). TOC samples were taken simultaneously with the OUR, centrifuged, acidified, stored at 4 °C and measured within 2 days. ATP samples were taken from the beaker before grey water and MP was added and after 30 minutes aeration.

3.4.5 Full cycle batch experiment (two variations and pre-testing)

In order to mimic the conditions of the pilot plant (where the sludge comes from), two beakers - one blank, one spiked (100 µg DEP/L) were ran with anaerobic, anoxic and aerobic conditions. They both contained activated 1.33L activated sludge and 0.67L grey water. 1 L k1 media from the aerobic tank of the pilot plant was added in the aerobic phase. The media has the following properties:

- 9.1mm diameter, 7.1mm length
- 500m^2/m^3 bulk
- 335m^2/m^3 (67% filling)

Throughout the three conditions, the beakers were stirred with magnet stirrers at 200 rpm. The time schedule of changing conditions and taking samples can be seen in Figure 9. For anoxic condition, sodium nitrate has been dissolved in MQ and added after two hours. Ammonium was added in the form of dissolved ammonium chloride after 3.5 hours.

In order to test the set-up and see, whether the same processes (nitrification, denitrification) are taking place on bench scale than on pilot scale, pre-tests were conducted to determine the necessary nitrate and ammonium dose and the influence of the k1 medium on nitrification. Instead of 10 mg/L nitrate and 8 mg/L ammonium, 1 mg/L nitrate and 0.8 mg/L ammonium were dosed. (The result of this pre-test showing that medium is needed for nitrification, is presented in Appendix F, p.95)

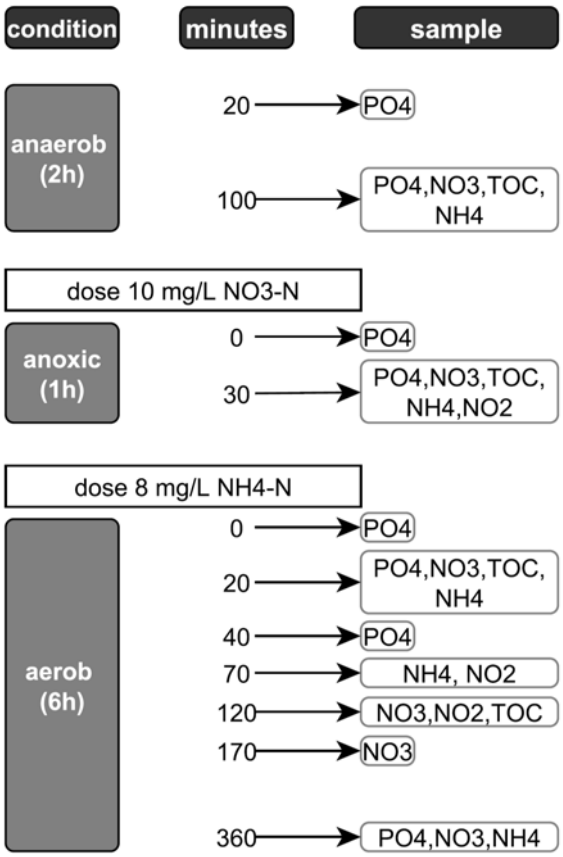


Figure 9 - batch experiments to mimic full cycle (pilot plant)
Two beakers (one blank and one spiked with 100 µg/L DEP) were put on a magnet stirrer with a time laps. The mixed liquor contained 1,33L aerobic activated sludge from the pilot plant and 0,67L grey water. The aerobic sludge was stirred for 20 minutes before the start of the experiment (i.e. before spiking and adding grey water) to establish anaerobic conditions. By dosing nitrate, anoxic conditions were established after 1h. By dosing ammonium and aerating with 200 L/h air, aerobic conditions for denitrification were established. After the depicted times, samples were taken for the parameters shown on the right side. For the PO4, NO3 and TOC sampling, 100mL of the mixed liquor was centrifuged with 130 000 rpm for four minutes and then filtered over 0.7µm filters.
K1 medium from the aerobic tank of the pilot plant has been transferred to the beaker at the beginning of the aerobic phase (i.e. after 3h). Selected TOC samples were later analysed with size exclusion chromatography (LC-OCD).
NO3-N and NH4¤-N doses varied; there were also experiments with 1 mg/L NO_3-N dosed after 2h and 0.8 mg/L NH4¤-N dosed after 3h.

3.5 Micropollutant sample preparation

In order to measure the low concentration of DEET and DEP after the aeration test and the full cycle tests, the samples have to be freed from interferences by the GW matrix and up-concentrated 200 times (DEP) and 500 times (DEET) with a reverse phase SPE unit followed by sample evaporation. Figure 10 gives an overview of the necessary steps of sample preparation.

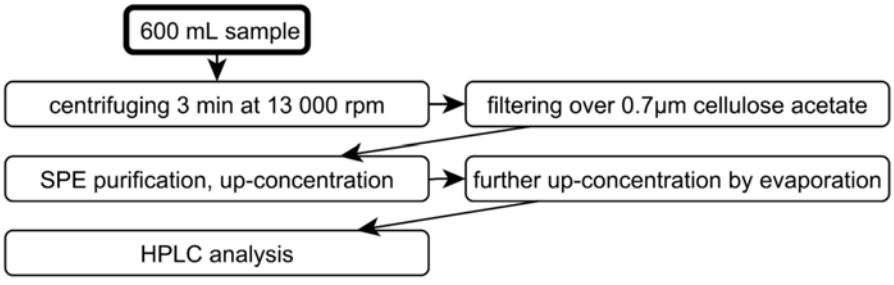


Figure 10 - overview sample preparation

The glassware was prepared by washing with MilliQ, ethanol, in an ultrasound bath and an acid bath. Plastic vessels and lids were avoided if it was possible. Since the development of the SPE

method was subject of a preparatory project and thus part of the thesis, SPE theory and an evaluation of the accuracy of the method is given in 0, p.84)

3.5.1 SPE method procedure

The SPE unit "Visiprep DL" was supplied by Supelco. The pump ("Laboport" N810.3 FT18 by KNF Neuberger GmbH) is connected to the SPE via a vacuum pump trap kid (Supelco). The used cartridges ("Bond Elut C-18") are produced by "Agilent". They have a capacity of 6mL and contain a hydrophobic silica based sorbent as a solid phase (500mg). The pump connected to the vacuum manifold bottom was operated in a way that keeps the vacuum in the SPE chamber from 0 to -20 kPa. The cartridges were conditioned, washed and eluted one by one by controlling the flow rate with the flow control valve on the manifold lid (individually for every cartridge). During sampling, the individual flow controls of the cartridge were fully opened and the flow rate was regulated globally via the vacuum bleed valve. Washing and sampling solution was discharged after passing through the cartridge. The elution was captured in glass tubes. The tubes were placed under the cartridges in a collection rag inside the vacuum manifold glass chamber. Due to a lack of alternatives, the 3mL and 1mL marks on the collection tubes had to be drawn manually prior to the collection of the elution. For elution, 3mL acetonitril (acn) was loaded onto the cartridge. The used solvents, concentrations and volumes are summarized in Table 6.

Table 6 - SPE: Summary of solvents, concentrations and volumes.
Used cartridges: Agilent, 6ml capacity, 1mL bed volume. The flow rate describes the flow through the cartridges. (Explanation of the 4 steps can be found in appendix 0, p. 83)

Step	Purpose	Solvents	Flow rate	volumes
1) Conditioning	Removal of impurities, activation of cartridge	Acetonitril (acn) MeOH MilliQ (MQ)	1-2 drop per second	5mL 10mL 10mL
2) Sampling	Bringing the analyte to the solid phase	[-] spiked GW sample	2 drops per second	600ml
3) Washing	Removal of undesired matrix compounds	20% MeOH, 80% MQ	1 drop per second	6mL
4) Elution	Detaching analyte, 200x up-concentration	Can	0.5 – 1 drop per second	3mL

3.5.2 Further up-concentration by evaporation

The SPE achieves an up-concentration of 200 times, which is sufficient for DEP. In order to up-concentrate DEET from the low relevant concentration to the high concentration needed for

HPLC measurement, a 500 times up-concentration was found to be necessary. This was achieved by evaporating the acn of the elution solution (see Figure 11).

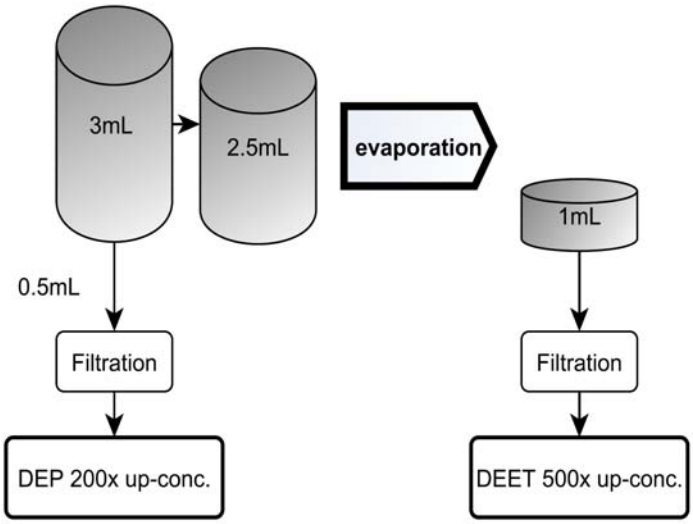


Figure 11 - Evaporation procedure:
DEP sample taken out of the eluted analyte before evaporation, DEET afterwards. Hence, for DEP with the method, a 600mL/3mL=200 fold up-concentration can be achieved. For DEET a 600mL/3mL X 2.5 = 500 fold up-concentration could be achieved.

Since DEP evaporates easily, first 0.5mL of the 3mL analyte elution was taken out and filtered through a syringe filter ("Acrodisc" LC 13mm, 0.45µm PVDF membrane, provided by Pall Life Science) into an HPLC vial. Then the collection tubes are placed back into the rag and into the vacuum chamber of the SPE. With the pump connected to the SPE, air was pumped through the lids (without cartridges on top) into the tubes containing the elution. The elution was evaporated down to 1mL (see Figure 11) before filtering with a syringe filter into a HPLC vial.

3.6 Sample analysis

3.6.1 Standard parameters

The following cuvettes provided by Hach Lange were used:

- LCK339 for nitrate
- LCK348 for phosphate
- LCK 341 for nitrite
- LCK 304 for ammonium.

3.6.2 Micropollutants

An HPLC system by "Agilent" 1200 series with a C-18 column ("Eclipse XDB", size: 4.6 x 150mm, solid phase particle diameter: 5µ) with a UV detector phase was used. The methods for the two compounds differ slightly with respect to flow rate of the liquid phase, gradient of the solvents and injection volume of the sample. Details of the method are displayed in Table 7.

Table 7 - HPLC method for DEP and DEET analysis

	DEET	DEP
Flow rate liquid phase	0.5 - 1 mL/min	1 mL/min
Solvent	35% MQ, 65% acn	50% MQ, 50% can
Wavelength of signal	226 nm	221 nm
Time of peak	7 min	4.3 min
Injection volume sample	25µL	
Pressure	300 bar	
Temperature	25°C	
Lamp	UV	
Width of slit (B_W)	4 nm	

The solvents are pumped through the column with a gradient. This gradient is depicted in Figure 12.

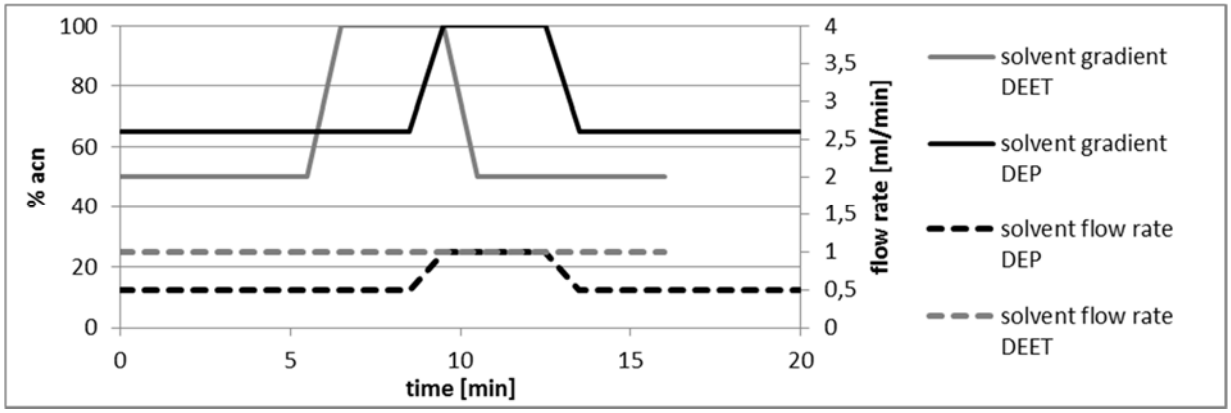


Figure 12 - HPLC method:
solvent gradients and solvent flow rates for measuring DEET and DEP. The other component of the solvent is MQ.

For the HPLC, 2mL vials (by Agilent) were used. Due to cost cuts, those vials, as well as their caps and Teflon lids had to be cleaned and re-used (though they are meant for single use). The washing procedure consisted of flushing the vials more than three times with MilliQ, exposing them to ultra-sound (in an ethanol-MilliQ bath) for 25 minutes and finally rinsing them with MilliQ again.

In order to use the above described methods for quantification, calibration curves had to be determined. The standards used to derive the calibration curve were created by spiking 600mL recycled grey water (filtered over 0.45µm) with known concentrations of MP. They were up-concentrated and measured with the HPLC in the above described way. The parameters describing the method accuracy are summarized in Table 14.

Table 8 - evaluation of method limit of quantification (LOQ), limit of detection (LOD), STD (10 samples).

	LOD [mAu]	LOD [µg/L]	LOQ [mAu]	LOQ [µg/L]	Recov.[%]	R^2 calibration curve
DEET	14.79	0.26	49.29	0.795	72.5	0.9988
DEP	9.22	1.01	30.72	2.12	103	0.9994

The derivation of the limit of quantification and limit of detection as well as the calibration curves are displayed in 0, p. 84.

3.6.3 Size exclusion chromatography (LC-OCD)

For a more detailed analysis of the different TOC fractions, a combination of liquid chromatography and organic carbon detector (LC-OCD Model 8, provided by "DOC Labor Huber", Karlsruhe, Germany). As a liquid chromatography, the instrument uses a separate HPLC column. For oxidation of the carbon to CO_2 the HPLC is followed by a Graentzel thin film reactor. An infra-red detector quantifies the amount of CO_2 thereafter. For a detailed list of column properties, pumps and other system components, see the provider's specification (Huber 2015). The analysis of data has been carried out by the software ChromCalc (by DOC Labor Huber). For integration borders of the chromatograms, standard settings of the software were used.

4 Results and discussion

This chapter is structured in the following way; first, an overview is presented about how much MP is removed from the beaker (4.1). Then, experiments are presented that evaluate the contribution of evaporation (0) and adsorption (4.3) to the removal. Those results are discussed immediately, since they tackle only one mechanism and thus can stand on their own. In the light of these results, total MP removal can be discussed in 4.4, since by then it will be clear by which pathways the MPs are removed.

In the second part of the chapter, the influence of MP on the performance of the bacteria will be presented and discussed.

4.1 Total MP removal results

This chapter presents the total removal of the two compounds from the beakers after 3.5h and 6h. While reviewing these results, one has to bear in mind that the overall removal of the MP is due to several mechanisms (see 2.4). Accordingly, the section 4.1 does not include a discussion part, since a discussion of total removal has to take into account all possible removal mechanisms.

4.1.1 Results total DEET removal

As shown in Figure 13, the percentage removal of DEET ranges from 11.7 to 29.4 %. Removal after 6h is slightly higher for all concentrations than removal after 3.5h with the exception of beaker 3 (second bar: 10µg/L DEP 10 µg/L DEET). It is highest for low DEET concentrations and lowest for high DEET concentration. For 3.5h retention time, lowest average percentage removal is achieved with a high DEET and a high DEP concentration. For 6h retention time, lowest average DEET removal is obtained in the beaker with a low DEP and a low DEET concentration. After 6h, beakers with the same DEET concentration, but a different DEP concentration are more similar than after 3.5h. Seemingly, for a short retention time, the DEP concentration has a higher influence on the DEET removal than for a long retention time. However, no definite influence of DEP on DEET removal can be formulated, since the error bars for the calculated removal in those beakers suggest a high range of possible outcomes.

Absolute removal [µg/gSS] (see Figure 14) ranges from 0.12 µgDEET/gSS to 0.4 µgDEET/gSS. It is highest for high DEET concentrations and lowest for low DEET

concentrations. The removal from the beaker with medium DEET concentration lies in between the removal from high and low concentration beakers. After 6h more DEET is removed from the beakers than after 3h with one exception; absolute removal from beaker three (second bar: 10µg/L DEP 10 µg/L DEET) after 3.5h is higher than after 6h. (Analog to percentage removal, compare with Figure 13.)

The removal rates [µg/gSS/h] (see Table 9) range from 0.035 to 0.11 µg/DEET/gSS/h for 3.5h retention time and from 0.28 to 0.07 for 6h retention time. The hourly removal is higher for the lower retention time. This implies that in each beaker, removal in the beginning of the aeration is faster than in the end. The increase of the rate (from 3.5h to 6h) is not dependent on the DEET concentrations.

- $k_{6h}(b1) = 0.76 \cdot k_{3.5h}(b1)$
- $k_{6h}(b2) = 0.51 \cdot k_{3.5h}(b2)$
- $k_{6h}(b3) = 0.72 \cdot k_{3.5h}(b3)$
- $k_{6h}(b4,b5) = 0.88 \cdot k_{3.5h}(b4,b5)$

This underlines the suggestions that DEET removal is not significantly dependent on concentrations.

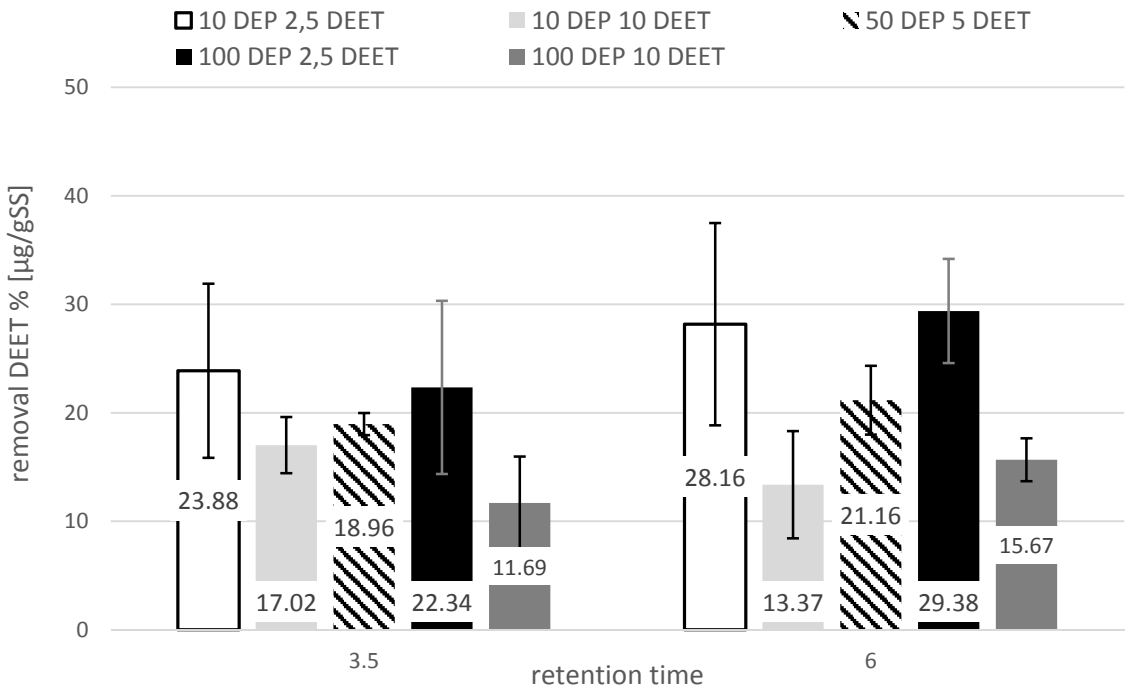


Figure 13 - % removal DEET

Total removal from beaker after 3.5h and 6h. Blanks are not plotted. Comment on Figure 13 to Figure 14: Removal is calculated based on initial and residual concentrations divided by SS concentrations [gSS/L] in the beaker. Error bars represent standard deviations of three repetitions.

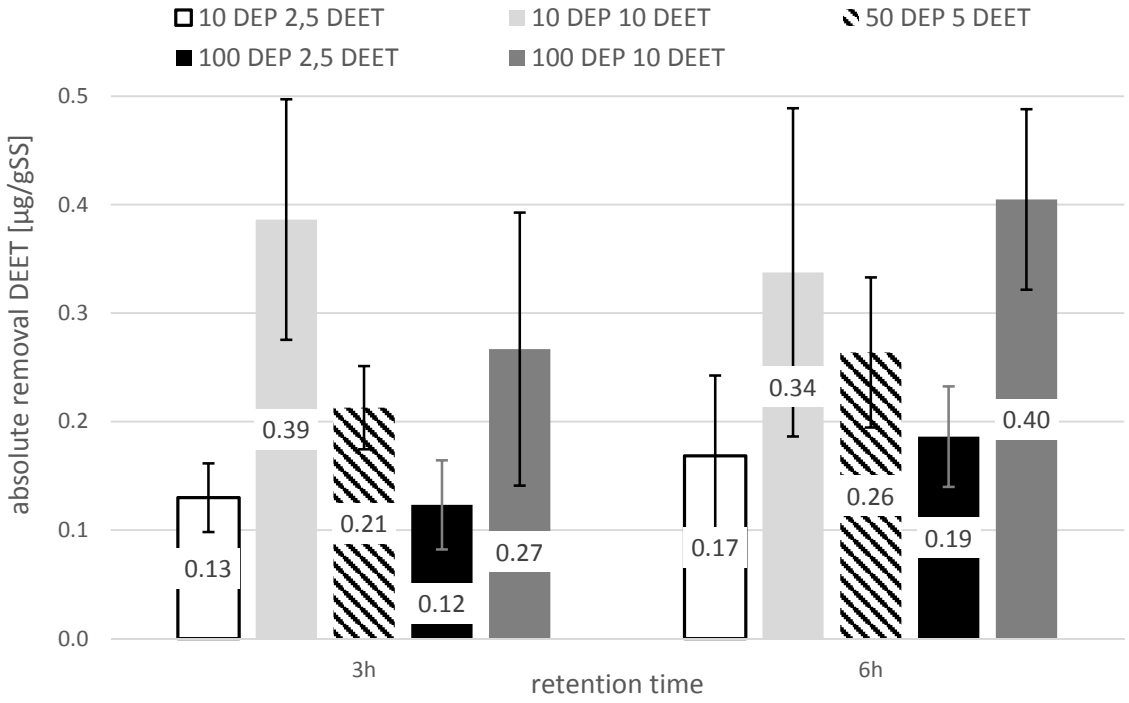


Figure 14 - absolute DEET removal [µg/gSS]

4.1.2 Results total DEP removal

Percentage removal of DEP (see Figure 15) ranges from 86.8% to 100% for 3.5h retention time and from 96.1% to 100% for 6h retention time. For the beakers with higher DEP concentrations (50 and 100 µg/L), total percentage removal is higher after 6h retention time than after 3.5h. Percentage average removal is highest for low DEP concentration and lowest for higher concentrations. The removal from the beaker with a medium concentration (50 µg/L) lies in between the removal of high and low concentrations. The percentage removal of DEP with a high concentration of DEET present is not different from percentage removal with a low DEET concentration present. Accordingly, DEET does not influence percentage DEP removal.

Absolute removal of DEP [µg/gSS] ranges from 2.2 to 19.2 µg/gSS (3.5h) and from 2.3 to 24.4 µg/gSS (6h) (see Figure 16). The absolute removal is highest in beakers with high concentrations and lowest in beakers with low concentrations. After 6h not more DEP is removed from beakers with low concentrations than after 3.5h. In contrast, in beakers with higher DEP concentrations, around 20% more DEP is removed during the additional 2.5h.

Rates (see Table 9) range from 0.64 to 5.49 µgDEP/gSS/h (3.5h) and from 0.36 to 4.07 µgDEP/gSS/h (6h). The rates refer to total removal – they include adsorption and degradation. Rates increase for both retention times with increasing concentrations. Two beakers with the same DEP concentration show different rates depending on the DEET concentration: the lower the DEET concentrations, the lower the rates. However, error bars suggest that this difference could be neglected. Rates calculated on basis of the 3.5h aeration experiments are higher than rates calculated on basis of 6h. This implies that removal in each beaker is faster at the beginning than in the end. The difference between the rates ($k_{3.5h}$ and k_{6h}) grows bigger with increasing concentrations:

- $k_{6h}(b1) = 0.61 \cdot k_{3.5h}(b1)$
- $k_{6h}(b2) = 0.64 \cdot k_{3.5h}(b2)$
- $k_{6h}(b3) = 0.71 \cdot k_{3.5h}(b3)$
- $k_{6h}(b4) = 0.73 \cdot k_{3.5h}(b4)$
- $k_{6h}(b5) = 0.74 \cdot k_{3.5h}(b5)$

This means that the lower the DEP concentration, the faster the removal in the beginning compared to the removal in the end. However, also this kinetic observation does not allow for conclusions concerning the mechanism of removal.

Table 9 - removal rates k [µg/gSS/h]
Rates are calculated based on initial and residual concentrations divided by SS concentrations [gSS/L] and by the retention time. Standard deviations of triplicates are indicated with ±.

		DEET		DEP	
beaker	dose	$k_{3.5h}$ [µg/gSS/h]	k_{6h} [µg/gSS/h]	$k_{3.5h}$ [µg/gSS/h]	k_{6h} [µg/gSS/h]
1	10 DEP 2.5 DEET	0.037 ± 0.009	0.028 ± 0.012	0.642 ± 0.097	0.385 ± 0.042
2	10 DEP 10 DEET	0.110 ± 0.032	0.056 ± 0.025	0.635 ± 0.091	0.405 ± 0.044
3	50 DEP 5 DEET	0.061 ± 0.011	0.044 ± 0.012	2.852 ± 0.199	2.028 ± 0.224
4	100 DEP 2.5 DEET	0.035 ± 0.012	0.031 ± 0.008	5.444 ± 0.863	3.986 ± 0.339
5	100 DEP 10 DEET	0.076 ± 0.036	0.067 ± 0.014	5.491 ± 0.569	4.071 ± 0.068

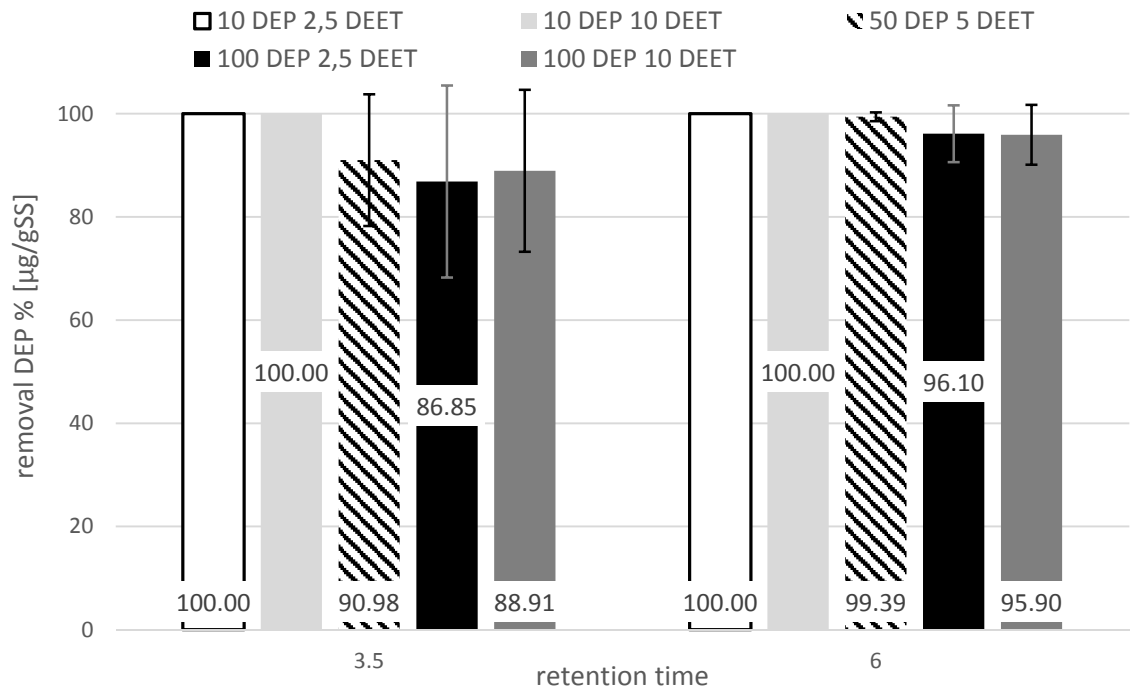


Figure 15 - % removal efficiencies DEP
Comment on Figure 15Figure 13 to Figure 16Figure 14: Removal is calculated based on initial and residual concentrations divided by SS concentrations [gSS/L] in the beaker. Error bars represent standard deviations of three repetitions.

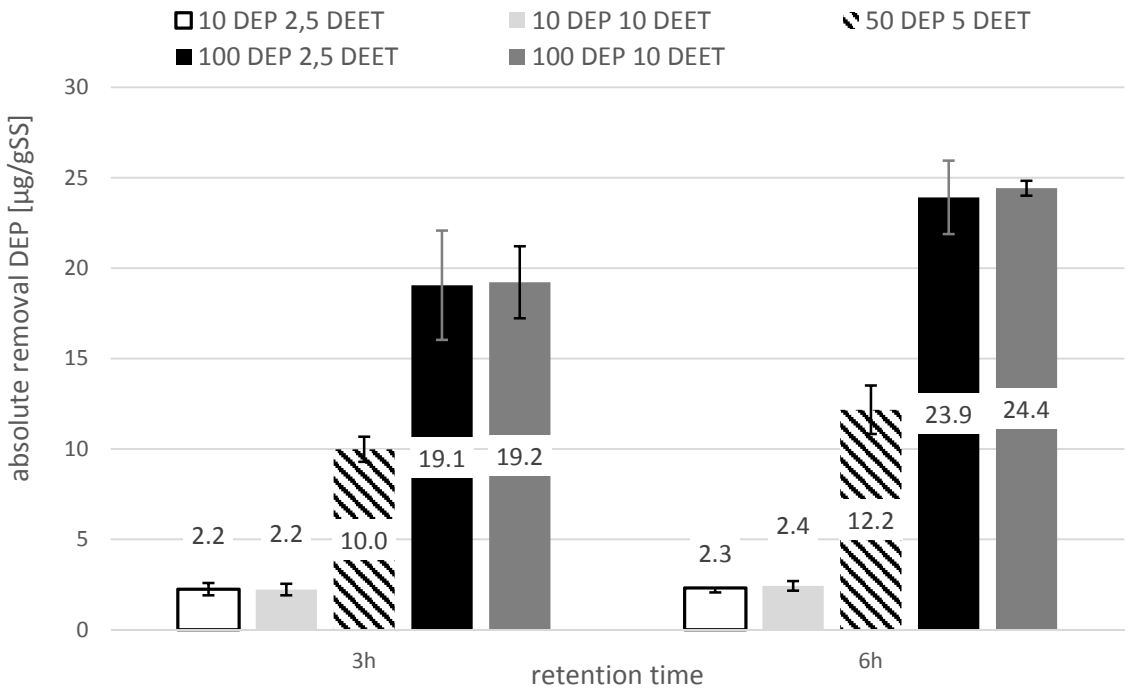


Figure 16 - absolute removal [µg/gSS] DEP

After experiments with oxygen uptake and ATP measurements, residual DEP has been quantified, too. The results in Table 10 show the dosed amount of DEP and the residual concentration DEP (both referred to gSS). To calculate a removal rate k [µg/gSS/h] does not make sense for this set-up, since the short retention time leads to a distortedly high k.

It can be seen in Table 10 that after 30 minutes, around one third is removed from the beaker with 100 g/L DEP and thus significantly less than after 3.5h. The very high DEP concentration (10 mg/L) is hardly removed when considering percentage removal. Considering removal per gSS, from the beaker with the high concentration more is removed (around 200 µg/gSS).

Table 10 - DEP concentrations [µg/gSS] after 30 minutes of aeration
Values and errors are based on duplicates.

target conc DEP [µg/L]	target conc [µg/gSS]	residual conc [µg/gSS]	removal % [µg/gSS]
100	27.55±0.91	19.38±0.47	29.52 ±4.03
10 000	3276.36±329.41	3063.57±557.80	3.81±26.7

4.2 Evaporation

Evaporation of DEP was observed during sample preparation, thus it had to be checked, whether the compounds also evaporate from the beaker. This section presents removal of the compounds from MilliQ during 6h of aeration.

4.2.1 Results

Figure 17 and Figure 18 show initial concentrations of DEET and DEP in MilliQ and concentrations measured after 6h aeration. No significant amount of neither DEET nor DEP is lost during aeration. The opposite is the case; the average concentration in beakers with 2.5µg/L DEET and 10 as well as 100 µg/L DEP is even higher after evaporation than initially. Only for one beaker (i.e. two replicates) containing 10 µg/L DEET, the concentration after evaporation is 5% lower than initially. However, taking into account the standard deviation as well as method inaccuracies, this is not a significant decrease.

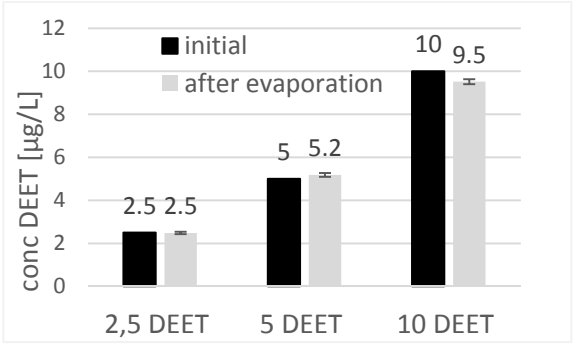


Figure 17 - evaporation DEET

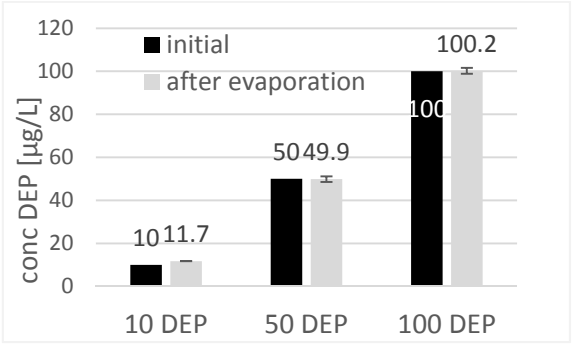


Figure 18 - evaporation DEP

Six beakers were spiked with 2.5, 5 and 10µg/L DEET and 10, 50 and 100 µg/L DEP and aerated for 6h. Error bars indicate the standard deviations of two replicates. Note the difference of scale due to differences in initial concentrations of the two graphs.

4.2.2 Discussion

The alleged increase of MP concentration in the beakers with 5 µgDEET/L and 10 and 100 µgDEP/L is probably due to dosing or method inaccuracy. Another possible reason is the evaporation of MilliQ, which leads to a total volume decrease and thus to a higher MP concentration.

Studies suggest DEP volatilizes during the activated sludge process (Dargnat et al. 2009, Tran et al. 2015). In addition, also in the course of the presented thesis/project work, volatilization of DEP had been witnessed; DEP disappeared during the evaporation step as part of the sample preparation. Apparently, it evaporates together with highly volatile substance

such as acetonitrile (as in the sample preparation). There are no highly volatile components in the mixed liquor in the batch experiments. Thus, the contribution of evaporation to the total removal of MPs from sludge can be neglected.

DEET has a higher vapor pressure than DEP (see Table 1). Thus, it should more readily evaporate. This is, however not supported by the result of the evaporation experiment. Studies show the transition of DEET into the vapor phase: When applied to skin, a mass transfer coefficient can be quantified (Santhanam, Miller and Kasting 2005). However, with respect to the batch experiments, this coefficient is not relevant, since it depends on skin penetration and co-ingredients of the insect repellent lotion. Apart from that, it is negligibly small (2.6 cm/h). Hence, also for DEET evaporation is not a major removal pathway during the batch experiments.

4.3 Adsorption

Two kinds of experiments have been carried out to quantify adsorption; 1. sludge was spiked and directly afterwards centrifuged (‘immediate removal’). 2. Sludge was inactivated chemically ($HgCl_2$) and thermally (pasteurization). Though pasteurization is often suggested as an inactivation method (e.g. in Fan et al. (2014) and citations therein), it has not been chosen as the final method for this study, because it alters adsorption properties and thus may distort results. Hence, the results are only presented in the Appendix C, p. 88.

4.3.1 Results immediate removal

As visible in Figure 19, 6.6 µg/gSS DEET (i.e.1.4 to 3.5 %) in the first five minutes. The standard deviation of the five repetitions are small (1.88 µg/gSS, which is 0.25% of the residual). (The exact time between spiking and centrifuging cannot be given, since the time for weighing/decanting etc. ranges between three and five minutes.)

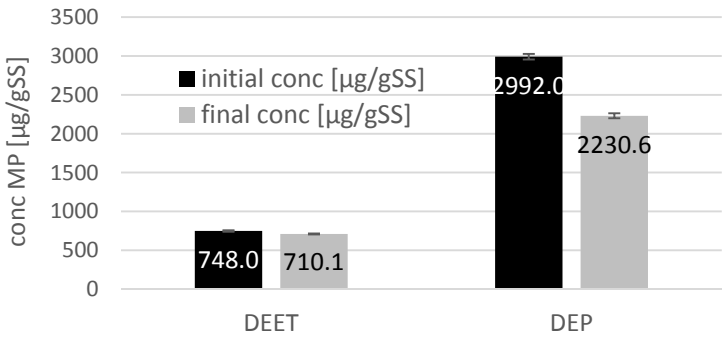


Figure 19 - removal in first five minutes
Mixed liquor is centrifuged immediately after spiking. Concentrations are referred to the SS content of each beaker. The error bars represent standard deviations of five repetitions. (Two blank samples were also run to account for a possible background contamination. Blanks are not displayed.) No SPE used.

In contrast, 245.4 µg/L DEP was removed from the beakers (i.e. 24.4%) within the first minutes: The standard deviation is small (7.34 µg/gSS, which is 0.24% of the 1%).

4.3.2 Results inactivation with $HgCl_2$

The results from the inactivation with 30 mg/gSS $HgCl_2$ do not fully support the results from the immediate removal (see 4.3.1 above): Figure 20 shows that no DEET has been removed after 6h retention time on the shaker. DEP has decreased by less than 2 µg/gSS, i.e. 6.6 %. Taking into account the errors, maximal 2.75µg/gSS adsorbs. This is only one fourth of the removal that had been observed by measuring a high spiked concentration during the first five minutes (4.3.1)

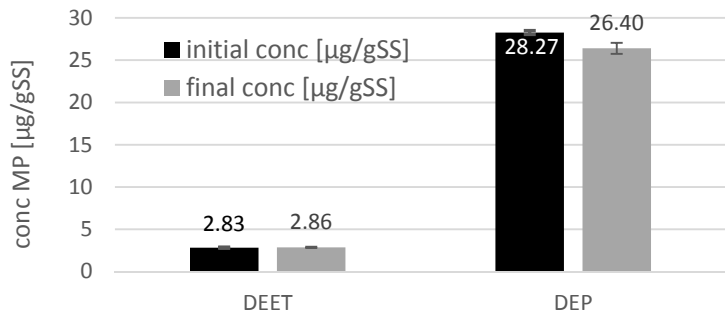


Figure 20 - removal after inactivation with 30 mg/gSS HgCl2, 6h retention time
Values and error bars (STD) are derived from three replicates. SPE was necessary because of low MP concentrations.

Based on the removal per gSS, K_P values can be calculated by applying equation (0.1) and modifying it in the following way, in order to quantify the concentration of MP in the solid phase:

$$K_P = \frac{\left(C_{inititial} - C_{final}\right)\left[\frac{\mu g}{kgSS}\right]}{C_{final}\left[\frac{\mu g}{L}\right]} \quad (0.12)$$

Where

K_p	is the solid-water distribution coefficient
C	describes concentrations of DEET and DEP
Numerator	describes the concentration in the solid phase
Denominator	describes the concentration in the liquid phase.

Modifying equation (0.2) and estimating the carbon content of the sludge to be 0.53·VSS/SS according to (Henze 2008) there is also an alternative derivation of K_P based on C-content and literature K_{OC} values (Keeley 1990):

$$K_p = 0,53 \cdot \frac{VSS}{SS} \cdot K_{OC} \quad (0.13)$$

Results are presented in Table 11. Here, the K_P for both pollutants are calculated with the above mentioned equations. K_P values for DEP are higher than for DEET. The two ways of calculating lead to very different results; the measured K_P is 5-8 times higher than the K_P based on C-content. For DEP, this difference is one order of magnitude, respectively. Apart from that, the standard deviation is high for DEET. However, one should bear in mind that the solid-water distribution coefficient is valid for equilibrium, which might not be reached after 6h and would partly explain the scatter in the data.

Table 11 - calculated water-solid distribution coefficients (K_p)
Values in the first column are calculated with eq.0.12. Values in the 2nd column are based on eq. 0.13, assuming the K_{OC} values given in the third column. Errors represent standard deviations from the 3 beakers.

	K_p measured	K_p based on C-content and K_{oc}	K_{oc} used
DEET	2.02±2.86	0.346±0.49	2.48 (NLM 2001)
DEP	19.18±2.45	1.12±0.008	2.65 (Jun Sekizawa 2003)

4.3.3 Discussion adsorption experiments

The aim of the adsorption experiment was not to characterize general adsorption coefficients for MP and this specific sludge. The aim was merely to quantify maximal adsorption [µg/gSS] for the setting of the aeration experiment. Maximal adsorption has been chosen, because the desired pathway for MP degradation is mineralization by microbes and thus a prevention of detrimental effects of the MP in the environment. Degradation to toxic metabolites is not

desirable. However, it is out of the scope of this thesis to quantify the conversion of the MP into toxic metabolites - other HPLC and SPE methods would have been needed. Adsorption onto the sludge is also not desirable. In the case of a full-scale grey water treatment plant, adsorption would shift the MP load onto the solid phase from which it might leak again in the aquatic environment, e.g. during sludge stabilization. In order to be on the safe side, a minimum degradation should be quantified by subtracting maximal removal by adsorption from total removal rates.

The results of the first adsorption experiment (4.3.1) have to be handled with care; they just give an estimation that DEP is likely to adsorb fast, DEET probably adsorbs either slowly or not. To describe and quantified adsorption and thus enable a comparison, however, the equilibrium between occupied adsorption spaces and concentration in the liquid phase has to be reached. (Thomas and Crittenden 1998) This might have not been the case after only a few minutes. Accordingly, the results obtained from 4.3.1 only give an indication.

Results from 4.3.2 are more reliably, since the rheological and thus adsorption properties have not been altered as severely as during pasteurization. Furthermore, the retention time Coherently, those results are chosen to quantify maximal adsorption. Despite the difference in spiked concentrations, one can compare the results from 4.3.1 with 4.3.2 qualitatively. It can be concluded that DEP is hardly adsorbing (4.3.2), i.e. the removal witnessed during 5 minutes aeration (4.3.1) is due to biological processes. Then biological degradation must be fast. With respect to DEET, the results from the immediate removal and the chemical inactivation are coherent; nothing is removed after 5 minutes i.e. nothing adsorbs and degradation does not take place in the first five minutes. Also the mercury inactivation suggests no adsorption.

The measured K_P values make sense, since they reflect a higher adsorption of DEP to sludge than DEET. This means, the produced results are coherent. But as soon as literature values are included to calculate the distribution coefficient, results differ strongly. The following paragraph evaluates first the measured DEET adsorption and then DEP adsorption in the context of literature.

K_P values for DEET found in literature are several times higher than the one determined during the 6h experiment with inactivated sludge: Stevens-Garmon et al. (2011) determined K_P values for 3 different activated sludges and found K_P values of 1.62 L/kgSS, and 2 L/kgSS. Another study suggests a sorption coefficient K_P = 1.91±0.147 L/kgSS (Hyland et al. 2012). Here, the sludge has been inactivated by freeze-drying, which might have increased the sorption

capacity. Both of the studies, however, are more accurate, since the time for the partitioning equilibrium is determined. In contrast, in this project equilibrium time had been neglected. Yet, the measured K_P values lie in the range of literature values. However, the difference between the K_P values in Table 11 indicate that it is apparently invalid to calculate K_P with the help of literature K_{OC} taken from equilibrium studies (like in Table 11, column 2). Furthermore, Yang et al. (2011) suggest that DEET is not removed by treatment with granulated activated carbon. This supports the assumption that DEET is not amenable for adsorption. Accordingly, removal must be due to degradation.

Comparing the observed DEP adsorption to literature, similar problems arise as for DEET; often, the experimental set-up differs, e.g. the concentrations loaded onto the sludge are much higher or experiments are conducted for equilibrium retention times. Fang and Zheng (2004) derived Freundlich coefficients and exponents (k = 1,203 and n = 0,7176) from experiments where between 0.5 and 10 mg/L DEP has been dosed into mixed liquor. They conclude that 0.73mg DEP adsorbes onto 1gSS and 14.3mg DEP adsorb onto EPS (both in equilibrium). These values are several orders of magnitude higher than the removal quantified in the presented project (2 µg/gSS). This might be due to the high initial doses used by Fang and Zheng (2004). Yet, the isotherms derived in this study should be valid also for lower concentrations of DEP in the liquid phase. Using their coefficients and equation (0.3), an expected load of 32 µgDEP/gSS can be calculated for an initial concentration of 100 µg/L in the liquid phase. This is still higher then what had disappeared from the liquid phase in this project. Comparing the observed removal of DEP (6.6%) to Julinová and Slavík (2012), again the literature values for removal are much higher (24.5% - 46.2%) then the observed removal. This might be due to a higher retention time in the cited study (24h) or differences in the inactivation method (Julinová and Slavík (2012) used sodium azide.) At any rate, this project could not support the statement “the most promising adsorbent […][for phthalates] appears to be the biomass of activated sludge.” (Julinová and Slavík 2012). Even if this were true, this would not be “promising”, since it would just shift the problem of a trace organic pollutant load from the liquid to the solid phase – a problem that would then still have to be dealt with.

To conclude the discussion; the derived values do not correspond well with literature values. However, that is not crucial, since the aim of the adsorption experiment was, to quantify adsorption in this specific set-up and subtract this from total removal. This was achieved; adsorption of DEET is negligible, adsorption of DEP is 2.75 µg/gSS at maximum.

4.4 Discussion total removal

With the help of the adsorption experiments, it can be derived that the removal of DEET (11.7 – 29.4%, i.e. 0.12 – 0.4 µg/gSS) is due to biodegradation or transformation. In contrast, DEP removal is partly due to adsorption. Accordingly, between 80.25 and 93.4 % of DEP is removed (i.e. 0.2 – 22.4 µg/gSS). The following section should evaluate, if this is realistic in comparison with other studies.

4.4.1 DEET

The observed total removal is in the range of values found in literature; Bernhard, Müller and Knepper (2006) report 0% to 50% removal of DEET during the activated sludge process in a sewage plant. In contrast, (Luo et al. 2014) suggests a removal efficiency of more than 74% during waste water treatment. In Kagle et al. (2009) studies are summarized that estimate the biological transformation of DEET in an activated sludge process to be between 37 and 90%. Knepper (2004) observed, only for concentrations of DEET higher than 1 µg/L in the influent, degradation can be observed during waste water treatment. Yet, other authors (Sui et al. 2010) measured initial concentrations of 0,6 -1,2 µg/L and still report a removal of 76% during secondary treatment and 0 % removal during primary treatment. 0% removal during primary treatments supports the suggestion that DEET is not adsorbing to particles or biomass. The variation of the reported data can be due to differences in the hydraulic regime of the tanks, or to variations in redox conditions of the sludge, microbial community, sludge retention times or varying initial concentrations. In the light of this large range of reported removal efficiencies, the observed removal of about 1/10 to 1/3 of initial concentration seems realistic.

The question remains, whether this removal is also sufficient, if the treated water should be used for high body contact application. If one assumes the load into a grey water treatment plant is 2.5 µg/L DEET, after the treatment (according to Figure 13) around 60% of the DEET load will left. If the water will be reused for showering, a new load of DEET will be washed off the skin and contribute to the load of the 2nd cycle. For the 5th cycle, the load will already be 7 µg/L. Since the percentage removal decreases with increasing concentrations (see Figure 13), DEET will up-concentrate in this system. One might argue that the risk connected to high body contact with DEET should not be overrated. After all, dermally applied commercial mosquito repellent crèmes contain up to 100% DEET as an active ingredient (Costanzo et al. 2007) – much more than what would be present in the recycled grey water. This remark, however, is not valid, since

DEET has been chosen as a model compound partly because of its suspected adverse effects. One cannot research a specific MP because it is suspected to be harmful, but then not consider it harmful anymore, if it is not sufficiently removed in the assessed treatment. The only valid conclusion, coherent with the motivation ("DEET might be a harmful substance") and the aim ("the grey water should be used for high body contact again") is, the presented biological grey water treatment is not sufficiently removing DEET.

4.4.2 DEP

Stales et al. (1997) summarize reported degradation efficiencies that range between 90 - 100% for wastewater and activated sludge (0.001-5 mg/L DEP, 1-7 days retention) under aerobic conditions. Removal under anaerobic conditions ranges from 0 to 70% in sludge and sediments (50-200 mg/L, 50-200 days retention). A pure culture (*Variovorax sp*) has been documented to degrade 100 mg/L DEP within 30h, using DEP as a sole energy and carbon source (Prasad and Suresh 2012). However, one has to bear in mind that a pure culture consists of organisms adapted to DEP degradation. So they most likely degrade DEP more efficiently than the mixed culture from the sludge used in this batch experiment. Apart from that, a pure culture of *Bacillus subtilis* was reported to reduce 50 mg/L DEP in 4h by more than 75%. In this study, a removal > 99% was only achieved by adding a surfactant and after 12 hours (Navacharoen and Vangnai 2011). The authors concluded DEP was co-metabolized with easily available carbon sources and good electron donors (citrate, glucose). Also sources of nutrients (yeast extract) were added, which improved biodegradation. Results by Navacharoen and Vangnai (2011) are more comparable with the batch experiment than those by Prasad and Suresh (2012) since the grey water used in the experiments also contains other carbon and nutrient sources. Summing up, the observed removal of 86 – 100% is realistic.

Is this removal enough? From 36 beakers, in 30 beakers removal was complete (taking into account all replicates, doses and retention times). That is to say, that it might be a flaw in the experiment causing the incomplete removal. At any rate, even if the incomplete removal was accurate, it is likely that microbes adapt to DEP and metabolize it more efficient later. Such an adaption has been reported by Prasad and Suresh (2012). The maximum 6.6 % of DEP, which adsorbs to the sludge, is not posing a risk onto the environment. After a lag phase, bacteria are likely to evolve that are capable of digesting DEP also in a soil-like matrix (Amir et al. 2005, Kapanen et al. 2007). To conclude; the examined system and set-up is sufficient to remove environmental relevant concentrations of DEP from grey water. Up-concentration due to

multiple reuse is not likely, because bacterial adaption might even increase the treatment efficiency.

4.5 Effect of DEP on Oxygen uptake rate (OUR)

Even if DEP is efficiently removed, there is still a risk connected to DEP in grey water: DEP might be harmful for the microbial community and inhibit bacterial processes needed for nutrient removal.

Oxygen consumption is an indicator for bacterial growth, cell maintenance and product synthesis (Garcia-Ochoa 2009, Riedel et al. 2013). It can be used to estimate the effect of DEP on bacterial communities (Hamon et al. 2014, Mohan et al. 2006, Garcia-Ochoa et al. 2010). OUR was measured 30 seconds, five minutes and 30 minutes after the beakers had been filled with sludge and spiked greywater. DEET is neglected, since results of the precedent project suggested that the impact of DEP on the microbial activity is more significant. Adenosine-5-triphosphate (ATP) has been measured in the same time. Since ATP is present in living bacterial cells and nearly always involved in metabolic reactions, it can help to estimate the amount of biomass in the beaker (Hwang and Hansen 1998). Thus, measured at different times, it can indicate microbial growth.

4.5.1 Results

Figure 21 shows the dissolved oxygen (DO) concentration 30 seconds after spiking. There are differences between the shapes of the curves; DO decreases fastest in the beaker with the highest DEP concentration (10 mg/L) and slowest in the two blanks. In this set-up, also two medium concentrations of DEP were tested. The curves for beakers with the two medium concentrations DEP (100 and 1000µg/L) lie in between the flattest and the steepest curves. This difference can also be quantified by calculating the specific oxygen uptake rate (SOUR), seeFigure 24 shows the level of ATP after two times in 5 beakers with three different DEP concentrations. The graph is based on data provided in the Appendix E, p. 94. The concentration of ATP after 5 minutes decreases with increasing DEP concentration. The sample 10 000µg/L DEP (2) is an exception. After 30 minutes, there is no correlation between the concentrations of DEP and the ATP level.

Table 12. The oxygen uptake rates (SOUR) are calculated on base of the linear curve sections (7 mg/L – 2 mg/L) of the curves and the SS content of each beaker. SOUR for the blank beaker

is lowest, (36.32 ± 0.29 gO_2/h/gSS), i.e. a low amount of oxygen is consumed per gram SS per hour. The SOUR for 10 000 µg/L is highest (29.06 gO_2/h/gSS) and the SOUR for 100 µg/L DEP lies in between the SOUR of the high and the low DEP concentration (54.17 gO_2/h/gSS).

After 5 minutes of aeration, there is no significant difference between the DO curves of beakers with different DEP concentrations. Also the SOUR cannot fully correlated with DEP concentrations; the SOUR for the blank is lowest, but the SOUR for the beaker with 100 µg/L is highest. The SOUR for the beaker with the high DEP concentration lies in between the blank and the 100 µg/L DEP spiked beaker. Considering the errors, however, the difference between the SOUR might be insignificant.

Likewise, the difference in oxygen consumption between the beakers is less apparent after 30 minutes compared to after 30 seconds. The curve for the blank2 exhibits a bump at 4 minutes. A foregoing blockade of the magnet stirrer or interference with atmospheric oxygen can cause such an irregularity. (Appendix D, p.90 shows more examples of how variations in the experimental procedure lead to different shapes of DO-plots.). The calculated SOUR show a tendency; they increase from 8.38 ± 0.61 gO_2/h/gSS to 11.49 ± 2.75 gO_2/h/gSS with increasing DEP concentrations. In short, for measurements after 5 seconds, a correlation between high SOUD and high DEP concentrations can be seen. For measurements at a later time, the correlation is less significant. Yet, there is still a tendency that higher DEP concentrations cause a higher SOUR.

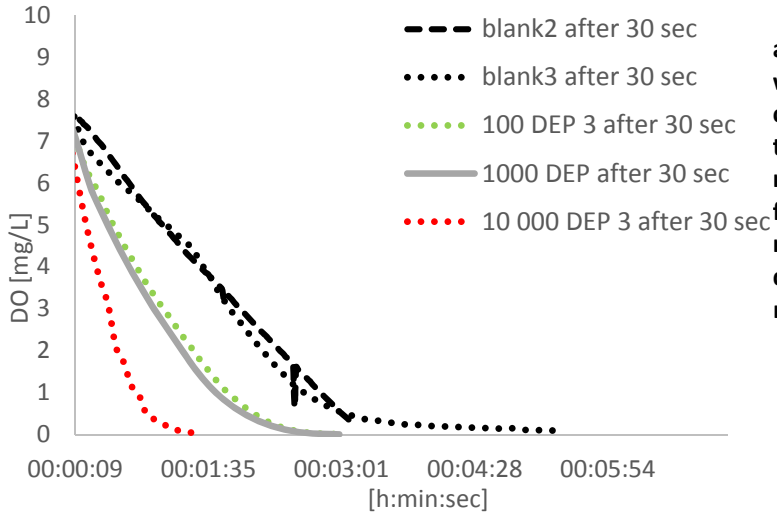


Figure 21 - OUR 30 sec
after mixing with spiked grey water, dissolved oxygen concentration (DO) was measured throughout the five following minutes. 100, 1000, 10 000 stands for the dose of DEP in µg/L. The numbers 2 and 3 after sample description stand for different replicates.

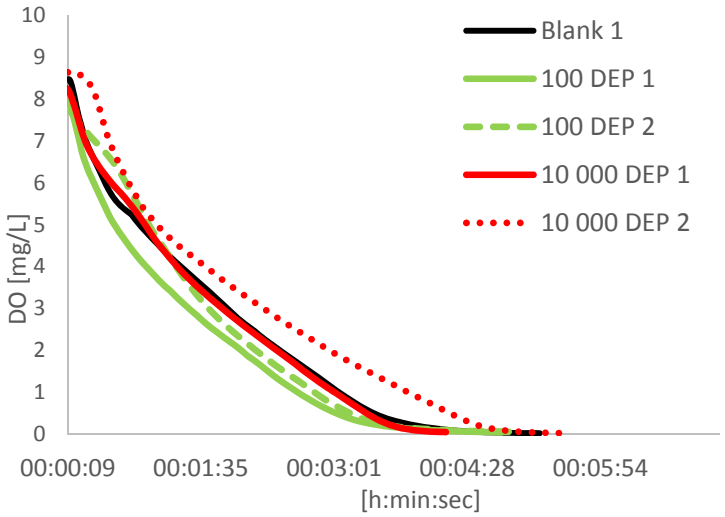


Figure 22 - OUR 5 min
after mixing with spiked grey water. after mixing with spiked grey water, air diffusor was taken out an oxygen concentration (DO) was measured in the five following minutes. 100, 1000, 10 000 stands for the dose of DEP in µg/L. The numbers 1 and 2 after sample description stand for different replicates.

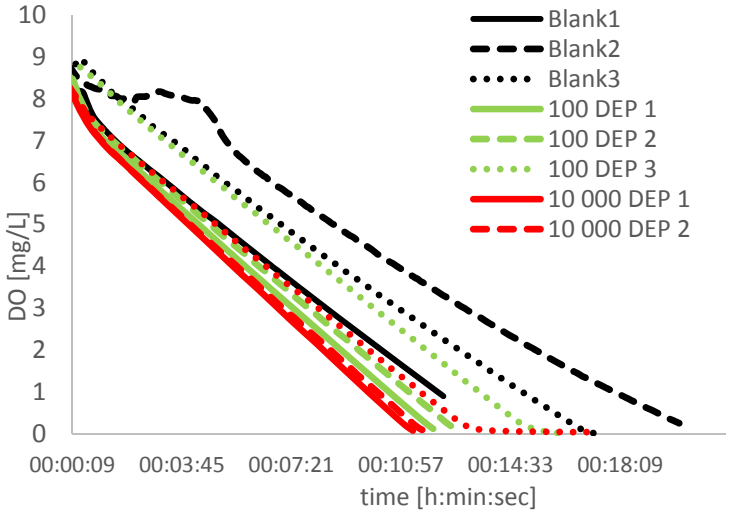


Figure 23 - OUR 30 min
after mixing with spiked grey water. Oxygen concentration (DO) was measured throughout the following 20 minutes. 100 and 10 000 represent the DEP dosage in µg/L. The numbers (1, 2, 3) after the sample description represent different replicates. Note the different scale of the y-axes of this figure.

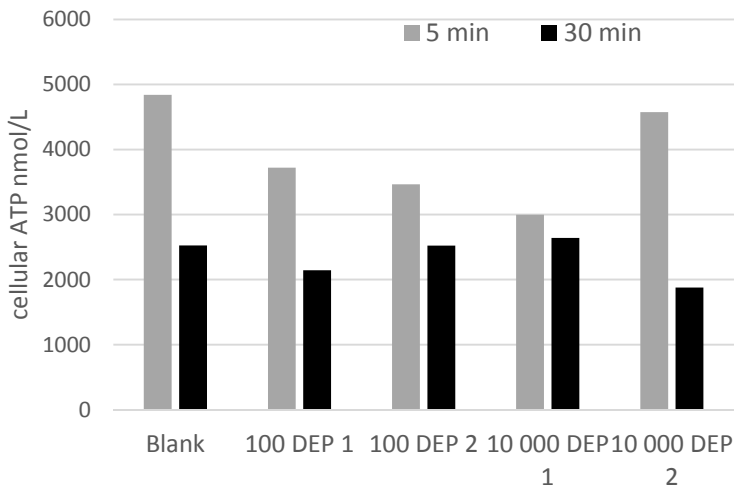


Figure 24 - ATP values from samples 5 and 30 minutes after mixing sludge with spiked grey water.

Figure 24 shows the level of ATP after two times in 5 beakers with three different DEP concentrations. The graph is based on data provided in the Appendix E, p. 94. The concentration of ATP after 5 minutes decreases with increasing DEP concentration. The sample 10 000µg/L DEP (2) is an exception. After 30 minutes, there is no correlation between the concentrations of DEP and the ATP level.

Table 12 - specific oxygen uptake (SOUR)
SOUR is calculated by determining the slope of the OUR curves between DO(7mg/L) and DO(2mg/L) and dividing this slope by the SS content. Values for SOUR $_{30min}$ are based on triplicates. Values for SOUR $_{5min}$ and SOUR $_{30\ sec}$ are based on duplicates (if an error mentioned) or singular trials (if no error mentioned)

Sample	SOUR $_{30\ sec}$ [$mgO_2/gSS/h$]	SOUR $_{5min}$ [$mgO_2/gSS/h$]	SOUR $_{30min}$ [$mgO_2/gSS/h$]
Blank	36.32 ± 0.29	38.24	8.38 ± 0.61
100µg/L DEP	54.17	53.87 ± 6.82	9.66 ± 1.14
10 000µg/L DEP	129.06	44.64 ± 1.53	11.49 ± 2.75

4.5.2 Discussion

It is difficult to compare the SOUR with literature values for typical SOUR for certain microorganism. Firstly, because the SOUR varies throughout different stages of microbial growth and increases throughout the exponential growth phase (Garcia-Ochoa et al. 2010). In the presented experiment, however, it is not possible to determine the growth stage of the bacteria, since too little is known about the bacteria (e.g. bacterial community, yield on oxygen, specific growth rate etc.). Secondly, the DO decline in the beaker is not only dependent on bacterial respiration, but also on the oxygen transfer to the cell (Garcia-Ochoa et al. 2010). The latter depends on the hydraulic regime and operation conditions and differs between the presented set-up and experiments in other studies. SOUR also depends on the carbon source and thus differs from study to study. However, the SOUR values for certain microorganisms

summarized in Garcia-Ochoa et al. give a rough indication that the calculated OUR and SOUR values are realistic.

Apart from comparing obtained absolute values for OUR and SOUR, the differences between the two diagrams (after 5 minutes and after 30 minutes) are of interest: oxygen decrease happens faster shortly after mixing with grey water than after 30 minutes. This is also mirrored in the high SOUR for 30 seconds/5 minutes and the low SOUR for 30 minutes after mixing. The global difference between Figure 21 and Figure 23 can be explained with the enzymatic reaction rates and their dependency on substrate concentration: the higher the concentration of substrate, the higher the rate of enzyme catalysis (see 2.5.2, p.16). Since respiration demands a number of enzymatic reaction for the electron transport chain (Madigan 2015e), a high substrate concentration (grey water) after 30 seconds or 5 minutes leads to a higher O_2 consumption than the low grey water concentration after 30 minutes. Since the substrate level after 30 seconds is similar to the level after 5, no big difference in the shape of the curves (Figure 21 and Figure 22) and $SOUR_{30sec}$ and $SOUR_{5min}$ can be observed.

The high SOUR at high DEP concentrations (see Figure 21) is more challenging to explain; either DEP enhances bacterial metabolism in general. That is, however, unlikely, since enzyme inhibiting effects of DEP have been reported (N. Premjanu 2014, Acros 2015) Or there is a specific strain of bacteria very active in the beginning that metabolizes DEP and accounts for a high oxygen consumption. This is more plausible, since biological degradation of DEP has been reported for a number of bacterial strains (Sompornpailin 2014) (see also section 2.8 and citations therein). However, studies report an adaption time, the bacteria need, to be able to metabolize DEP (Boonnorat et al. 2014). In the presented batch experiments the bacterial communities comes from the pilot plant, i.e. there was no previous exposure to DEP, apart from the low DEP content of the grey water. So an adaption to DEP and hence an improved degradation is unlikely. Further studies are required.

SOURs shortly after spiking are effected by DEP, while after 30 minutes no difference can be detected. These are two possible explanations: DEP might increase bacterial catabolism or even reproduction in the first minutes. After DEP is consumed (e.g. as a carbon source (Prasad and Suresh 2012)), the bacterial activities even out in beakers because the level of DEP is zero in both. The second possible explanation addresses the case in which DEP is not degraded entirely after 30 minutes; if only one strain of bacteria is responsible for metabolizing DEP and this strain is outcompeted later by other strains, respiration would be higher in the beginning

(with this strain still active) than after 30 minutes. There is, however no good reason for a bacterial strain to be outcompeted, while its substrate (grey water with a high content of DEP) is still present. Thus, that scenario is only plausible, if this strain needs grey water as an obligatory primary substrate. Grey water then depletes before the end of the 30 minutes aeration. Hence, after that bacterial strain died, respiratory activity is the same in all beakers, even before DEP is metabolized completely. And as Table 10 indicates, DEP indeed is not metabolized completely.

There are two phenomena in the ATP measurements that can be discussed: the difference between the ATP level after 5 and 30 minutes. And the decrease in ATP_{5min} with increasing DEP concentration (leaving the exception 10 000µg/L DEP(2) aside. The difference of the ATP level after 5 minutes and after 30 minutes suggest that the microbial community decreases in this time interval. This is surprising, since after the feeding with grey water, the bacteria was expected to grow. This growth was estimated to last longer than 30 minutes, since the generation time of e.g. e-coli strains is 20 minutes under optimal conditions (Madigan 2015b). Accordingly, after 30 minutes, the microbes (most likely including slower growing species like nitrifiers) should still be in their exponential growth phase. In contrast, a lower ATP level after 30 minutes compared to after 5 minutes suggests, the microbial population in the beakers declines. On the other hand, substrate might already be depleted within the first 30 minutes. In this case, exponential growth cannot be expected during this time and the ATP results make sense. However, the results differ, depending on the method (i.e. the dilution) used to obtain them (see Appendix E). Apart from that, ATP levels should differ in one or two orders of magnitude, in order to formulate a trend and not –like here- by only 50-60%. This might indicate that the ATP results should not be overrated.

However, in combination with the SOUR results, the ATP results make sense. Looking at the decreasing ATP_{5min} level, one might conclude that DEP is hampering microbial growth. The SOUR results imply DEP is increasing metabolic activity. This is not a contradiction: Oxygen consumption indicates under some circumstances also cellular stress (Novak 2014, 2015). This is for instance the case in the respiratory burst, where a phagocyte cell increases oxygen consumption in order to produce reactive radicals and other reactive oxygen species ($O_2^{\bullet -}$, H_2O_2, NO•, ROO•) that kill an ingested pathogen. (Madigan 2015f). This is not a plausible scenario for what happens in the beakers, since presumably there are no organisms with an immune system present in the tanks. Yet, it illustrates, how and why cells might take up an increased amount of oxygen. Not only in phagocytes, but in every cell (including bacteria) radical oxygen

species (ROS) are produced with the help of enzymes in the course of the respiratory chain. They are used for the physiological control of cell functions. If the generation of ROS exceeds the consumption, this poses harmful oxidative stress upon the cells, which damages e.g. proteins, lipids and thus the cell membrane. (Valavanidis et al. 2006, Elisa Cabiscol 2000) DEP has been shown to cause oxidative stress in zebrafish cells (Xu et al. 2013). Furthermore, it causes a rise in lipid peroxides in the cell which are an indicator for an attack of lipids by radicals (Kang et al. 2010). Lipid peroxidation requires an increased up-take of oxygen (Marisa Repetto 2012). Apart from that, it triggers anti-oxidative reactions (i.e. the induction of neutralizing enzymes) to defend cells in other aquatic organisms against oxidative stress. (Chen and Sung 2005, Kang et al. 2010). But at the same time DEP hampers the activity of these enzymes (N. Premjanu 2014). Hence, applied to the beakers, the following scenario seems reasonable: As an environmental stress factor, DEP disturbs the cells and poses oxidative stress. This increased production of ROS demands a high uptake of oxygen, which can be an explanation for the observed increased SOUR in the spiked beakers. Even though the bacteria induce enzymes that neutralize the radicals, the cells are impaired, since DEP can inhibit these mechanisms. Hence, the cell dies or becomes less active. That would explain the lower ATP activity with high DEP doses.

To sum up: there is a theoretically reasonable explanation for the observations, but the data does not provide for a sound verification for this. The only conclusion to be drawn is that DEP is not impeding microbial activity in a fatal way; for both parameters, the blank did not differ from the spiked beaker dramatically. There is a possibility that DEP decreases microbial activity slightly, and at the same time increases oxygen demand. This, however, needs to be verified in further studies. More sensitive parameters than SOUR and ATP should then be chosen to study DEP's effect.

4.6 Effect of DEP on P uptake and release

In the pilot plant, grey water goes through anaerobic, anoxic and aerobic conditions. In the full cycle experiments, this was mimicked in order to observe differences in nutrient removal between beakers spiked with 100 µg/L DEP and blanks. During all those runs, the removal of DEP was complete.

While developing the set-up for the full cycle batch experiment (see 3.4.5, p.33) nitrate and ammonia doses have been varied. During these trials, P in phosphate (PO_4-P) was measured. In

one of the runs, no medium was transferred from the pilot. Due to these variations, the results cannot be handled as triplicates. Yet, they are presented together in the following section.

4.6.1 Results

Figure 25 shows P-uptake and -release in two beakers (blank and 100 µg/L DEP) during three different experiments. In the first phase, the concentration of P is increasing. After 2h, nitrate was added. Depending on the nitrate dose, in the 2nd phase of the experiment (between 1h40min and 2h30 min), the P concentration increases further (with 0.8 mg/L additional NO_3-N present) or decreases (with 3.4 and 0.8 mg/LNO_3-N added). After switching on the aeration after 3h, the P concentrations slopes rapidly in all beakers. The P-concentrations in the experiments where less nitrate was dosed, are nearly twice as high as in the experiment, where 8 mg/L NO_3-N was dosed – even before the dosage. (For this, compare the second data point of each series, at 1h40min.).

The P-uptake by the bacteria in the aerobic phase (i.e. the decrease in P-concentration in the beaker) seems slowest for the beaker with 3.4 mg/L NO_3-N, since the slopes of the grey curves after 2h30min are flattest. This, however, might be due to the long time interval before the last measurement. Most likely, an earlier measurement than 5h20min for the series 5) and 6) would have also resulted in a low concentration. Then the slopes of these curves would be similar to the other slopes of series 1) to 4).

There is a slight difference in P concentration between the blank beaker and the 100DEP beaker in all three runs. For the time t=0, the P concentration can be assumed to be identical in both beakers, since the dose of grey water is the same and DEP does not add P to the mixed liquor. After 20 minutes, the P concentration in the beakers with DEP is slightly increased. Shortly before dosing nitrate, the P concentration in the DEP containing beakers is 12% (series 1 and 2), 7% (series 3 and 4) and 4% (series 5 and 6) higher than in the blanks. In anoxic conditions (series 1 and 2), the difference between the blank and the spiked beaker increases further during the anoxic P uptake. For the low dosages of nitrate, in this time interval, the difference stays constant (series 1 and 2). In beakers 5 and 6 it reverses: a higher concentration of P is measured in the blank beaker at the beginning of the aerobic phase (comparing data points at 2h30min).

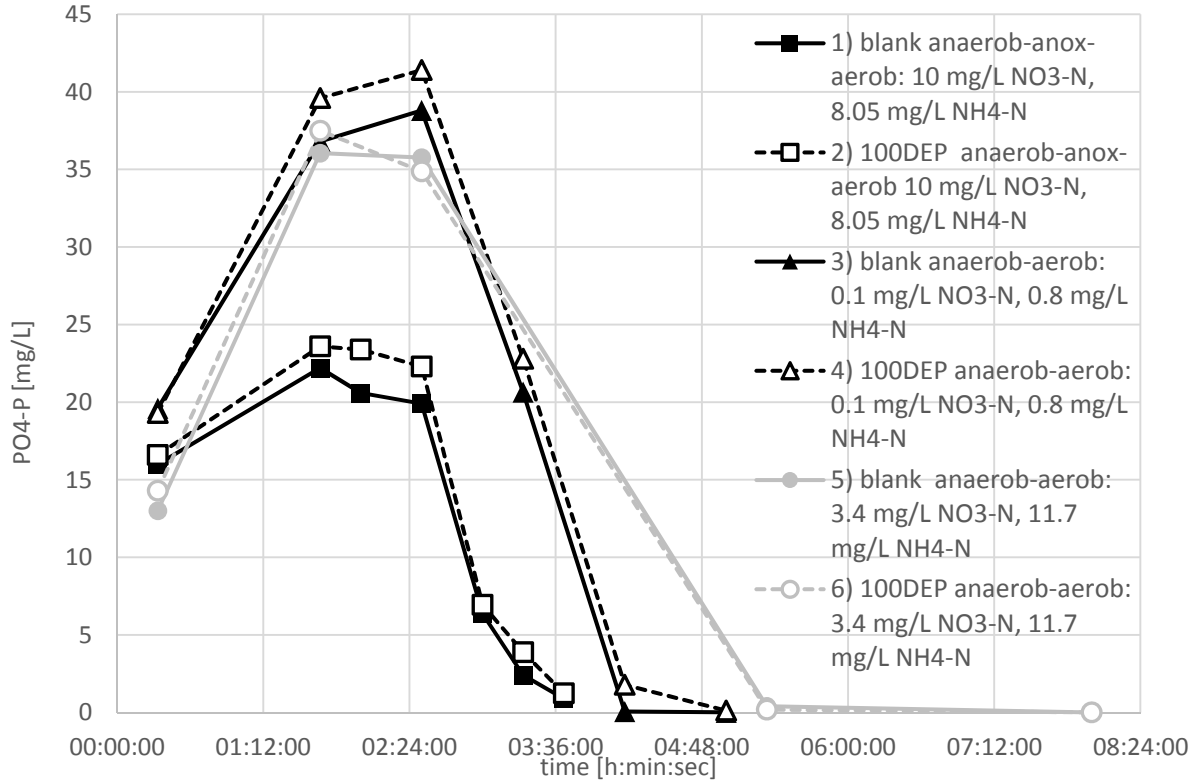


Figure 25 - P uptake and release during 3 full cycle experiments with different nitrate and ammonia doses. Nitrate was dosed after 120 minutes, ammonium after 180 minutes. The specific nitrate and ammonium doses (given in mg/L N) are listed as part of the data series' names. The last 4 data series (triangle and circle) are named anaerob-aerob, since the nitrate dose was too low to establish anoxic conditions. The data for series 5 and 6 is obtained from a run without medium.

4.6.2 Discussion

Four observations need to be discussed; firstly, the global shape of the curves (fast increase – slow increase/slump – fast decrease). Secondly, the Differences in P-uptake, dependent on the nitrate dose. Thirdly, the overall low P-concentration in series 1 and 2 and finally, the difference between spiked and blank beakers.

The shape of the curves can be explained with the theory given in 2.6.3, p. 20 about phosphate accumulating organisms (PAO). In the anaerobic beginning of the run, PAO hydrolyze the poly-P they had stored and release phosphate ions, since grey water is a more preferable nutrient source. A decrease of the P concentration towards the end of the runs is a sign for a P-uptake by the PAO; P-uptake enables them to build polyphosphate, since with an ongoing depletion of grey water as a nutrient source, they need an alternative way of storing energy. P-uptake during aerobic conditions is faster than during anoxic conditions (series 1 and 2), since only a few species of PAO are active in anoxic conditions, while all PAO can take up P under aerobic conditions. (Carvalho et al. 2007) During the aerobic phase, P assimilation can

be estimated by looking at the TOC removal. Precedent studies with the pilot plant have shown that with this specific grey water under purely aerobic conditions, 0.0171 mg P are removed per mg TOC removed. Hence, the following P assimilation can be estimated in the beakers: beaker 1: 0.0342 mg/L, beaker 2: 0.378 mg/L, beaker 3: 0.0855 mg/L and beaker 4: 0.041 mg/L. This implies P assimilation is higher for beakers in DEP. This conclusion is not valid, since removal of TOC happens faster without DEP and thus, the TOC removal in beakers 1 and 3 is low during the aerobic phase, because TOC was already depleted then. Accordingly, the P assimilation is low for the blanks. TOC removal in the later phase is higher for spiked beakers, which artificially increases the calculated P assimilation. Hence, the numbers given for assimilation are just an indication for the order of magnitude, but do not give additional information about microbial processes.

If not enough nitrate is dosed (as in curves 3 to 6), the electron acceptor needed for P uptake is missing (Kerrn-Jespersen and Henze 1993). Accordingly, with a high dose of nitrate (series 1 and 2), the P concentrations slumps after nitrate is dosed, because then an e-acceptor is available. In contrast, in the beakers 3 and 4, P-release lasts until the aeration is switched on, providing O_2 as an electron acceptor (see also Figure 5.).

The third observation, the low level of P throughout the run with a high nitrate dosing, is difficult to explain. Since the same conditions should govern all the beakers before the nitrate dosing (i.e. in the first 2h), the first 2 data points on each curve should be similar. This is not the case – the P-release even during the first 200 minutes is higher in beakers 3 to 6. Though it seems, the P-release correlates with the amount of nitrate dosed, this cannot be a causality, since nitrate is dosed later. A difference in the microbial compositions in the sludge be an explanation. This is, however, unlikely since only 3 days were between the experiments 1, 2, and 3, 4. In this time, major differences in PAO biomass are not realistic, since the operation of the pilot did not change. The other possibility would be a change in the grey water concentrate composition and P content; maybe in series 1 and 2, flock building and precipitation had caused a lower P- content in the concentrate dosed into the beaker. Yet, this is improbable, because the production of the synthetic grey water follows a strict recipe and has been carried out routinely by the same person for all the series. Furthermore, the grey water was homogenized before used in the experiments. The last possible reason includes nitrite; Nitrite can influence P-uptake, since it can also serve as an electron acceptor and enable nitrite denitrifying P-uptake (Li et al. 2006). Its presence would decrease P-release. Unfortunately, nitrite samples have only been taken in the anoxic and aerobic phase of the cycle, since this parameter was expected to be only

relevant in the context of nitrification. But in the anoxic phase, where the nitrite values are not yet influenced by nitrifying bacteria, the values for series 1 are more than twice as high (2.66 mg/L) as those obtained from a pre-test (1.25 mg/L similar conditions, samples taken at similar times, see Appendix F). This would imply that the initial nitrite level was higher in the beakers 1 and 2, which could have inhibited P-release. Nevertheless, one can doubt that 1.4 mg/L nitrite in difference can cause a difference in P-release of around 16 mg/L. (The nitrite difference will be explained in 4.7)

Similarly, the fourth observation is subject to speculation; DEP might hamper overall P-removal, since the P concentrations in the spiked beakers are higher than in the blanks in beakers 1 to 4 throughout the whole run. One might suggest that P-release is increased under the influence of DEP, since at the end of the P-release (after 2h) the difference between blank and spiked beakers is most significant. On the other hand, the similar final concentration in P suggests that the increased P-release is compensated by an increased P-uptake towards the end of the experiment. The detailed effect of DEP on P-uptake and release (e.g. a disruption of cell membranes by metabolic products of DEP (Cartwright et al. 2000) or an effect of ROS in the P metabolism) would be mere speculation. The only definite conclusion to be drawn from the data is that DEP in the tested concentration does not inhibit P removal from grey water.

4.7 Effect of DEP on nitrification and de-nitrification

The data presented in this chapter has been obtained from the same experiments as the P removal results. The numbers of the beakers (i.e. the data series) correspond to those in chapter 4.6, since the concentrations of different nutrients were measured in the same beakers. (See also Figure 9). In this following chapter, also beakers are considered, in which too little nitrate was dosed to establish anoxic conditions. Though de-nitrification did not work in these beakers and hence it cannot be considered, a “full cycle run”, these runs still give additional information about the DEP influence on nitrification.

4.7.1 Results

Figure 26 shows concentrations of nitrate and ammonium (measured in mg N/L) in spiked and blank beakers during 2 runs. For beaker 1 and 2 (rectangular series), anaerobic, anoxic and aerobic conditions were established. In beakers 3 and 4 (triangle series), only anaerobic and aerobic conditions were established.

Series 1 and 2: The nitrate concentration in the spiked and in the blank beaker is low in the beginning. After spiking 10 mg/L NO_3-N after 2h, the concentration slumps from 10.1 to 1.5 (1.6 for the spiked beaker) mg/L NO_3-N within the anoxic phase. The concentration of ammonium measured after 2h30min (i.e. in the middle of the anoxic phase) is around 1.5 mg/L NH_4-N in both beakers, since the grey water contains urea, proteins and other nitrogen containing biomolecules that are broken down to ammonium during the anaerobic phase. After the dosage of 8 mg/L NH4-N after 3h, the ammonium concentrations decreases fast down to 0.05 mg/L within the next 4h15min.

In beakers 3 and 4, the initial nitrate level is low. It remains low until 2h30min, since the nitrate dose after 2h is negligible. After switching on the aeration (after 3h), the nitrate level rises significantly to 3.8 mg/L in the following 3h. In the last 3h of the cycle, the nitrate concentration increases only slightly and reaches in the end 5.3 mg/L in the blank and 4 mg/L in the spiked beaker. Before switching on the aeration, the ammonium level lies between 2.44 mg/L at 2h and 3 mg/L at 2h30 min. 20 minutes after switching on the aeration (i.e. at 3h20min), the ammonium level is still in this range. In the following 50 minutes, however, it slumps by more than one third down to 0.99 mg/L in the spiked beaker and to 0.4 mg/L in the blank. After 5h, it levels out at 0.1 mg/L.

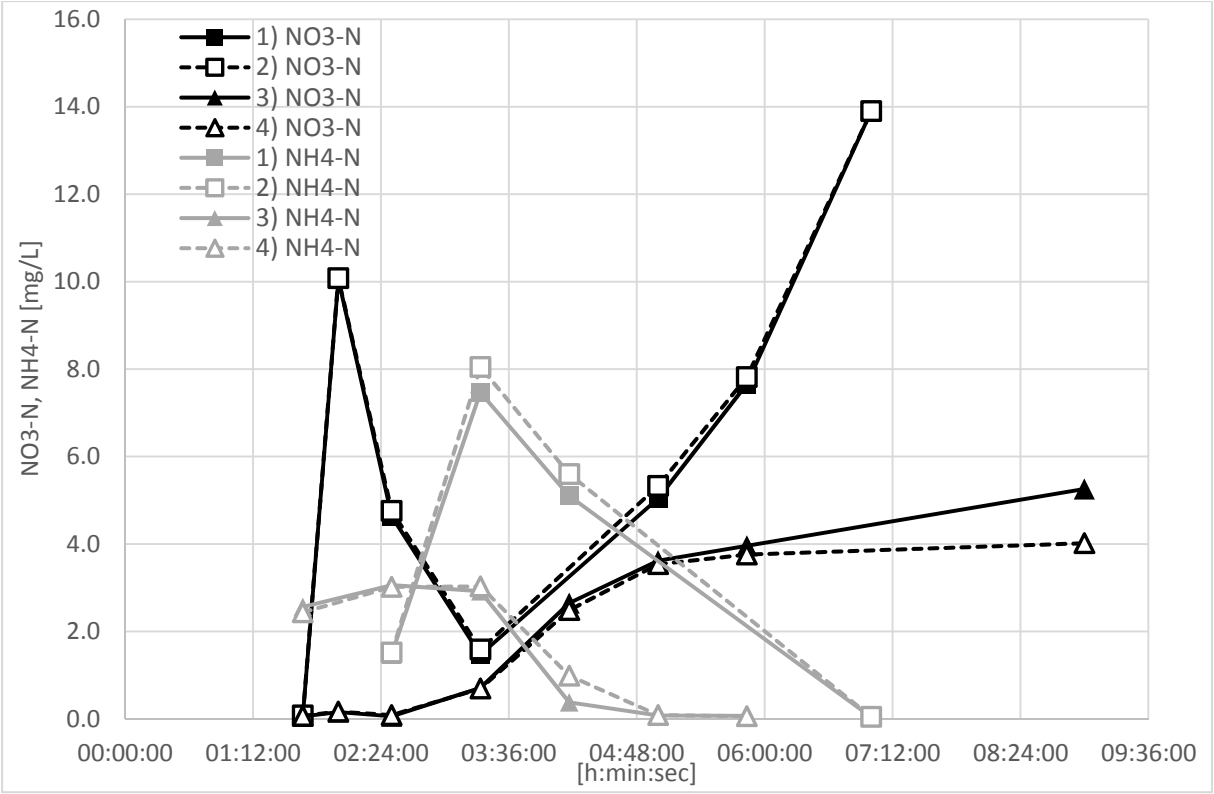


Figure 26 - Nitrification and de-nitrification during 2 full cycle experiments
Number 1 and 2 represent beakers where 10mg/L NO3 has been added after 2h, and 8 mg/L NH4 has been added after 3h. Beaker 1 is a blank, 2 contains 10 µg/L DEP. Number 3 and 4 represent beakers where 1mg/L NO3 has been added after 2h, and 0.8 mg/L NH4 has been added after 3h. Beaker 3 is a blank, 4 contains 10 µg/L DEP. So beakers 1 and 2 go through anaerobic-anoxic-aerobic conditions, beakers 3 and 4 only through anaerobic and aerobic conditions. The black curves show the nitrate concentration, the grey curves the ammonium concentrations (given in mg N/L).

4.7.2 Discussion

Three observations should be explained: Firstly, the rise and fall of the nitrate and ammonium concentrations. Secondly, the difference between the curves, when anoxic conditions are established (beakers 1 and 2) in contrast to when only anaerobic and aerobic conditions govern the beakers. Thirdly, the difference between spiked and blank beakers. (Similarly to the first 3 discussion topics in section 4.6.2, the first 2 topics here are rather an application of established knowledge. Only the last topic, the difference between spiked and blank beakers introduces genuine and new information. This is the reasons, why the first 2 observations are discussed only shortly.)

The ammonium concentration in all the beakers decreases after introducing oxygen into the system, because with oxygen as an electron acceptor, nitrifying bacteria convert ammonium to nitrite and nitrate (see 2.6.1). Accordingly, the nitrate concentration rises in the beakers to roughly the same extend as ammonium decreases. However, not all the ammonium is converted

into nitrate, but around 4 to 5 mg N is also assimilated per mg COD removed. Accordingly, considering the TOC removal during the anoxic phase (see 4.8.1, p.67) and the TOC/COD ratio previously derived for this pilot plant, the following amount of assimilated N can be estimated: Beaker 1: 0 mg/L N, beaker 2: 0.48 mg/L, beaker 3: 0.48 mg/L, beaker 4: 1.44 mg/L.

After a high dose of nitrate (beakers 1 and 2), nitrate serves as an electron acceptor for denitrifying bacteria. It is subsequently reduced to NO_2, NO, N_2O and to N_2. The plots do not show, however, which of these reduced nitrogen compounds is produced. Not all the nitrate is converted to dinitrogen, but also to nitrite. This can be proven with nitrite samples after 2h30min, where 2.66 mg/L NO2-N was measured in the blank. 2.79 mg/L NO2-N was measured in the spiked beaker (not depicted in Figure 26). This also explains, why in last 6h the nitrate level in beaker 1 and 2 increased by 12 mg/L; while the ammonium level reduced by only around 8 mg/L: This means, during the anoxic phase, ammonium was reduced to nitrite and this nitrite was responsible for the production of 2.8 mg/L nitrate in the aerobic phase. This would be in coherence and even support the theory in section 4.6.2 that nitrite hampers P-release. Since some PAO are also capable of de-nitrification (Lee, Jeon and Park 2001), a correlation between the performance of these two processes is likely. The rest of the 1.2 mg/L N missing for a correct mass balance must than go on the account of measuring inaccuracies.

The most striking difference between beaker 1 and 2 compared to beaker 3 and 4 are the overall higher levels of nitrate and ammonium and the more significant changes in concentrations within one beaker. This is trivial, since less nitrate and ammonium was dosed. In beakers 3 and 4, too little nitrate was dosed to observe denitrification. Accordingly, also less nitrite was produced and that is why for these beakers, the ammonium decreases in the last 6h to the same extend than nitrate increases. There is a difference in the ammonium level at 2h30min between the beakers with anoxic conditions at that time (1 and 2) and beakers with anaerobic conditions (3 and 4): In beaker 3 and 4 the level of ammonium is higher. This might be due to fluctuating levels of nitrogen species in the tanks of the pilot plant and thus different initial levels

There is a visible difference in ammonium levels between spiked and blank beakers for both runs. Apparently, DEP is affecting nitrification; there is less removed from spiked beakers. In addition to that, in beakers 3 and 4, also less nitrate is produced in spiked beakers. In the first half of the aerobic phase, the difference is negligibly small, but in the last 3 hours of the run, the spiked beaker contains only 86% of the nitrate concentration measured in the blank. However, this difference between nitrate levels cannot be observed in beakers 1 and 2. Here,

the nitrate level in the spiked beaker is even slightly higher than in the blank in the first 2h of the aerobic phase. In the end, spiked and blank beaker contain the same concentration of nitrate. To conclude; there is no fatal consequence of 100 µg/L DEP on nitrification or de-nitrification. There might be a slight impairment of nitrification, but this needs more repetitions to be verified.

4.8 Effect of MP on TOC removal

Measuring TOC throughout a batch experiment is another strategy to estimate microbial activity, since components of TOC serve as a substrate (carbon and energy source) in aerobic respiration. The following section presents TOC results obtained during and after the 3.5h (6h) aeration experiment as well as during and after the full cycle experiments.

4.8.1 Results

Figure 27 displays plots of residual TOC over time for the 6 beakers. The decrease of TOC is fastest in the first 15 minutes; TOC decreases by 40% in beakers with a high DEP concentration and by 70% in beakers with a low DEP concentration and in the blank. In contrast, after 15 minutes until the end of the experiment (6h), only 10 mg/L TOC was removed, i.e. no significant amount. For the three times (15 minutes, 3.5h and 6h), the residual TOC is highest in beakers spiked with 100 µg/L DEP. Here it ranges from 41 mg/L after 6h to 52 mg/L after 15 minutes. The residual TOC in beakers with a low DEP concentration ranges between 23 mg/L and 27 mg/L for the three times. In the beaker with a medium concentration of DEP, residual concentrations range between 30 and 39 mg/L for the three times. The difference between the blank beaker and the beakers with a low DEP concentration are not significant. In beakers with a high DEET concentration, the residual TOC is higher than in beakers with the low DEET concentration (keeping the DEP concentration constant). There is one exception: in beakers with a high DEP concentration after 3.5h, the increase of the DEET concentration does not influence the TOC. However, compared to the differences in TOC due to DEP, the influence of DEET seems negligible.

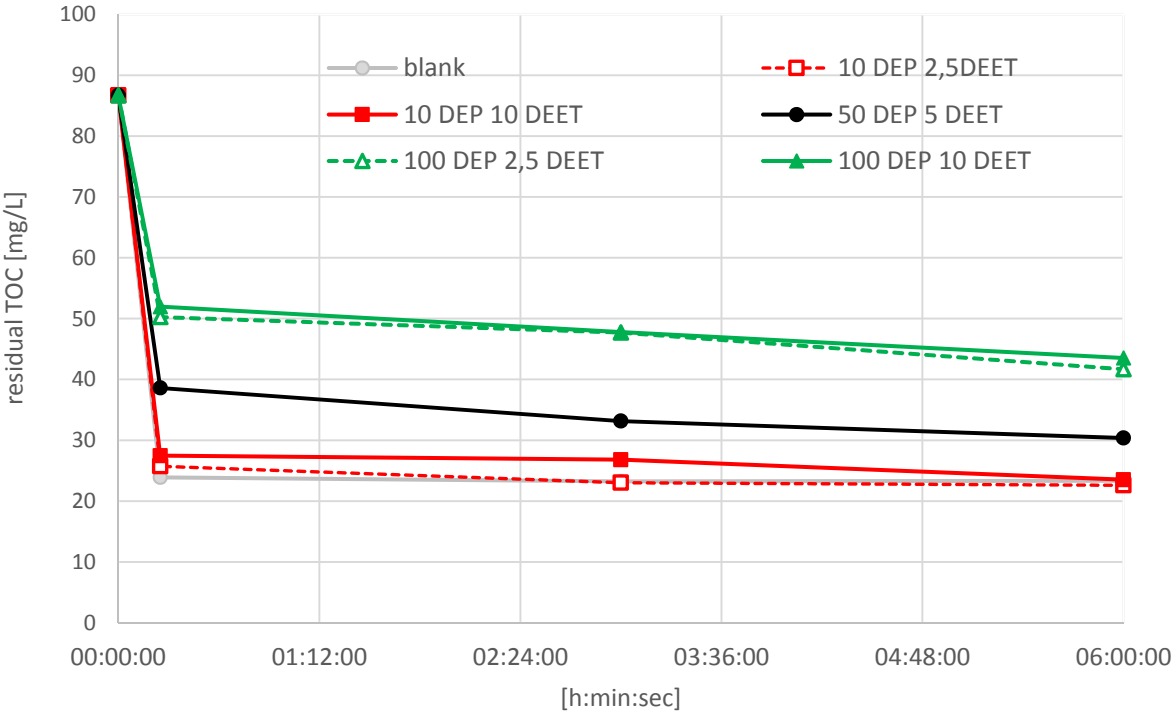


Figure 27 - residual TOC [mg/L] during aeration experiments (overview)

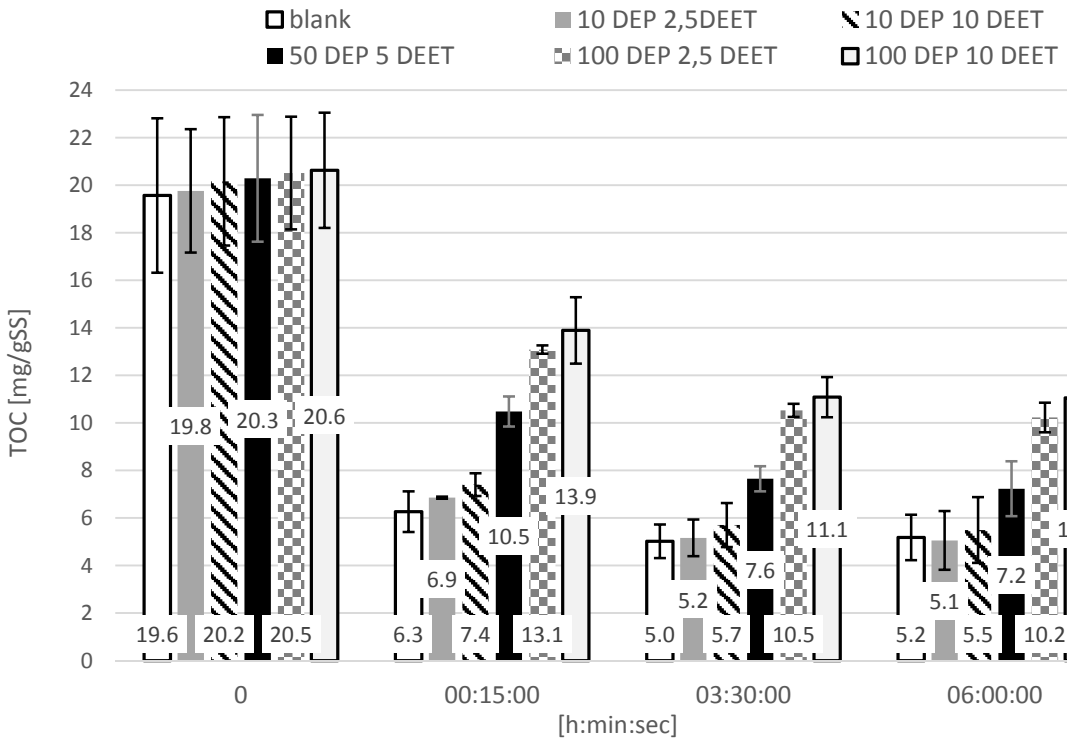


Figure 28 - residual TOC [mg/gSS] during/after aeration experiments
Residual TOC is divided by the SS content [gSS/L] of the respective beaker. The value t=0 has been calculated based on the carbon content of the raw grey water and on measurements of filtered sludge from the pilot plant. Error bars indicate the standard deviations of the repetitions. Initial concentration varied because of varying SS content. The values for averages (Figure 27) and averages and standard deviations (Figure 28) is based on the following number of repetitions: 6 (0h), 2 (0.4h), 4 (3.5h) and 3 (6h).

In Figure 28, the TOC is referred to gSS in the respective beaker. Data from three replicates is included. A pattern can be observed even after 15 minutes:

The higher the DEP concentration and the higher the DEET concentration, the higher is the average residual TOC/gSS in the beaker for each time. Only for 6h retention times, the residual average TOC for the beaker "10 DEP 10 DEET" is lower than in the beaker "10 DEP 2.5 DEET. The influence of DEET, however, is not as significant and can be neglected, considering the standard deviations. In contrast, the influence of high DEP concentrations on high residual TOC is consistent and important to notice.

As already indicated in Figure 27, main removal of TOC takes place in the first 15 minutes: within 15 minutes, between 68 and 72% of TOC is removed from beakers with low DEP concentrations. The percentage removal increases in the next 6h only slightly: 73 to 74% TOC is removed from those beakers after 6h. From beakers with high DEP concentrations, between 40 and 42% TOC is removed after 15 minutes and 50 to 52% after 6h. I.e. time matters more for TOC removal from beakers with high DEP concentration than for low DEP concentrations.

Residual TOC has also been measured during the full cycle runs. The previously presented experiments indicate that the influence of DEP on TOC removal is more severe than the influence of DEET on TOC. Accordingly, in order to look deeper in the mechanisms of the DEP influence, only DEP was spiked in the full cycle experiments, DEET was omitted.

Figure 29 shows the residual TOC [mg/L] of 6 beakers at different times during three experimental days. Solid lines represent the blanks, dotted lines represent the spiked beakers (100 µg/L DEP). The series of the three days differ in terms of nitrate and ammonium dosing (analogously to section 4.6 and 4.7). Hence, only in beaker 1 and 2 anoxic conditions were established in between the anaerobic and aerobic phase. The TOC concentrations for the spiked beakers are significantly higher than in the blanks for all experimental set-ups, throughout all times. The highest TOC concentrations (for blank and spiked beaker) are observed in the run without medium. In the beakers with a high dose of nitrate and ammonium (10 mg/L and 8 mg/L, respectively), TOC values are lowest. Series 3 and 4 (triangles) imply that the difference in TOC concentrations between a spiked and a blank beaker decreases with increasing retention time. This is less visible for the other beakers, but becomes apparent when looking at the TOC differences after 1h40min and after 5h, 8h or 9h: At the beginning, the TOC difference between beaker 1 and 2 is 36mg/L. The difference between 3, 4 and 5, 6 is around 26 mg/L and 22 mg/L. After 5h, the difference is 18 mg/L (1 and 2) and 19 mg/L (3 and 4). After 8h, the difference

between beaker 5 and 6 is 25 mg/L TOC and after 9h, the difference between beaker 3 and 4 is less than 10 mg/L. For the beakers without medium, however, the decrease in residual TOC difference is negligible. Apart from beaker 5 and 6, the TOC curves for the spiked beakers are steeper than for the blanks. This implies that TOC is removed from the blank beakers faster or earlier than from the spiked. In beakers 3 and 4, TOC decreases between 2h30min and 3h20min is more rapid then in the other time intervals. This is most likely due to the aeration, switched on after 3h. In the other beakers, the start of the aeration is not visibly influencing the shape of the curves. In this experiment, the maximal decrease of removal efficiency is witnessed; taking the first data point of series 3) as 100% possible removal in this time (1h40min), then under the influence of DEP, only 52.3 % of TOC removal is achieved.

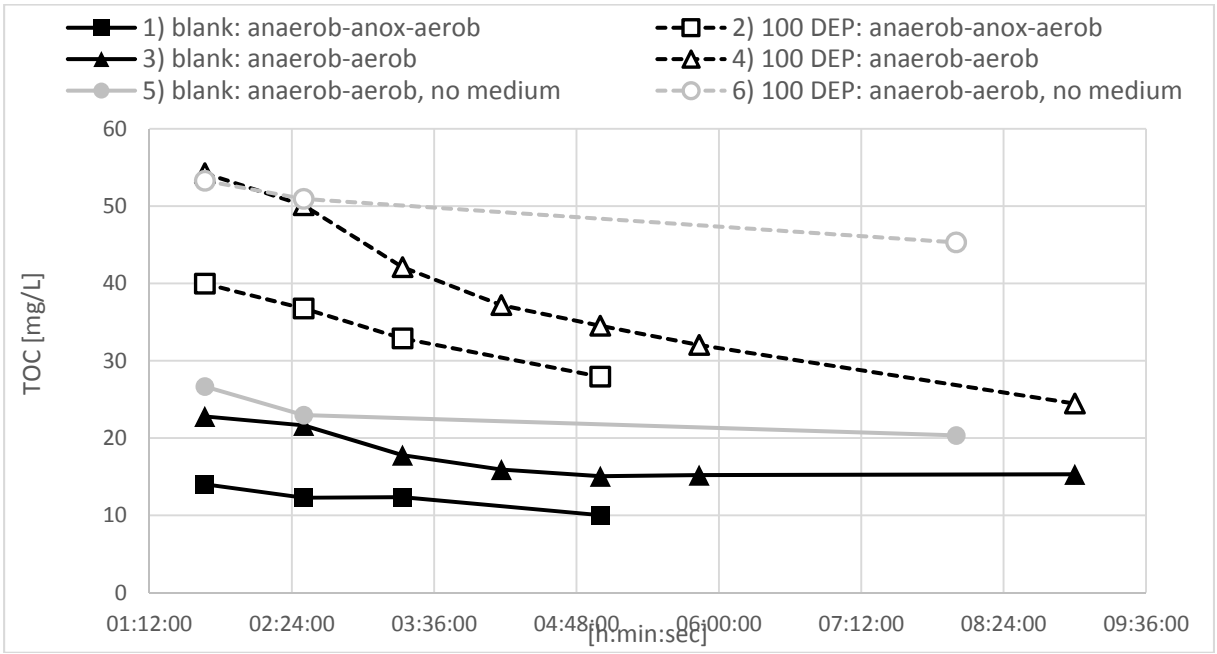


Figure 29 - residual TOC during full cycle experiments
In beakers 1 and 2, 10 mg/L NO_3-N was dosed after 2h and 8mg/L NH_4-N was dosed after 3h. Series 3 and 4 represent beakers, in which 1 mg/L NO_3-N was dosed after 2h and 0.8 mg/L NH_4-N was dosed after 3h. In beaker 5 and 6 (no medium), 3.4 mg/L NO_3-N and 11.7 mg/L NH_4-N was added. Solid lines represent blanks, dotted lines represent beakers spiked with 100 µg/L DEP. The initial TOC level (at 0:00:00) is 86.7 mg/L TOC.

Also during the OUR experiments, TOC has been measured after 5 minutes of aeration and after 30 minutes of aeration in a blank and two spiked beakers (100 and 10 000 µg/L DEP). Figure 30 shows the results for the blank and the beaker with 100 µg/L DEP. Figure 31 shows the same data series as Figure 30, but the values for the beaker containing 10 000 µg/L DEP are included. (The values are depicted in 2 different graphs, because next to the high TOC values for 10 000 µg/L spiked DEP, the details of the other series are invisible.) As it can be derived from Figure 30, the difference between the residual TOC in the blank and the spiked beaker is obvious after 5 minutes; then the TOC in the blank is 32% of the TOC in the spiked

beaker. After 30 minutes, the TOC in the blank is 29% of the TOC in the spiked beaker. Figure 31 presents high residual TOC values for 10 mg/L spiked DEP. The experiment has been repeated and resulted in similar values (see error bars). The TOC values for a dosage of 10 mg/L DEP are nearly a 100 times higher than the TOC values of the beaker containing 100 µg/L. They are around 250 times higher than in the blank after 5 minutes and 300 times higher than in the blank after 30 minutes.

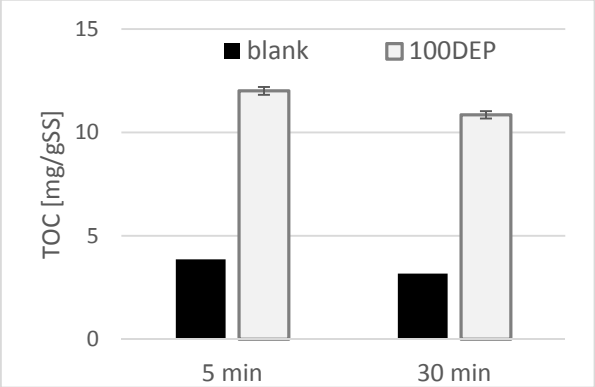


Figure 30 - residual TOC [mg/gSS] after 5 and 30 min aeration (blank and 100 µg/L DEP)

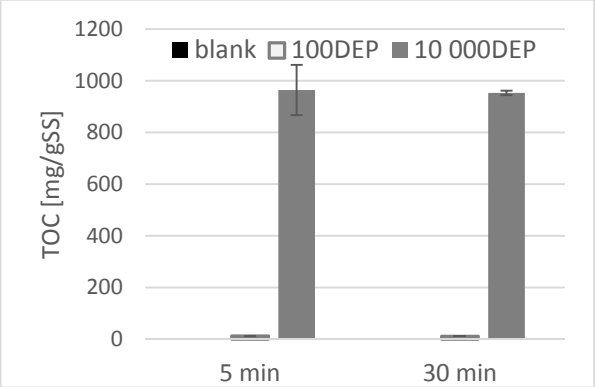


Figure 31 - residual TOC [mg/gSS] after 5 and 30 min aeration (blank, 100 and 10 000 µg/L DEP)
A dosage of 10 mg/L DEP accounts for 6.48 mg/L carbon, i.e. less than 2 mg/gSS carbon

What does the high residual TOC in the spiked samples consists of? To answer this question, the next section is dedicated to the size exclusion analysis of the residual TOC. Some of the TOC samples presented in Figure 29 have been analyzed with a liquid chromatography organic carbon detector (LC-OCD) to estimate what the TOC consists of. Each graph (Figure 32 and Figure 33) represents one full cycle experiment. Figure 32 shows results from a run, where little nitrate and ammonium was dosed (1 mg/L nitrate after 2h, 0.8 mg/L ammonium after 3h). Figure 33 shows a run, in which 10 mg/L nitrate and 8 mg/L ammonium was dosed. (The experiments correspond to series 1, 2, 3 and 4 in Figure 29.) Each chromatogram, i.e. each line on a graph, represents one TOC sample taken at a different time from a spiked or a blank beaker. The peaks/sections in the chromatograms represent size fractions of dissolved organic carbon (DOC). Yet, since a ratio TOC/COD has been derived for this system, the terms TOC and COD are exchangeable here. And since the samples taken from these experiments at specific times had been introduced as “TOC samples” in the previous chapters, they are still referred to as such in the following. However, to be accurate, TOC samples have been analyzed for COD and COD fractions.

From left to right, the fraction is becoming smaller. The area under the curve indicates the concentration of this size fraction, i.e. the percentage of this fraction of total C in the sample. The black vertical lines indicate the integration borders, the software ChromCalc uses to quantify the fractions. However, assigning bio-chemical properties to the size fractions might be arguable, since the sample matrix might differ from what ChromCalc had been programmed for. Nevertheless, for the sake of coherence, the size fractions are named according to ChromCalc and the producer of the LC-OCD (S. A. Huber 1996). Left of the first line, biopolymers (BP, >> 20 000 m/mol) are located. The next size fraction are larger humic substances. They are eluted between 30 and 45 minutes and have an estimated molar mass of 1000 g/mol. These are followed by hydrolyzed building blocks (BB) of humics. After building blocks, low molecular weight (LMW) acids are eluted. LMW neutrals as the smallest fraction come last.

Figure 32 shows the chromatogram of the full cycle run, where 1 mg/L nitrate has been dosed after 2h and 0.8 mg/L ammonium after 3h. A substantial amount of medium sized COD components (LMW acids and neutrals) remains in all the samples. There is a tendency that the signal for this COD fraction decreases with increasing time. (Exceptions are the samples after 1h40 min and the blank after 2h30min.) Until an elution time of around 70 minutes, there is no general difference between samples from the spiked beaker and the blank. After that – between minute 70 and 80 – the samples from the spiked beaker (dotted lines) show a significantly high signal; the quantified amount (see Table 13) of LMW neutrals in the COD of the spiked beakers is 6.5 times higher than in the COD of the blank. (1h40min). The peak in the samples taken from the spiked beaker decrease with time; after 9h, the amount of LMW neutrals in the spiked beaker's COD are 3.1 times higher than in the blank beaker.

Figure 33 shows chromatograms of a full cycle, where 10 mg/L nitrate and 8 mg/L ammonium were dosed. Compared to Figure 32, the signals for big and medium sized TOC molecules in all the samples are lower. This global difference between Figure 32 and Figure 33 is probably due to higher denitrification efficiency in exp. II due to a higher ammonium dose. This might have consumed additional carbon. (That is not important for identifying the residual TOC left after DEP spiking, hence it will not be subject in the discussion.) For the blanks, also the signals caused by small molecules are low. In the spiked beaker, however, high signals for LMW neutrals are detected; the concentration of this COD fraction in the spiked beaker is more than 8.5 times higher than in the blank. The signal decreases with time, so that after 9h, the amount of LMW neutrals detected in the spiked beaker is 6.1 times higher than in the blank.

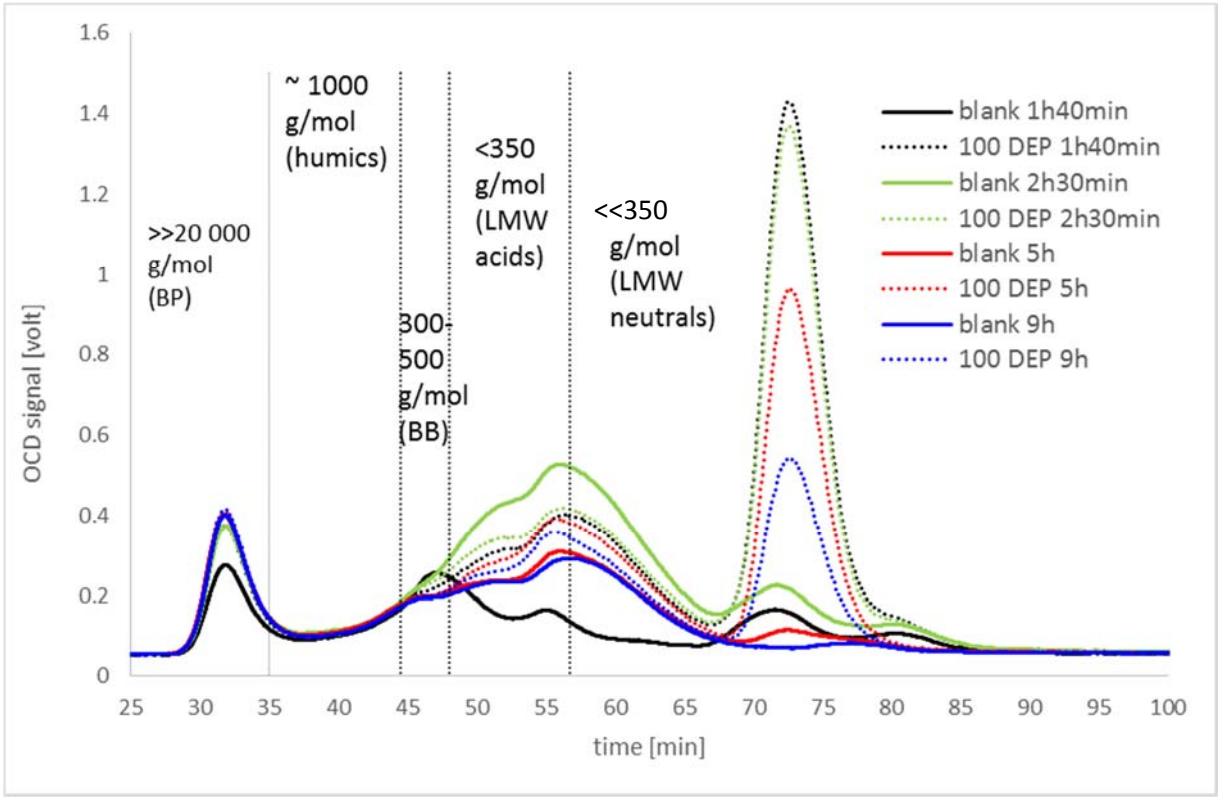


Figure 32 - LC-OCD analysis of residual TOC of blank and spiked beaker (100 µg/L DEP), measured at 4 different times. (Exp. I)
Anaerobic - aerobic, dosage of 1 mg/L nitrate, 0.8 mg/L ammonium. BP=Biopolymers, BB= building blocks (hydrolyzed humics), LMW= low molecular weight

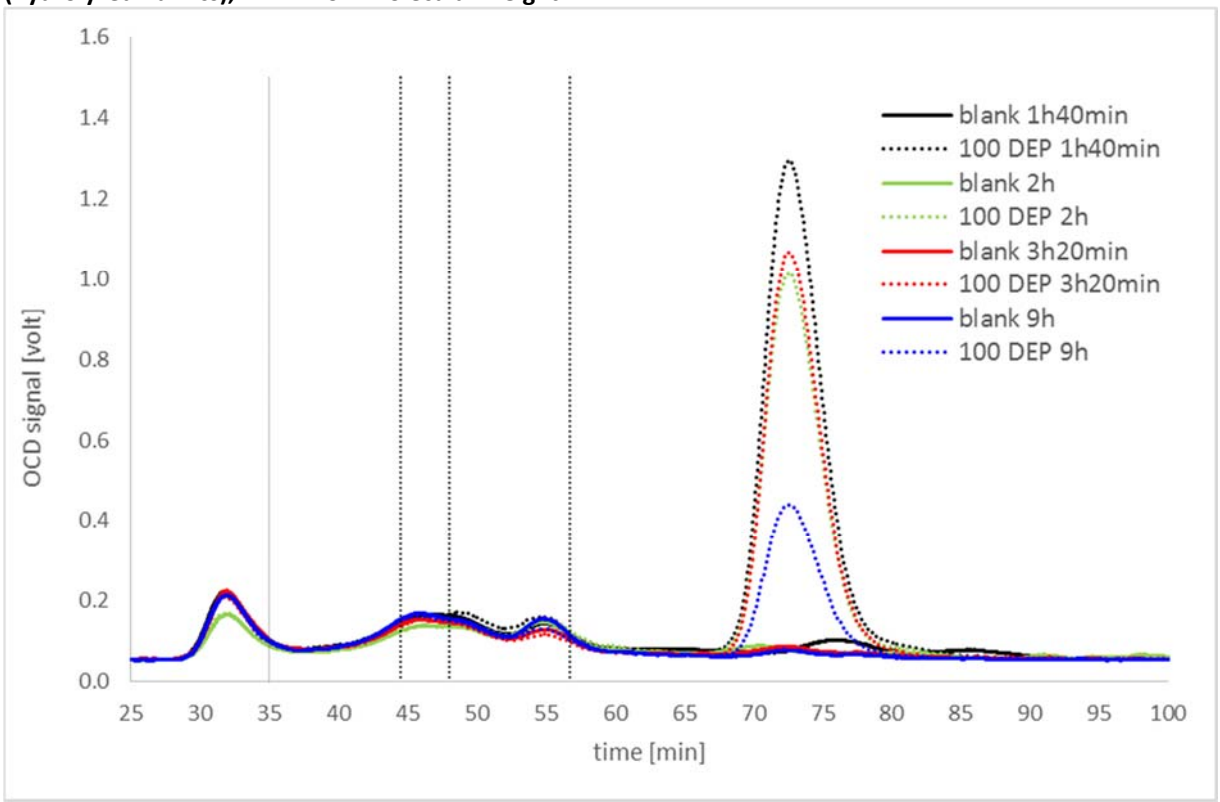


Figure 33 - LC-OCD analysis of residual TOC of blank and spiked beaker (100 µg/L DEP), measured at 4 different times. (Exp. II)

Anaerobic - anoxic- aerobic conditions, dosage of 10 mg/L nitrate, 8 mg/L ammonium.
Table 13 - LOC-OCD results
Size exclusion analysis of two full cycle runs (integration of the chromatograms in Figure 32 and 33.): low dosage of nitrate/ammonium (exp I) and high dosage of nitrate and ammonium (exp II). TOC sample taken at different times have been analyzed for fractions of DOC according to molecular weight. Carbon content of fractions are given in percent of total dissolved carbon DOC and in ppb. LMW=low molecular weight.

Exp. I) anaerobic - aerobic, dosage of 1 mg/L nitrate, 0.8 mg/L ammonium

		Biopolymers BP (>>20 000 g/mol)	Humic substances HS (~1000 g/mol)	Building blocks BB (300-500 g/mol)	LMW acids (<< 350 g/mol)	LMW neutrals (< 350 g/mol)
blank	ppb-C	4429	4684	4307	n.q.	5723
1h40min	% DOC	23.1%	24.5%	22.5%	--	29.9%
100 DEP	ppb-C	6000	6486	12333	63	37195
1h40min	% DOC	9.7%	10.4%	19.9%	0.1%	59.9%
blank	ppb-C	6156	6970	17541	368	13874
2h30min	% DOC	13.7%	15.5%	39.1%	0.8%	30.9%
100 DEP	ppb-C	5991	5732	13996	294	35576
2h30min	% DOC	9.7%	9.3%	22.7%	0.5%	57.8%
	ppb-C	6338	6239	8502	n.q.	5810
blank 5h	% DOC	23.6%	23.2%	31.6%	--	21.6%
100 DEP	ppb-C	6320	5524	11156	844	24281
5h	% DOC	13.1%	11.5%	23.2%	1.8%	50.5%
	ppb-C	6255	5572	8264	198	4723
blank 9h	% DOC	25.0%	22.3%	33.0%	0.8%	18.9%
100 DEP	ppb-C	6494	5746	9784	708	14788
9h	% DOC	17.3%	15.3%	26.1%	1.9%	39.4%

Exp. II) anaerob - anoxic - aerobic, 10 mg/L nitrate, 8 mg/L ammonium

		Biopolymers BP (>>20 000 g/mol)	Humic substances HS (~1000 g/mol)	Building blocks BB (300-500 g/mol)	LMW acids (<< 350 g/mol)	LMW neutrals (< 350 g/mol)
blank	ppb-C	3251	4146	2190	9	3216
1h40min	% DOC	25.4%	32.4%	17.1%	0.1%	25.1%
100 DEP	ppb-C	3143	4146	2801	27	27532
1h40min	% DOC	8.3%	11.0%	7.4%	0.1%	73.1%
	ppb-C	2169	3464	1965	n.q.	2342
blank 2h	% DOC	21.9%	35.0%	19.9%	--	23.7%
100 DEP	ppb-C	2312	3863	2074	80	21528
2h	% DOC	7.7%	12.9%	6.9%	0.3%	72.1%
blank	ppb-C	3242	4012	1593	n.q.	2035
3h20min	% DOC	29.9%	37.0%	14.7%	--	18.7%
100 DEP	ppb-C	2944	3638	1407	n.q.	21719
3h20min	% DOC	9.9%	12.3%	4.7%	--	73.2%
	ppb-C	3139	4406	1710	334	1416
blank 9h	% DOC	28.5%	40.0%	15.5%	3.0%	12.9%
100 DEP	ppb-C	3009	4089	1636	n.q.	8615
9h	% DOC	17.4%	23.6%	9.5%	--	49.8%

4.8.2 Discussion

The results are consistent: throughout 10 different retention times, 4 DEP dosages, five different set-ups (including replicates), DEP leads to an increased residual TOC value. Compared to DEP, the influence of DEET is minor and is thus not discussed in detail.

The spiked compounds could increase the carbon content in the solution because they contribute with carbon bound in the molecule. This is, however, impossible, since even the very high concentration of DEP (10 mg/L) contributes with 6.48 mg/L C. In contrast, the difference between the residual TOC in the beakers is 500 times higher than these 6.48 mg/L. The same is true for the metabolites; regardless of the molecules' degradation pathway (compare 2.7.2 and 2.8.2); none of the possible metabolites can cause such a high TOC difference, since also the metabolite's concentration would range between 10 and 100 µg/L (or 10 mg/L). The little amount of spiked DEP also rules out the idea that it might serve as a solemn carbon source (as shown in Cartwright et al. (2000)) instead of TOC. It is impossible that the microbes ignore TOC and digest DEP instead – there is not enough DEP present. DEP might also inhibit TOC degradation; it could be metabolized as a competitive inhibitor (see 2.5.3). That means it binds onto reactive sites on catabolic enzymes that would have otherwise been occupied by TOC. DEP as an alternative carbon source or as a competitive inhibitor is a reasonable explanation why DEP is removed to a large degree during the aeration experiment and completely during the full cycle experiments; and since adsorption is low – only 2 µg/gSS – it must have been degraded.

Another possible explanation is that DEP inhibits bacterial metabolism not by blocking active sites, but non-competitively preventing TOC utilization; DEP has been reported to inhibit chitobiase, an enzyme needed to break down chitin. The repression of this enzyme affects arthropods and mollusks negatively. (Zou and Fingerman 1999) However, the organisms in the cited studies were exposed to doses of DEP that are orders of magnitude higher than the doses in the presented experiments. Though chitobiase is expressed in bacteria (Joshi et al. 1989, Kourtev, Ehrenfeld and Huang 2002, Toratani et al. 2008) and in fungi (St. Leger, Cooper and Charnley 1991) it might not be relevant in activated sludge, since there is no chitin in the mixed liquor that could induce its expression. This enzyme is, however, is also used to measure general enzyme activity that could also be related to C, N and P cycling (Kourtev et al. 2002). Hence, these studies support the idea that DEP has detrimental effects on metabolism. Hence, it is

realistic that similar, more relevant enzymes also are inhibited which then suppresses catabolic reactions.

It is out of the scope of this project to measure the influence of DEP on one specific enzyme. But also more generally speaking, DEP is likely to have a non-specified impact on bacteria; microtox results presented by Acros (2015) imply that DEP has a negative effect on vibrio fischeri (EC50=112 mg/L, 30 minutes exposure). In Addition, DEP inhibits the activity of antioxidant enzymes glutathione peroxidase, glutathione reductase and superoxide dismutase. Those enzymes protect cells from endogenous oxidative stress. (N. Premjanu 2014) It is very plausible that an impairment of these enzyme activity causes a strong damage in the cells, since DEP has been proven to cause oxidative stress (Kang et al. 2010). DEP might not only cause oxidative stress (see 4.5.2), but can also prevent the cell's defense against it. To sum up; taking into consideration the reported negative effect of DEP on cells, it seems likely that DEP inhibits microbial degradation of TOC.

Nevertheless, all the so far discussed reasons – co-metabolism, inhibition, and detrimental effects on enzymes – could only explain why TOC is not removed from the beakers, but stays the same. However, even assuming all of it happens at the same time, it cannot explain, why the residual TOC after 10 mg/L DEP dosage (see Figure 31) is much higher than what initially had been in the beaker (taking into account the TOC contribution of the grey water, sludge and spiked amount of DEP). I.e. the above-mentioned reasons cannot explain an increase compared to initial TOC.

These are other possible reasons for the TOC increase, which will be elaborated in the coming paragraphs; the microbes might increase EPS production. More specifically, they might release damaged cell material (e.g. lipid peroxide produced under oxidative stress) or lyse. To judge, which of the reasons is most likely, the LC-OCD results are discussed.

The LMW molecules detected with the LC-OCD are most likely responsible for the high residual TOC levels measured in the beakers. These molecules could be part of additional EPS excreted by the microbes. EPS contains molecules of a wide range of sizes, but Stewart et al. (2013) show with an LC-OCD analysis that LMW neutrals are the main fraction of EPS. Pasquini et al. (2013) reported that already 0.5 µg/L of MP induce an increased production of bound EPS in activated sludge flocks. Though they did not study DEP but other MP contained in household chemicals, it is likely that the microbes show a similar response to DEP. An increased production of EPS can be interpreted as indicator of bacterial sensitivity towards toxic agents (Avella et al. 2010), since EPS is a buffer against environmental changes (Wingender,

Neu and Flemming 1999). Apart from bound EPS, microbes also produce loosely bound EPS and soluble substances (soluble microbial products, SMP). EPS and SMP contain chemically similar compounds. In activated sludge both EPS and SMP contain lower molecular weight molecules (<1000 g/mol) like polysaccharide. Biopolymers >20 000 g/mol account for around 1/3 of EPS and SMP. (Tsai, Chang and Lee 2008) Accordingly, the observed increase in LMW molecules in the presented study can also mean an increased SMP production. This is even more plausible than an increase in EPS, since bound EPS forms the matrix of the biofilm and assures its functional integrity (Wingender et al. 1999). In the presented study, there was a carrier present; hence the analyzed suspended sludge might have contained more SMP than EPS, since EPS would be merely present in the biofilm on the carrier. Aquino and Stuckey (2004) report an increase of SMP and EPS in an anaerobic bioreactor after exposure to chromium and chloroform. They also witnessed an "overwhelming" increase in volatile fatty acids, which they do not consider part of EPS/SMP. This supports the thesis that overproduction of EPS/SMP is a sign for microbial sensitivity. The EPS overproduction might prevent the microbes from negative impacts by DEP. This is likely, since DEP is fully degraded, despite high residual TOC.

On the other hand, the volatile fatty acids could be secondary products of lipid peroxidation of the cell membrane (Marisa Repetto 2012). This can be a sign of a harmful effect on the microbes. Kang et al. (2010) confirm that DEP increases the production of lipid peroxides. Putting this together, it leads back to the theory presented in 4.5.2 (p.56) that DEP triggers oxidative stress. Oxidative stress creates shorter lipids out of the cell wall's phospholipids. Lipid peroxidation can also lead to the formation of other volatile hydrocarbons (Frankel and Tappel 1991). Those might then be detected as LMW molecules in the residual TOC after DEP spiking. Lipid peroxidation could also trigger cell lysis (Marisa Repetto 2012). Lysis products would additionally contribute to a high residual TOC. Accordingly, the TOC results - especially the size fractionation with the LC-OCD – support the explanations for the previously observed high consumption of oxygen in spiked beakers.

To sum up this discussion: The high residual TOC might be an indicator for EPS overproduction and a successful microbial defense against DEP. However, it might also indicate an increase of damaged cell material or lysis.

At any rate, the phenomenon of strikingly high residual TOC containing LMW molecules might be relevant for a large-scale application. In full-scale, even low concentrations of DEP could impair the TOC removal capacity of the treatment system.

5 Conclusion

Concerning the removal of DEET and DEP during the 3.5h and 6h aeration experiments, the following conclusions can be drawn:

- Depending on the spiked concentration, between 11.7% and 29.4 %, i.e. between 0.12 µg/gSS and 0.4 µg/gSS of DEET are removed. DEET is not removed by evaporation or adsorption.
- At minimum, 11.7% (13.4 %) DEET, i.e. 0.12 µg/gSS (0.17 µg/gSS) is removed during 3.5h (6h) aeration.
- At minimum, 86.9% (100%) DEP, i.e. 2.2µg/gSS (2.3 µg/gSS), is removed during 3.5h (6h) aeration. 6.6% DEP, i.e. 2 µg/gSS, adsorb onto sludge.
- Given the possible adverse effects of DEET and the risk of up-concentration during multiple grey water reuse for high body contact applications.
- In contrast, DEP removal is sufficiently high to prevent up-concentration.

As for the impact of DEP on microbial processes, the findings can be summarized as follows:

- DEP does not significantly influence nitrification or de-nitrification.
- DEP does not impair biological P removal.
- Oxygen uptake rates (OUR) and ATP measurements in spiked beakers suggest that DEP causes an increased oxygen consumption and a decreased metabolic activity. This, however, has to be verified with more replicates.
- Beakers exposed to >50 µg/L DEP for >15 minutes contain significantly more residual TOC than blanks. 100 µg/L DEP cause between 9 mg/L and 39 mg/L higher TOC concentrations, depending on the retention time. At maximum, the TOC removal efficiency of the system is impaired by 47.7%.
- Size exclusion analysis shows that this residual TOC in spiked beakers consists of neutral low molecular weight (LMW) components. These components might be products of lipid peroxidation of cell walls or products of cell lysis, both due to oxidative stress. That would be coherent with the observation that DEP triggers an increased oxygen uptake. It would mean that DEP is harming the microbial community.
- There is a second interpretation of the high residual TOC levels in spiked beakers: The LMW neutrals could also indicate an overproduction of extracellular polymeric substances as a successful defense against the environmental stress factor DEP. That

would be coherent with the observation, that DEP is nearly fully degraded. It would mean that exposure to DEP is not fatal for the microbes.

6 Future work

A number of observations has not been elucidated completely with this thesis. The most interesting and relevant observation – that DEP hampers TOC removal – should be researched further:

The residual TOC after spiking with DEP should be analyzed in more detail. It should be cleared, whether it contains toxic DEP metabolites. This necessitates the development of a new SPE and HPLC method for.

It has to be studied whether DEP is harmful for the microbes or whether it triggers overproduction of EPS, without impairing microbial metabolism; to determine, whether the LMW neutrals are lipid peroxides, other products of cell damage or increased EPS, these compounds should be analyzed with an LC-OCD. Then chromatograms should be compared. Also, a method for EPS separation and quantification of the contained size fractions would be useful.

To verify the theory that DEP causes oxidative stress and leads to a higher oxygen consumption and cell damage, replicates of the OUR experiments are needed. ATP should be measured more often.

With respect to a full-scale application, it would be important to know, whether the microbial community adapts to the DEP dose and increases its TOC removal after adaption. Therefore, more full-cycle batch experiments could be carried out continuously in a row. DEP and grey water concentrate would have to be re-fed e.g. after 9h. TOC values in a blank and a spiked beaker should then be compared over a longer time period of e.g. several days.

DNA sequencing during the continuous exposure to DEP could be helpful to indicate, which enzymes are induced or repressed due to the presence of DEP. This would convey information about the damage to cells or anti-oxidative defense mechanisms.

However, one should bear in mind that any further research in this direction, if anything, only improves only the treatment. Non-technical solutions like limiting the consumption and emission of MPs also lowers their concentration in the aquatic environment. Instead of spending more time and research funds in technical solutions, avoidance strategies, production limits and changes in consumption habits should be screened for their potential to decrease MP concentrations.

Appendix A. Pilot plant flow chart

For a better understanding of how the sludge and the grey water used in this study were generated, a flow scheme is provided in

Figure 34.

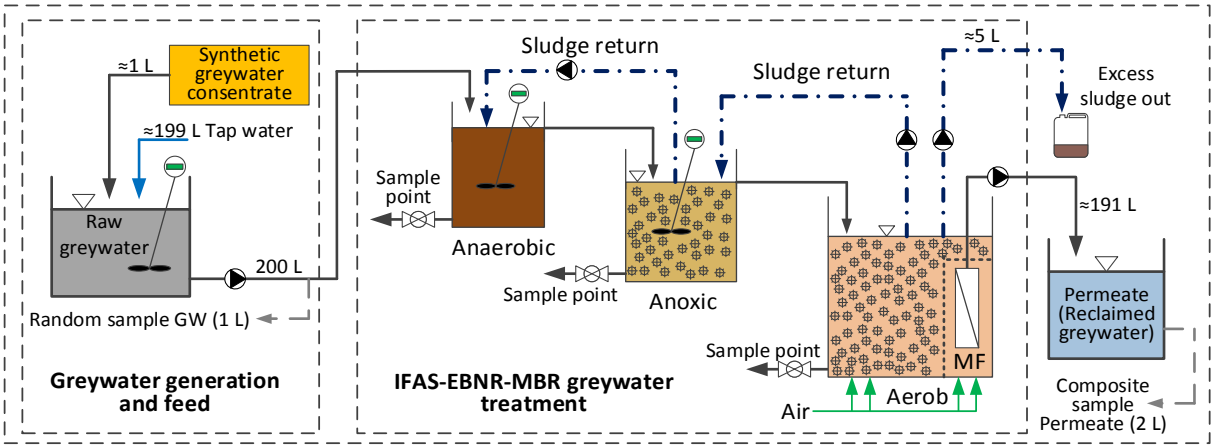


Figure 34 - flow scheme pilot plant

Grey water used in the experiments was either taken from the raw grey water tanks, or generated from the synthetic concentrate. Sludge was taken out of the aerobic reactor. The calibration curves were derived from standards made of permeate. (Drawing used by courtesy of Viggo Bjerkelund.)

Appendix B. SPE Method

As a preparation for the master thesis, a project has been carried out to develop a method for purification and up-concentration. After this solid phase extraction (SPE), micropollutants could be measured in the samples. Since the SPE method was a time consuming step during the practical part of the thesis and also one of the biggest challenges in the project, a longer appendix is dedicated to it.

Theory SPE

SPE is based on selective sorption of the analyte of interest: Big sample volumes are passed through a cartridge which contains a well-packed bed. In this bed, the analyte is retained, while some impurities of the matrix are flushed through. Yet, other impurities also interact with the solid phase in the cartridge bed and are also retained. Those can be washed out and thus separated from the analyte by washing the cartridge with solvents. This washing solution separates impurities from the solid phase, but not the analyte. Thereafter the cartridge is eluted with a solvent that interacts strongly with the analyte and detaches it from the solid phase. The elution solution is captured and analyzed. Eluting with a small volume (compared to the sample volume) allows for up- concentrating the analyte. The SPE also has a filtering effect by trapping large particles and inhibiting them from entering the analyte solution. (Majors 2010)

Figure 35 shows the procedure of SPE. Both DEP and DEET are sampled in the same cartridges and run through the same SPE procedure.

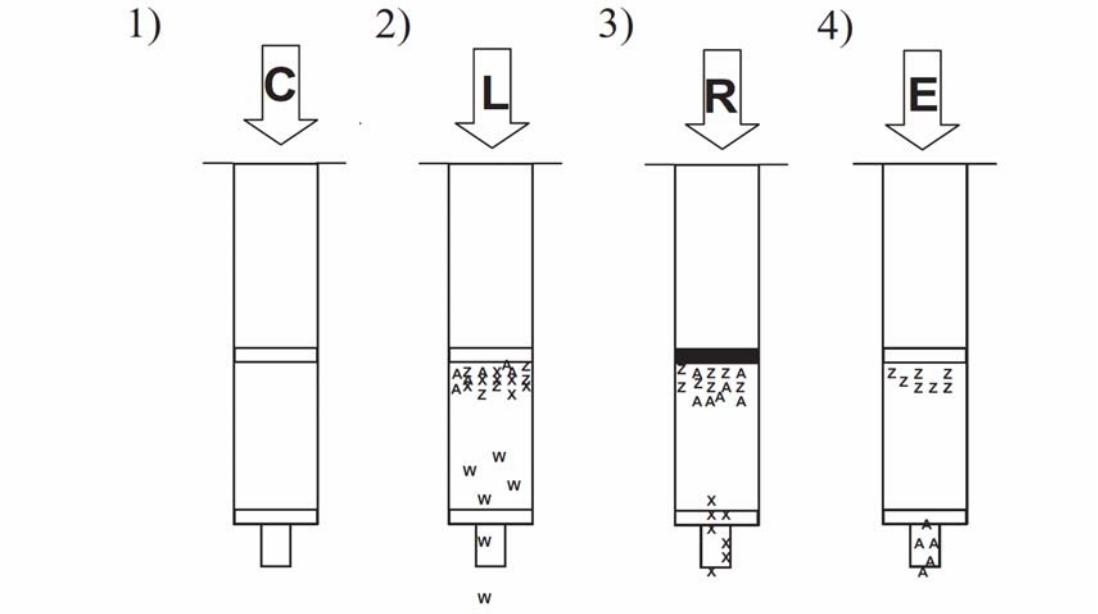


Figure 35 - SPE steps in the developed method:
1) Conditioning with conditioning solvent (C), 2) Sampling with sample (L) containing undesired matrix component (W-weakly retained, Z-strongly retained, X-medium retained) and analyte (A) 3) Washing with washing solution; undesired matrix component X is washed out 4) Elution with eluting solvent (E), adopted to analyte, which is washed out while Z is still retained. (Figure modified according to (Majors 2010))

SPE method development

To develop a method, first a cartridge type (i.e. a mode such as reverse phase, ion exchange etc.), and a type of solvent has to be chosen according to decision trees. These decision trees can be found in handbooks and brochures provided by equipment suppliers (Supelco 1998) or in analytical chemistry books (Majors 2010). After choosing the mode, the four steps (Figure 35) have to be optimized. Therefore, different interactions have to be evaluated: matrix-sorbent, sorbent-analyte, analyte-matrix interactions, as well as interactions between the solvents with all the other entities. The interactions are governed by chemical properties of the entities (pKa values, polarity, K_{OW} value, etc.), which cannot be fully elaborated in this context. It should, however, be stressed, that for evaluating these interactions and optimizing the four steps, tests are suggested in handbooks; gradually increasing washing volume and plotting the washed-out concentration with respect to volume helps to determine washing volumes. A similar breakthrough curve can be constructed for the solvent composition by increasing the amount of solvent (e.g. MeOH) in the washing solution gradually. (Majors 2010)

Method accuracy: Limit of quantification and detection

After the method had been developed, its accuracy and usability was evaluated. The limit of quantification (LOQ) and the limit of detection (LOD) was determined by running 10 RGW samples spiked with concentrations estimated to be close to the detection limit (3µg/L DEP and 0.75 µg/L DEP). A factor R has been determined according to:

$$R_{LOQ} = \frac{Average_{peak\_area}}{3 \cdot STD} \quad (0.14)$$

$$LOQ = 10 * STD[mAu] \quad (0.15)$$

$$LOD = 3 * STD[mAu] \quad (0.16)$$

With

Average peak area [mAu]	referring to the areas obtained from HPLC measurement
STD	being the standard deviation of these areas
R	3 < R < 10 for an accurate method

The obtained values for LOQ and LOD in signal areas (mAu) have been converted after the construction of the calibration curve into concentrations (µg/L). The parameters describing the method accuracy are summarized in Table 14.

Table 14 - evaluation of method: R, LOQ, LOD, STD (10 samples)

	STD [mAu]	LOD [mAu]	LOD [µg/L]	LOQ [mAu]	LOQ [µg/L]	R	Recov.[%]
DEET	4.93	14.79	0.26	49.29	0.795	2.8 *	72.5
DEP	3.07	9.22	1.01	30.72	2.12	6.23	103

*6.4 is the R value obtained with areas that are not adjusted for DEET background in the blank sample

After a satisfactory R was obtained for both compounds, the method was used to construct a calibration curve with 9 different concentrations of DEP and DEET (excluding blanks). Figure 36 shows the calibration curve. The high R values (R> 0.99) are satisfactory. In different grey waters, different backgrounds of DEET could be found in the blank sample. Thus, the areas used for the DEET calibration curve are the adjusted areas obtained after subtracting this DEET background. Accordingly, this calibration curve is only valid for signal areas that are likewise adjusted for the background.

Calibration curves

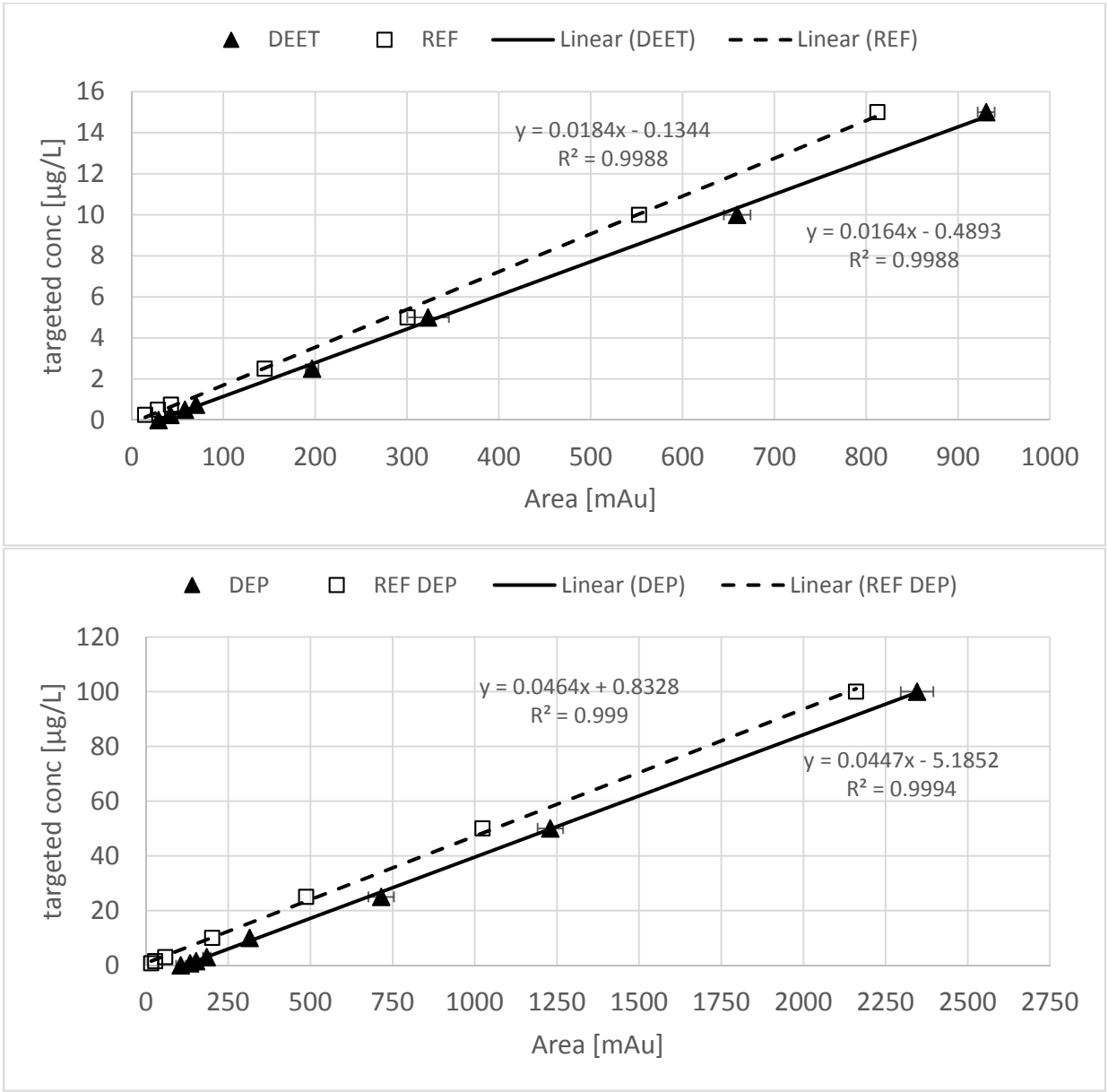


Figure 36 - Calibration curves DEET and DEP.
As references (REF), MQ was directly spiked with the compounds (without applying the method). The black curves were used as a basis to determine concentrations. They were created with standards in recycled grey water, which was up-concentrated and purified with the SPE method.

Appendix C. Adsorption (thermal inactivation)

In one of the inhibition experiments the sludge was heated in a metal bucket hanging in a water bath for 30 minutes, until 70□C were reached. 70□C were kept for 10 minutes.

Figure 37 and Figure 38 show percentage and absolute removal per gram suspended solids. For DEET the percentage removed decreases with increasing concentration in the beaker. The absolute removal in µg/gSS is in the same range for different concentration. In contrast, the absolute removal of DEP increases with increasing concentration. Similar to DEET, the percentage removal decreases with increasing concentrations. Thus, the adsorption of DEP is more concentration dependent than the adsorption of DEET.

However, the thermal inactivation must have altered the rheology of the sludge, as suggested in (Hamon et al. 2014); firstly, the sludge had changed its physical properties. It was harder to separate from the liquid phase by centrifugation compared to active sludge. Secondly, the total removal of DEET was higher with inactivated sludge (only adsorption) than with activated sludge (adsorption and biological degradation). This would not be possible, if the adsorption capacity of the sludge did not increase by heating it up.

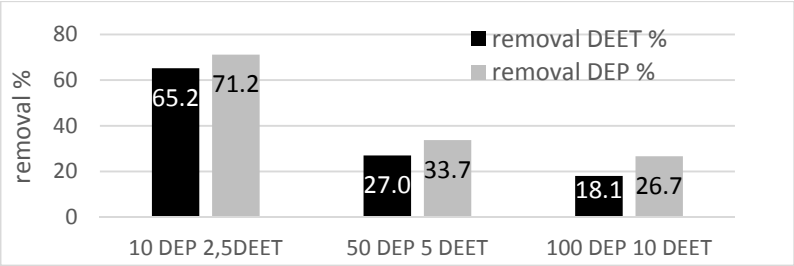


Figure 37 - adsorption after pasteurization percentage removal. Note: thermal inactivation alters the adsorption properties (Hamon et al. 2014).

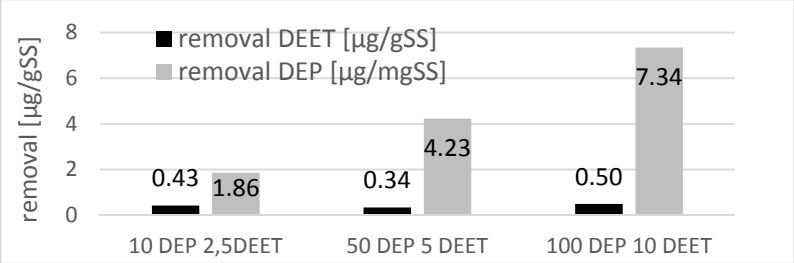


Figure 38 - adsorption after pasteurization [µg/gSS] removal. The adsorption µg/gSS of DEP is more concentration dependent than the adsorption µg/gSS DEET.

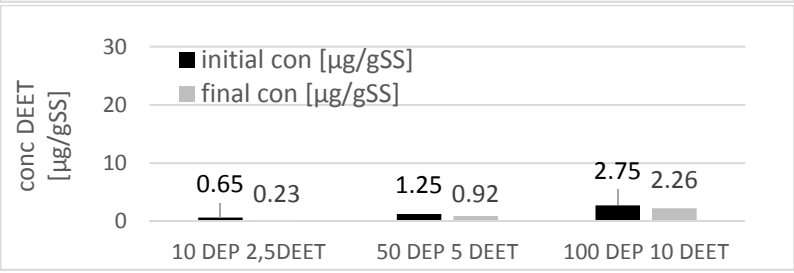


Figure 39 - adsorption initial and final conc. DEET
Enables comparison with Error! Reference source not found.

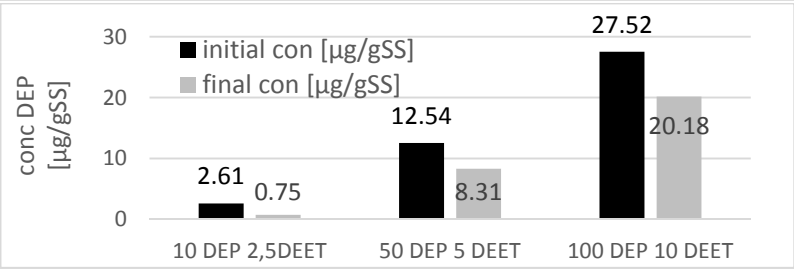


Figure 40 - adsorption initial and final conc. DEP
(Compare with Error! Reference source not found.**) Absolute reduction of DEP increases with increasing concentrations.**

Appendix D. Oxygen uptake rate (OUR) variation

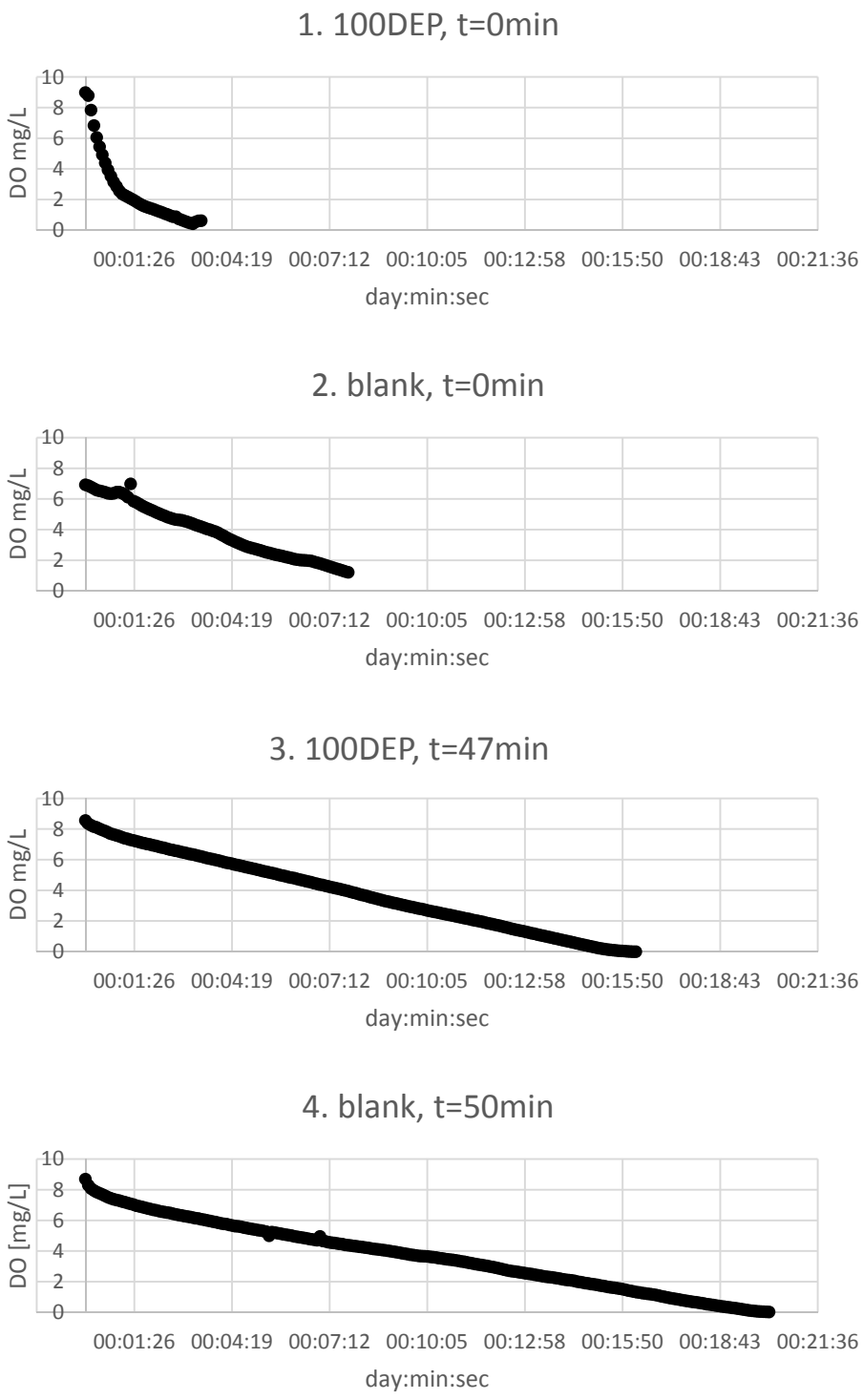
Apart from the OUR set-up described in 3.4.4 and its results presented in 4.5, a second variation of an OUR experiment has been carried out:

A blank beaker and a beaker spiked with 100 µg/L DEP were aerated for 12 hours. Initially, they contained 0.67L aerobic sludge and 0.33L grey water. After 6 and 12 hours, an amount of grey water concentrate (i.e. the ingredient of the pilot plant's grey water) was added to both of the beakers. This "re-feed" was equivalent to 0.33L grey water with respect to TOC, nutrients and salt content. In the experiment the concentrate was added twice ("first and second re-feed"). OUR was measured 5 seconds after adding grey water to the (spiked) sludge, after 50 minutes, after 6 hours (right after first re-feeding with grey water), after 9 hours, and after 12 hours (before second re-feeding as well as after second re-feeding).

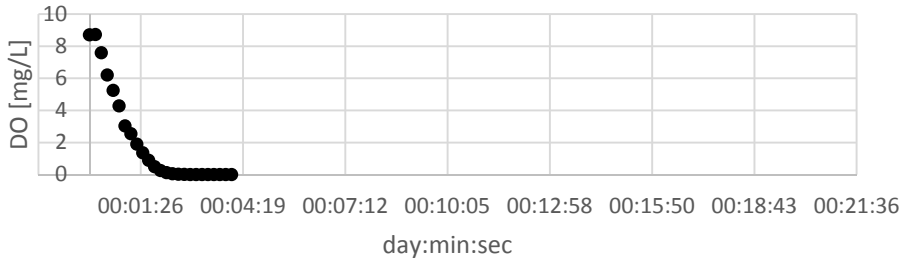
Figure 41 shows the DO plots over time. There is a difference between the shapes of the curves generated at different times; curves taken right at the beginning of the experiment and after feeding are steeper than curves taken later.

Flaws and slight variation influence the shape of the curves in the experimental procedure. Flaws could be incomplete mixing for some seconds, use of small beakers instead of Erlenmeyer flask and problems to seal the vessels immediately and completely with parafilm. Irregularities in plot 2, 7 and 9 were caused by problems with the magnet stirrer. Plot 10 is based on measurements taken in an Erlenmeyer flask, which was covered incompletely.

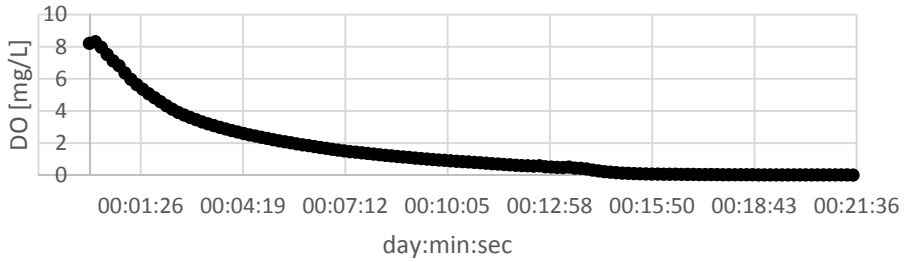
Figure 41 - OUR plots over 9h time with re-feeding



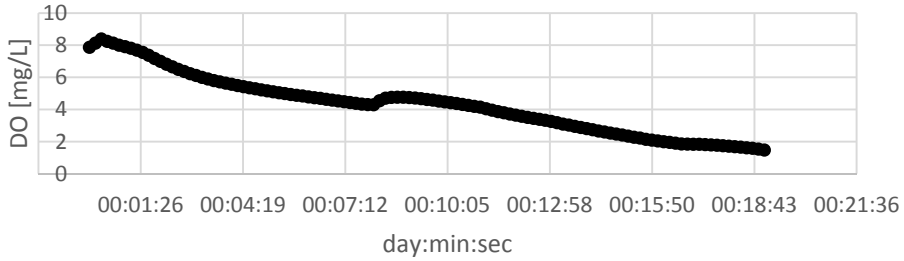
5. 100DEP, t=6h, 5min after grey water feeding



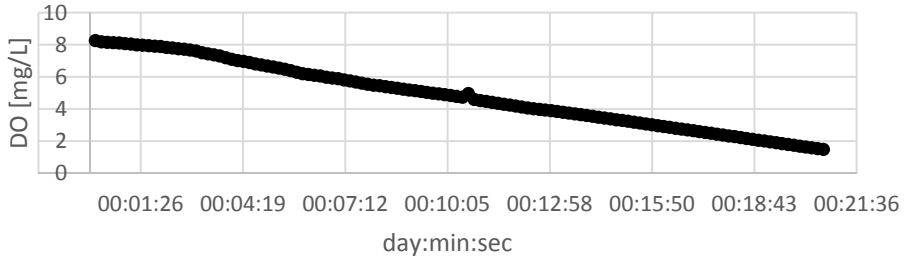
6. blank t=6h, 5min after grey water feeding



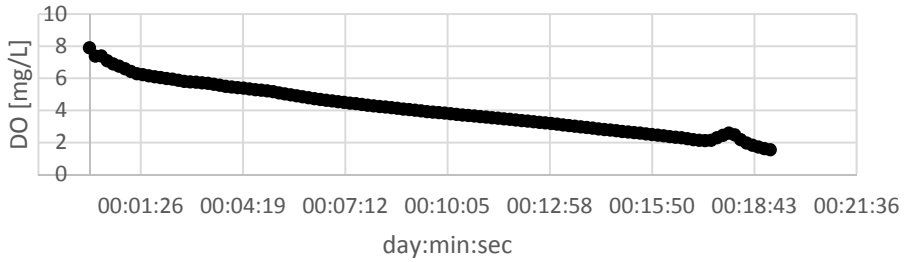
7. 100DEP t=9h, 3h after grey water feeding



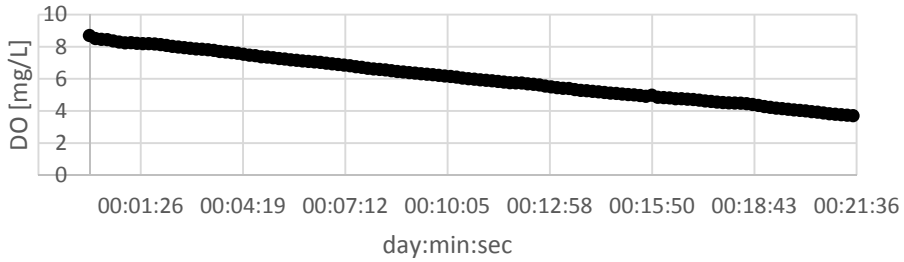
8. blank t=9h, 3h after last grey water feeding



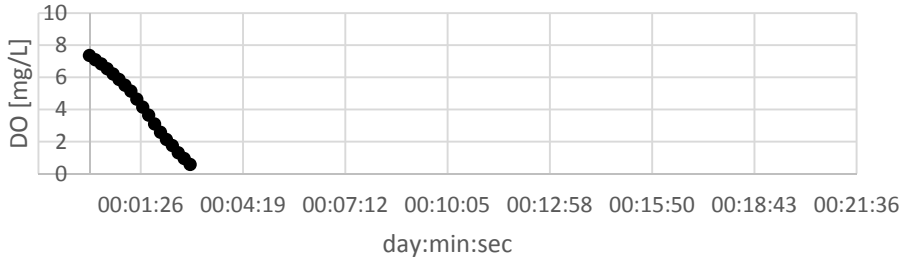
9. 100 DEP, t=12h, 6h after first feeding



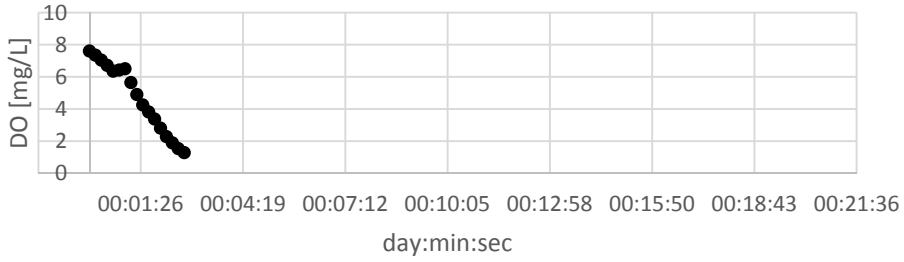
10. blank, t=12h, 6h after first feeding



11. DEP 100 t=12h, 30 sec after 2nd feeding



12. blank, t=12h, 30 sec after 2nd feeding



Appendix E. ATP during oxygen uptake

One sample was taken out from each beaker each time. The samples were diluted. For each dilution, 10 measurements were made. The results in Table 15 represent the averages and STD of those 10 measurements. The results in the boxes are used in the main report (section 0)

Table 15 - ATP measurements 5 minutes and 30 minutes after mixing sludge and spiked grey water

5 min – ATP in nmol/L							
Sample description	**Tot. ATP**	**STD (tot.)**	**dead cells**	**STD (dead)**	**ATP**	**STD**	**dilution used**
Blank	**4953**	**110**	**113**	**2**	**4841**	**110**	***1000**
	4934	**258**	**113**	**2**	**4821**	**258**	***1000**
	2524	84	77	1	2446	84	*10000
	3442	201	77	1	3364	201	*10000
100 DEP 1	**3842**	**386**	**120**	**2**	**3722**	**386**	***1000**
	2441	184	135	1	2307	184	*10000
	2457	97	311	16	2146	99	*100000
100 DEP 2	**3571**	**121**	**106**	**3**	**3465**	**121**	***1000**
	2317	122	161	7	2156	122	*10000
	2483	131	698	19	1785	133	*100000
10 000 DEP 1	**3092**	**276**	**94**	**13**	**2997**	**277**	***1000**
	2356	52	88	3	2268	52	*10000
	1929	403	114	14	1815	403	*100000
10 000 DEP 2	**4731**	**116**	**155**	**73**	**4576**	**137**	***1000**
	3240	94	97	2	3143	94	*10000
	2419	217	86	5	2332	217	*100000
30 min – ATP in nmol/L							
Blank	**2627**	**34**	**101**	**14**	**2526**	**37**	***1000**
	1860	51	87	4	1773	51	*10000
100 DEP 1	**2246**	**129**	**101**	**2**	**2145**	**129**	***1000**
	1765	127	108	2	1657	127	*10000
	1723	327	281	33	1442	329	*100000
100 DEP 2	**2614**	**89**	**92**	**0**	**2522**	**89**	***1000**
	1873	81	152	6	1720	81	*10000
	2126	90	749	37	1377	97	*100000
10 000 DEP 1	**2736**	**61**	**94**	**2**	**2641**	**61**	***1000**
	2079	117	92	3	1987	117	*10000
	1232	125	122	5	1110	125	*100000
10 000 DEP 2	**1980**	**62**	**103**	**3**	**1877**	**62**	***1000**
	1341	190	86	5	1254	190	*10000
	890	138	172	15	719	138	*100000

Appendix F. Influence of medium on nitrification

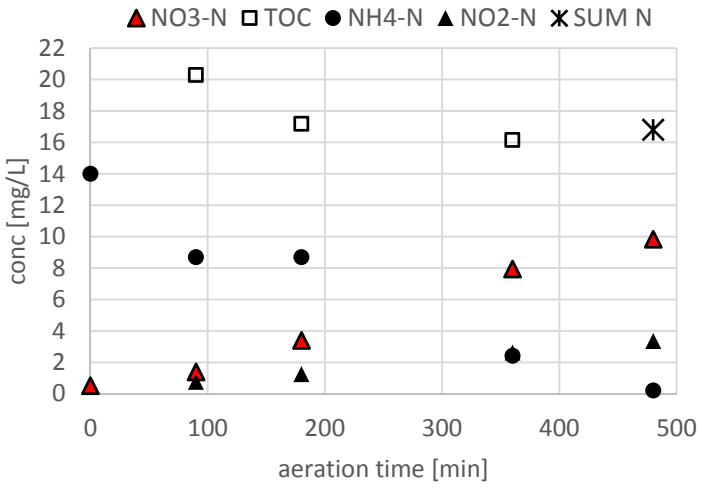


Figure 42 - aeration with medium
At the beginning, 12.7 mg/L NH4-N were dosed. The value NH4-N at t=O is calculated according to the ammonia content of the grey water and the dosed amount.
Note the high nitrite content (~3.4 mg/L) at the end of the experiment in both beakers.

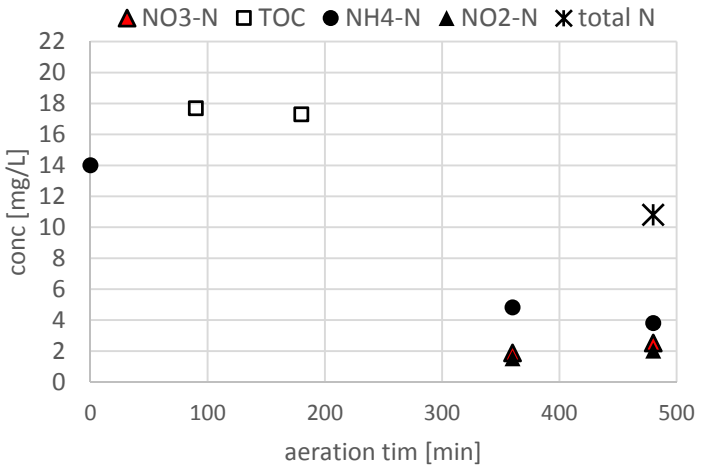


Figure 43 - aeration without medium. Note that the difference in total N implies inaccurate dosing of NH4 (lower in the beaker without medium) Accordingly, with the same dose of NH4-N, residual NH4 concentrations in this beaker would be even higher.

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