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# Assessment of Activity of Bacteria in Integrated Fixed Film Activated Sludge

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ASSESSMENT OF ACTIVITY OF BACTERIA IN INTEGRATED FIXED FILM ACTIVATED SLUDGE

BY

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# ABSTRACT

In this study, the feasibility of a manometric batch test method to measure biological activity of Integrated Fixed Film Activated Sludge (IFAS) microbial aerobic and anaerobic communities was investigated. Additionally, the substrate consumption ratio, the $N_2O$ emissions from the biological activity of the different microbial populations and the inhibitory effect of stormwater pollutants on the activity and $N_2O$ production were investigated as well.

The obtained results from the aerobic tests showed qualitative correspondence with trends described in the literature, but differed greatly in quantitative terms (1 to 2 orders of magnitude). The anoxic test did not produce interpretable results, because values recorded with the manometric method could not be transformed using the method that had been destined for the transformation, and the results were contradictory to what was depicted in the literature. The stormwater toxicity test results were scattered so that an interpretation did not seem feasible, because the values for the experimental duplicates varied so largely that no larger pattern could be established. The trend of the results obtained for the $N_2O$ production agree with previous reports, however, because of the unreliability of the fluid analysis results (for example in terms of $N_2O$ production per nitrogen) mass balances to corroborate them were not possible to achieve. Overall the experiments did not provide the results that were expected and significant improvements to the methods and a further investigation of the influencing factors are necessary to ensure that the proposed method provide more accurately results.

**ACKNOWLEDGMENTS**

I especially want to thank my major Professor, Dr. Vinka Oyanedel Craver, for all the help, inspiration and feedback she gave me during my masters research and the writing of the thesis. I also want to thank the members of my thesis committee, Dr Thiem, Dr. Thornber and Dr. Kennedy for the constructive comments on the thesis. Additionally I want to thank my Lab-mates Dounia and Nelson for their help and advice during the experiments.

**PREFACE**

This Thesis has been written in a Manuscript Format resembling a scientific paper and the IEEE citation style was used and therefor the references have been listed and numbered by appearance in the text.

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**MANUSCRIPT INTRODUCTION**

*Prepared for the submission to the ASCE Journal of Environmental Engineering*

**ASSESSMENT OF ACTIVITY OF BACTERIA IN INTEGRATED FIXED FILM ACTIVATED SLUDGE**

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## 1. INTRODUCTION

Wastewater treatment facilities remove pollutants and nutrients, before the contaminated stream is released into receiving water bodies, minimizing impacts in the environment. This is achieved through a chain of physical, biological and chemical treatments. The heart of most Wastewater Treatment Plants (WWTP's) treating municipal wastewater is the biological (or secondary) treatment stage (see Figure 1). In the secondary treatment units, conditions are established to support biological processes in aerobic (aerated) and anoxic zones. In the biological treatment, most of the nutrients, such as carbon, nitrogen and phosphorus compounds are removed from the wastewater by microorganisms that use these compounds as source of energy and matter for their cell metabolism and growth. In the aerobic zones, the microorganisms use the oxygen that is supply through aeration, to oxidize the
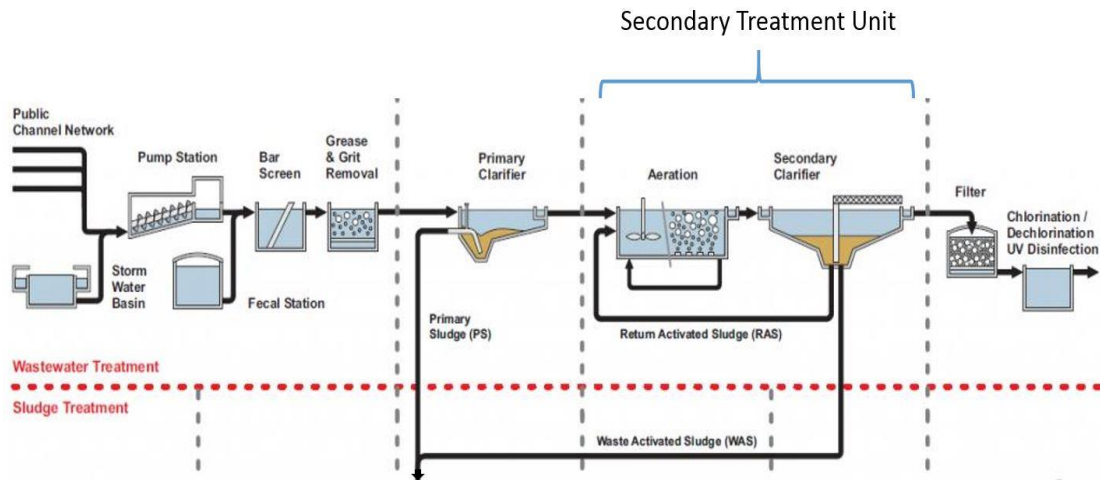


*Figure 1: Schematic of a common activated sludge process; Source: www.sswm.info*

substrates (carbon to $CO_2$ and ammonia to $NO_3$) and in the anoxic zones facultative bacteria reduce $NO_2$ and $NO_3$ to $N_2$ when using them as electron acceptors to respire organic carbons.

In coastal areas, nitrogen is the limiting nutrient for the growth of nuisance algae that can cause eutrophication. Because of eutrophication, low oxygen zones can occur, which have led to fish kills, closing of beaches and fishing grounds [1], [2]. In order to prevent these issues, the United State Environmental Protection Agency (USEPA) implemented programs with states to issue increasingly strict regulations for the

nitrogen concentration of WWTP's effluent [3]. During the last decades, several alternatives to enhance nitrogen removal have been developed. One of these technologies is the Integrated Fixed film Activated Sludge (IFAS) system [4], which is a hybrid process that increases the nitrification capacity by providing support media for nitrifying bacteria to grow along with suspended biomass in the aeration tank of WWTP (see section IFAS for more details).

This study was conducted in cooperation with the Narragansett Bay Commission (NBC), which is especially interested in high performing nitrogen removal processes for their two WWTPs, which are the largest in the State of Rhode Island and are located on the northern end of the Narraganset Bay. Due to the upcoming re-permitting of the plant, it is anticipated that stricter effluent standards for pollutants and nutrients will be set by the Rhode Island Department of Environmental Management (RI-DEM) as the permitting agency [5], [6]. To increase the performance of a wastewater treatment process it is important to adjust the process parameters (like aeration, solid retention time or hydraulic retention time) in a way that enables it to achieve the highest removal rates possible. In this case, the understanding how the components of the hybrid IFAS system (suspended and attached biomass) work and influence each other in the process of nutrient removal is needed. One way to characterize a process is measuring the biological activity of the microbial communities responsible for the different removal steps (carbon, nitrogen, and phosphorous removal, among other). The determination of the biological activity is important because the conventional biofilm describing parameters (like dry weight or biofilm thickness) do not always show linear correlation with its ability to consume substrates [7]. The biological activity can be measured via respirometric and molecular based methods, and by the measurements of substrate concentrations over time in continuous flow and batch experiments while manometric measurements of the gas phase in batch tests [7],[8], [9]. The molecular based methods assess the activity through the analysis of compounds produced by living cells. A prominent and accurate method is the analysis of the ATP content. ATP is

produced by active cells and disappears instantly when cells die and is therefore a good indicator how active biomass is. Its main disadvantage is the complexity of its extraction process. An advantage of the method is that the values stay constant after samples are frozen. Another method described as very sensitive and simple is the INT-dehydrogenase, which measures the activity of the electron transport system (ETS) through the reduction of an added compound (INT) by electron diverted from the ETS. The dehydrogenase analysis works best for population in a stable state and is widely applicable (wide temp. range, anaerobic and aerobic activity) although it does not distinguish between biological and chemical reduction of the INT. It has been characterized as simple, sensitive and rapid and therefore suitable for wastewater treatment plants [7]. "The most conventional technique for microbial activity determination […] is the measurement of the substrate removal rate" [7]. This can be measured through influent and effluent concentrations in continuous flow experiments or start and end (and timed) measurements in batch tests. The disadvantage of these tests is that limitations by oxygen or substrate availability have to be prevented by the experimental design.

Respirometric methods use different means to measure respiration activity in terms of oxygen uptake rate (OUR). The OUR is a fundamental physiological characteristic of culture growth [10] and is a frequently used parameter, even though its sensibility and reproducibility are low and a distinction between primary and secondary metabolisms is not possible [7]. OUR measures the oxygen uptake of a microbial community (or a pure culture) and is directly tied to the substrate consumption of aerobic processes, because the oxygen is necessary as an electron acceptor for the substrate oxidizing bacteria. During the exponential growth phase of the bacteria the OUR increases, because of the higher substrate consumption, and it decreases again in the stationary phase, because of the lower metabolic activity [10]. The sensitivity and reproducibility of the measurements can be improved using sensors and microelectrodes. Respiration rate can be measured using DO-probes [11], gas flow analysis [12] or manometric techniques [8]. The manometric method measures the

pressure drop in a closed system which in aerobic conditions can be correlated with oxygen consumption. This method has been also used to determine denitrification activity of biofilm from a post denitrification in Moving Bed Bio Reactor (MBBR) under anoxic conditions[8]. That study used the same principle, with the difference that the increase of pressure was allocated to the production of $N_2$.

Main objective of this study was to assess the use of a manometric method for measuring the respiration activity of the heterotrophic, nitrifying and denitrifying bacteria. Furthermore, nitrous oxide ($N_2O$) production was measured to determine the production of this gas associate with the different biological activities. Finally, the effect of stormwater pollutants on the different microbial populations was assessed in terms of activity and $N_2O$ production.

### 1.1. Background
#### 1.1.1. Nitrogen Removal

High nitrogen loads in the effluent of WWTP's can have negative effects on receiving water bodies. Nitrogen, in the form of nitrate, is the limiting nutrient for eutrophication in coastal waters, inducing rapid growth of algae biomass. When this biomass dies, high amounts of oxygen are used by bacteria to degrade this biomass, which can lead to anoxic (no oxygen but presence of other electron acceptors) conditions in the waterbody that are lethal to all aerobic aquatic life. In the past, eutrophication events in Narragansett Bay were mostly caused by effluent from the Providence wastewater treatment facilities and combined sewer overflow (CSO) form the Providence area [13], [14] (see section: Narraganset Bay Commission WWTP at Field's Point). In the majority of the CSO events, large nutrient loads are discharged into the receiving water bodies and can cause degradation of the water quality and eutrophication. A second negative effect is, that during storm events an increase in the influent flow to the WWTP occurs, reducing the hydraulic retention time in the biological stage, leading to incomplete treatment and increased pollutant concentrations in the effluent.

To address the concerning pollution of the Narragansett Bay, the Narragansett Bay Commission (NBC) enhanced the operation of the Field's Point wastewater treatment

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plant in several phases. In the early 1990s a planning process started to reduce the pollution from storm events, which lead to the construction of a three stage CSO abatement tunnel system, the last stage of which was finished in 2016 [14]. The tunnels capture the sewer overflow, to ensure that all stormwater gets stormwater treatment and none gets discharged untreated. In order to reduce the nitrogen discharge from the WWTPs effluent, enhanced aeration technology and the IFAS system were implemented in 2013[15].

The biological nitrogen removal process consists of two phases: nitrification and denitrification (see Figure 2). Nitrogen enters the treatment plant mostly in the form of ammonia (NH3), which is transformed by biological ammonification from organic
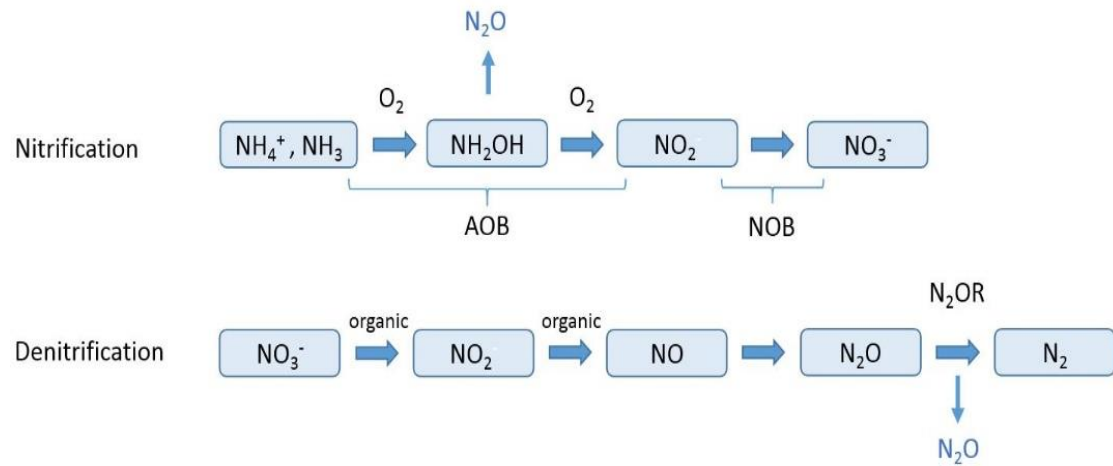


*Figure 2: Schematic of Nitrification and Denitrification; Source: H.Behrmann*

nitrogen (for example from fats and proteins) while the wastewater is transported in the sewer system to the wastewater treatment plant [16].

In the nitrification phase, the ammonia ($NH_3$) is oxidized to nitrate in a two-step aerobic process. First Ammonia Oxidizing Bacteria (AOB) transform it to nitrite ($NO_2$) followed by the transformation to nitrate by Nitrite Oxidizing Bacteria (NOB). The AOB first oxidize $NH_3$ to hydroxylamine ($NH_2OH$) using the enzyme ammonia monooxygenase (AMO) and then $NH_2OH$ to $NO_2$ using hydroxylamine dehydrogenase (HAO). NOB use a complex enzymatic chain reaction to oxidize $NO_2$ to $NO_3$[17]. Other microbes, which can oxidize ammonia are ammonia oxidizing archaea (AOA) and bacteria, which can oxidize $NH_4$ under anaerobic conditions using $NO_2$

(anammox), but neither of these species play a big role in classic wastewater treatment processes, because of the very specific metabolic environmental conditions needed by the anamox (anoxic, no carbon sources) and the low growth rate of the archaea [18]. Following nitrification is the anoxic process of denitrification, where heterotrophic chemoorganotrophic (bacteria that use organic carbon for growth and energy from the oxidation of chemical compounds) bacteria use the oxygen bound in the nitrate for their carbon assimilation and reduce the nitrate through the intermediates $NO_2$, $NO$ and $N_2O$ into molecular nitrogen ($N_2$). Strict anoxic conditions have to be established to ensure denitrification, because some of the intermediate steps are very susceptible to even very small amounts of oxygen (as low as 0.2 mg/l)[17]. There are also some autotroph bacteria capable of denitrification, among which some species are also nitrifiers (*Nitrosomonas eutropha & N.europaea*) [18]. If these species engage in nitrification under low DO levels, it is called nitrifier denitrification, which also brings some problems in terms of increased $N_2O$ production (see 1.1.3.Green House Gas production in Wastewater treatment plants).

### 1.1.2. The Integrated Fixed Film Activated Sludge (IFAS)

Heterotrophic and nitrifying bacteria compete for oxygen and space in the aerobic zone of WWTPs [19]. Heterotrophic bacteria grow faster than nitrifiers, so they win this competition [20]. Common measures to increase nitrification in an activated sludge process would be increased aeration and longer solids retention times (SRT) [21]. Since an increase of biomass concentration in the aeration
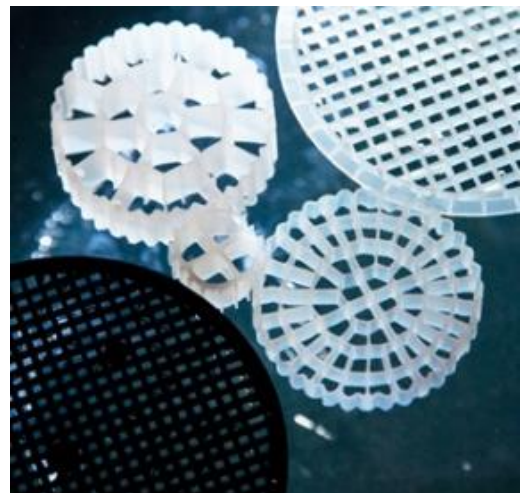


Figure 3: Picture of different IFAS media; Source: wateronline.com

tank is limited due to operational requirements (too high SRT decrease activity,

growth rate and gas production from sludge treatment) [18], the SRT cannot be drastically increased, if good settlement qualities of the sludge are to be maintained [21]. Both increased aeration and increased reactor volume entail high cost, due to increasing energy requirements (aeration) and/or investment in new technology [21]. Integrated Fixed Film Activated Sludge (IFAS) system were developed to address these issues. The IFAS is a hybrid system, which consists of suspended sludge and biofilm (see Figure 3) that co-exist in the same tank. This separates the bacteria populations. In this case, slow growing nitrifying bacteria can thrive in the biofilm while the suspended biomass allows facultative aerobic bacteria cycle between the aerobic and anoxic tanks [22]. Previous studies have found that the IFAS system yields higher nitrogen removal than conventional systems [20], [22], [23]. The main advantages of the IFAS system are the enhanced nitrification capabilities in less space and the increased process stability in terms of its resilience to low temperatures and temporary disturbances like hydraulic stress, toxins or changes in their environmental conditions [7]. Also, it offers the possibility to add more media to increase treatment capacity [18] with reported values up to 70% of the volume of the aeration tank [22], and it can be used for simultaneous nitrification-denitrification at low DO conditions [18]. The disadvantages of the system are the need for higher DO levels due to the higher biomass content and possible transport of oxygen to the anoxic tank, the use of propriety products (the media and technology are sold by AnoxKaldness, Veolia), the higher difficulty of maintenance, due to the necessity to remove and store the media when maintenance in the tanks is necessary, and additional hydraulic head loss in the WWTP by the flow resistance of the plastic media [18].

### 1.1.3. Green House Gas production in Wastewater treatment plants

Nitrous oxide ($N_2O$) is a powerful greenhouse gas (GHG) that is a by-product in the nitrogen and it has a 300 fold (265-310 reported range value) [24]–[26] global warming potential (GWP) of $CO_2$ and accounted for about 5% of the anthropogenic GHG emissions in the US [25]. Kampschreur et al. reported the contribution of

wastewater treatment to anthropogenic $N_2O$ emission is about 3.2% [27], but $N_2O$ from these facilities might account for up to 26% of the GHG emissions of the water supply and sanitation sector combined [17], [27].

In the context of biological nitrogen removal, $N_2O$ is produced in both parts (nitrification and denitrification) of the biological nitrogen removal process (Figure 2 and 4).
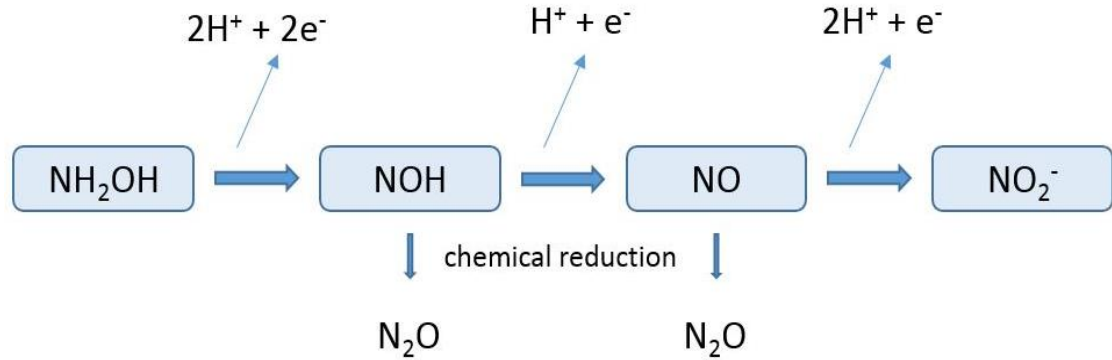


*Figure 4: Schematic of the Ammonia oxidizing process and the intermediates, which are chemically reduced to $N_2O$; H. Behrmann, adapted from Todt et al.*

The two main microbial communities responsible for nitrification are the ammonia oxidizing bacteria (AOB) and the nitrite oxidizing bacteria (NOB). Of these the AOB are mostly associated with $N_2O$ production, mostly through nitrifier denitrification [28] or higher nitritation rates than nitrification ones, which lead to accumulation of $NO_2$ and intermediates of the oxidation process. It has been suggested that during $NH_3$ oxidation, highly reactive intermediates are released by AOB, which then are transformed to $N_2O$ through chemical processes[17] (see Figure 4). Nitrifying denitrification is a process where otherwise nitrifying bacteria (like *Nitrosomonas europaea*) reduce $NO_2$ to NO, $N_2O$ and $N_2$ under low oxygen conditions. The main production path of $N_2O$ through nitrifying denitrification is during hydroxylamine oxidation (HAO) [28]. Nitrifying denitrification is considered a survival metabolism at low $O_2$ levels, and has been controversially discussed as a self-protection mechanism against $NO_2$ levels[17]. Main drivers of $N_2O$ emissions from AOB have been identified to be: nitrite accumulation [8] [16], low DO concentrations [17], [27], excess inorganic carbon concentration [17], low pH conditions [17], [27]. NOB have only

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been connected to $N_2O$ production under anoxic conditions, but their metabolism has scarcely been studied [17]. The main contribution to the $N_2O$ production by NOB is indirect, through their respiration by which they control the $NO_2$ accumulation, which causes increased $N_2O$ production by other bacteria. The accumulated higher concentrations of $NO_2$ can then inhibit other bacteria and also lead to incomplete nitrifier denitrification. The main factors cited for NOB inhibition are high $NH_3$ concentrations (although unspecific, because the inhibiting concentrations depend on the nitrite oxidizing species) and $HNO_2$, which is correlated to $NO_2$ accumulation at low pH[17].

In the denitrification process, $NO_3$ and $NO_2$ are used as electron acceptors in the absence of $O_2$ and thereby are reduced to $N_2$ through the intermediates NO and $N_2O$. When this process is not fully conducted, $N_2O$ is released. The crucial factor for this is the enzyme *$N_2O$ reductase ($N_2OR$)*, which accounts for the reduction of $N_2O$ to $N_2$. This enzyme is very sensitive to even very low concentrations of oxygen and is also inhibited by high $NO_2$ concentrations, likely through stress caused by $HNO_2$ and NO [17], [27]. Interestingly, the inhibitory effect caused by NO, unrelated to its origin, was found to be irreversible even if free NO only appeared temporary. Another factor observed to cause increase in $N_2O$ production from denitrification are low or very high COD:N ratios. At low COD:N (<3.5) ratios the $N_2O$ emissions increased when organic carbon became the limiting factor and the bacteria started to consume internal storage compounds. In other cases, the limited organic carbon can lead to an accumulation of $NO_2$ which then caused an increased on $N_2O$ production. At high COD:N ratios an enrichment of aerobically denitrifying organisms can occur which could be connected to increased $N_2O$ production.[27]

### 1.2. Main Objectives

The hypothesis developed for this study is that the Oxitop based manometric method can be used to assess the activity and greenhouse gas production of the different bacterial communities and the effects of inhibitory substances on the IFAS system. In other to probe this hypothesis, the main objectives of this work was the validation of

the manometric method, quantification of the biological activity of the heterotrophic, nitrifying and denitrifying bacteria in the IFAS system using manometric measurement methods. Additionally, the response of the hybrid systems components to disturbance by synthetic stormwater and the production of nitrous oxide ($N_2O$) emissions in the different processing steps were investigated as well.

## 2. MATERIALS AND METHODS
### 2.1. Materials

**Biomass**

The suspended sludge and the support media of the biofilm were taken from a model wastewater treatment plant in the URI Environmental Engineering laboratory, which mimics the process specifications of the NBC WWTP in Field's Point. The original
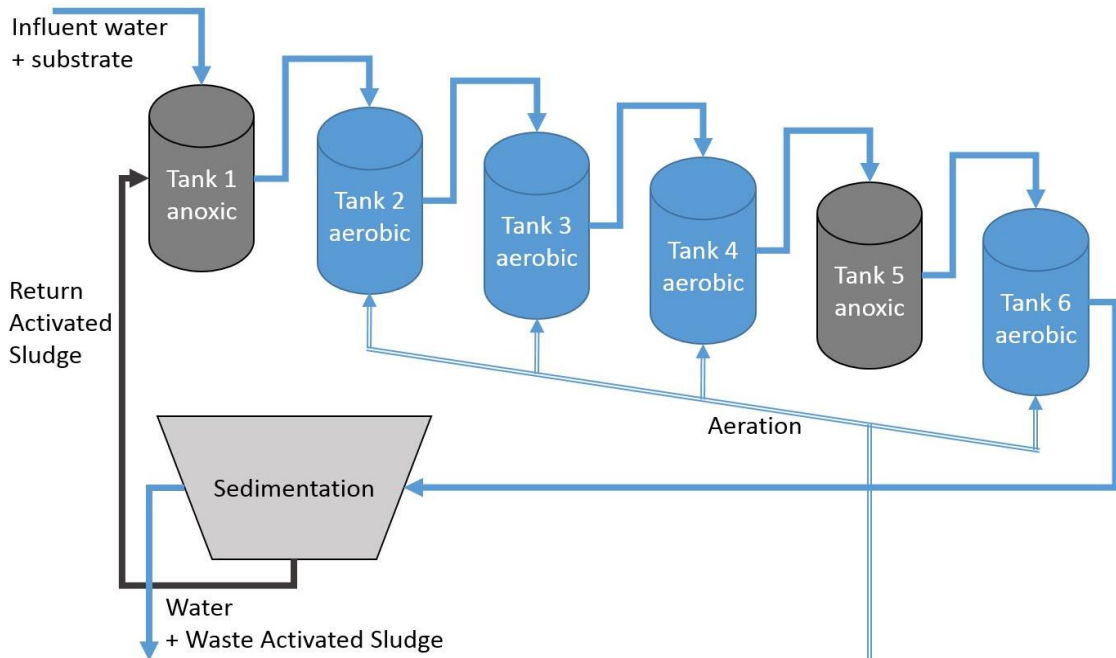


Figure 5: Schematic of the model wastewater treatment plant at the URI Environmental Engineering Laboratory; Source H. Behrmann

suspended sludge and biofilm support media used to start the model WWTP in the laboratory, came from the WWTP at Field's Point. The solids retention time in Field's Point is about two weeks, while in the model WWTP was set to 3 to 4 weeks in order tomaintain a proper MLSS concentration in the plant. However, long SRTs can reduce biological activity and aerobic stabilization of the sludge (if SRT > 40d [29]). The suspended sludge for the experiments was taken from Tank 6 (see Figure 5), since it had the lowest amount of substrate left from the feeding solution, compared to the other tanks. The Biofilm support media were collected from Tank 4 because they were abundant and the disturbance of the model WWTP was thereby minimized. The average solids retention time (SRT) in the model WWTP was between three and four

weeks, which is very long. In comparison the SRT at the Fields Point WWTP is around two weeks, which is also relatively long (compare [29]).

**Reagents and Solutions**

The substrate and nutrients concentrations in the liquid phase were analyzed using HACH kits TNT 821 (COD), TNT 831 ($NH_4$-N), TNT 835 ($NO_3$-N), TNT 839 ($NO_2$-N) and 880 (TKN)) and measurement were performed in a HACH DR 2800 Spectrophotometer.

A Phosphate Buffer Solution (PBS), which contained 5.6 g/l Potassium Phosphate Diabasic, 2.4 g/l Potassium Phosphate Monobasic (both Fisher Chemical) and 0.01 g/l EthyleneDiamineTetraaceticAcid (EDTA) (Sigma-Aldrich) was used to buffer changes in the pH throughout the tests.

Table 1 shows the concentrations of the substrates in the injected solutions and the target concentrations in the bottles, which were determined on the basis of literature values [28] [20] [8]

*Table 1: Substrate concentrations of the solutions that were injected in the experiments and the target concentrations in the experiments*

| Substrate | $C_6H_{12}O_6$ | $NH_4$-N | $NO_2$-N | $NH_4$-N + COD |
|---|---|---|---|---|
| Concentration in solution | $10000 \frac{mg\ C_6H_{12}O_6}{l}$ | $3000 \frac{mg\ NH_4-N}{l}$ | $2000 \frac{mg\ NO_2-N}{l}$ | $4000 \frac{mg\ NO_3-N}{l}$ $+ 16000 \frac{mg\ COD}{l}$ |
| Target concentration in the bottle | $100 \frac{mg\ C_6H_{12}O_6}{l}$ | $30 \frac{mg\ NH_4-N}{l}$ | $20 \frac{mg\ NO_2-N}{l}$ | $40 \frac{mg\ NO_3-N}{l}$ $+ 160 \frac{mg\ COD}{l}$ |

The Stormwater experiments were conducted, because at times of precipitation events, stormwater run-off from the catchment area is transported to wastewater treatment facilities, movilizing pollutants like heavy metals and PAHs. Because the difference between the average daily flow and the maximum treatment capacity of the Field's Point WWTP is about 40%, it was decided to calculate the maximum concentrations of pollutants to resemble a 40% stormwater additional flow in the biological treatment.

The stormwater solution was mixed adapting a recipe that was used before by Kasareni et al. [30] (see Appendix I). The concentrations in the recipe were defined to correspond to 100% stormwater. Therefore, the maximum concentration of pollutants in the bottles was set to be similar to those found at the maximum stormwater input to the WWTP. The pollutant concentrations for the injection mixture were then calculated to reach those corresponding concentrations in the bottle with an injection of 1ml.

## 2.2. Analytical methods

**Biomass concentration**

Total Solids (TS) concentration was chosen for normalization of the results due to values of activity are proportionally correlate with the biomass concentration. The TS of the suspended sludge was determined at the beginning of every experiment. When the suspended sludge samples were put in the bottles for the manometric measurement, a part of the prepared fluid was retained (see section methodology) to be used for analysis of the substrate concentrations and the determination of the TS. This was done in duplicates by weighing a sample of suspended sludge ($m_1$) in a container ($m_{container}$), drying it at 105°C for 24h and then weighing it again ($m_2$). The TS results then from Equation 1. The TS used for the calculation of the specific TS per bottle was the arithmetic mean of the results for the TS of the two samples. The TS per bottle was calculated by multiplication of the average TS and the weight of the sample in the bottle (see methodology).

*Equation 1*
$$TS = \frac{m_2 - m_{container}}{m_1 - m_{container}} \left[\frac{g}{g}\right]$$

For the biofilm total solid determination, the average amount of TS per support media was determined once by choosing 19 random media, drying them over 24h at 105°C, and weighing them. Then they were cleaned by sonication for about 2h with multiple changes of the cleaning fluid (DI-Water), dried again and weighed again. The average TS per media was then calculated by arithmetic mean of the 19 weight

differences which resulted in a number of 0.0502 g TS/support medium with a standard deviation of s = 0.0078g calculated with Equation 2, where n is the number of samples, $x_i$ is the weight of the dried biofilm on the specific sample and $\bar{x}$ the average weight of the dried biofilm per sample.

Equation 2
$$s = \sqrt{\left(\frac{1}{n} * \Sigma(x_i - \bar{x})^2\right)} = 0.0078\,g$$
(see Appendix III)

The Oxitop® Control System was used for the experiments, which consists of Oxitop® bottles, pressure sensor heads and a hand-held controller (WTW, Weilheim, Germany), a magnetic stirrer bar per bottle and an incubator. The experiments were conducted in 250 ml bottles (see Figure 6). Additionally to measuring head opening, the bottles have two side sockets, which were closed with septi and screwcaps allowing fluids and gas sampling, while keeping the system closed. Below measuring head sodium hydroxide solution container is placed in order to absorbs the $CO_2$ produced during
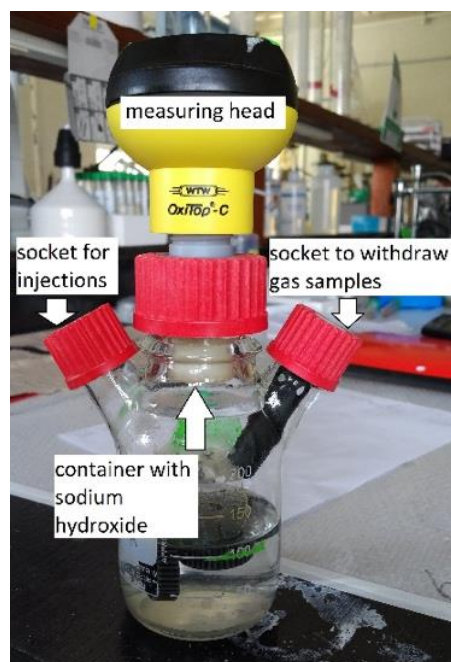


Figure 6: Oxitop Bottle; Source H. Behrmann

respiration. This step is needed in order to only record the pressure reduction due to oxygen consumption (heterotrophic and nitrifying activity) or pressure increase due to nitrogen production (denitrification).

To analyze the rate of the pressure change, the periods with the highest, stable pressure change after the injection of substrate were selected and the slope of the pressure change in the selected time frame was calculated. Figure 7 shows the image of a representative graph of the change of pressure over time in an aerobic experiment. The pressure at time t=0 is determined to be 0 by the measuring system.
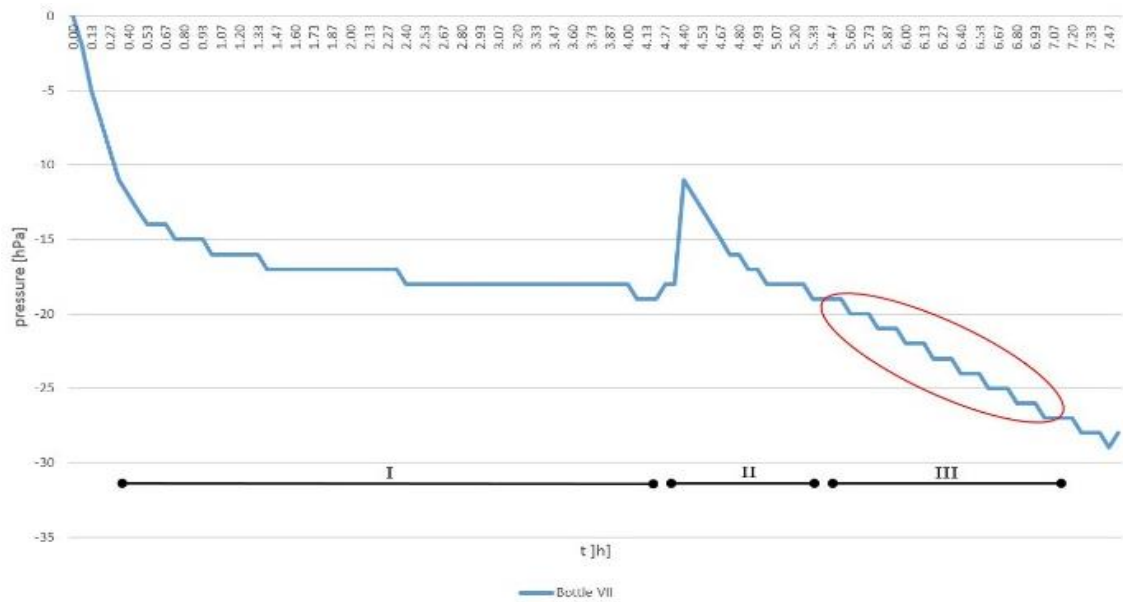
*Figure 7: Example of a standard graph of pressure over time for an aerobic experiment; Source H. Behrmann*

Different phases can be distinguished: first, there is a pressure drop right after the start of the measurements (start of phase I). This is probably due to the starting capture of $CO_2$ by the NaOH and the temperature drop, when the bottles were put into the incubator since most days the ambient temperature was warmer than 20°C. Then the period of acclimatization started (section I, in Figure 7) where the bacteria adapted to the new conditions. Slopes of the later part of the acclimatization period (in Figure 7 the second half of section I) were calculated but not used as control values, because they often significantly differed from the slopes of the control bottles in the later time frame. The second phase (section II, Figure 7) includes the substrate injection, which is clearly visible by the steep peak in the graph, and a following shorter phase of acclimatization. The third phase (section III, Figure 7) is the phase with the strongest pressure drop, attributed to respiration by the bacteria when substrate was injected. Part of the data from this phase was selected to determine the rate of the pressure depletion, which then allowed to calculate the substrate assimilation rates (see details on Appendix IV). The results of these mathematical determinations can then be compared to the results of the fluid sample analysis performed in during the beginning and end of the test. It was assumed that the

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values from the mathematic determinations are higher, because of the endogenous respiration of the bacteria mix. The endogenous respiration describes a process when cells consume their own tissue or the tissue of dead cells to gain energy for cell maintenance [18]. Values that can be found in the literature for the endogenous respiration are 0.037 $d^{-1}$ for heterotrophic bacteria, 0.008 for AOB and 0.005 for NOB [32].

**GHG production**

The gas samples that were taken at the time of injection and the end of each experiment were analyzed in Professor Mozeman-Valtierras Lab in the CBLS Department of URI using a Shimadzu GC-2014 Gas Chromatograph, which was calibrated with three samples each of three different standards with concentrations of 0.508ppm, 2.125ppm and 10.02ppm of nitrous oxide. The gas samples were analyzed for their $N_2O$ concentration. An analysis for $N_2$ was not possible, but the concentrations of $CO_2$ and $CH_4$ were also measured, although their calibrations were not as reliable as the one for $N_2O$. Also, it should be noticed that the $CO_2$ concentrations were not accurate, since NaOH was added to all bottles to bind $CO_2$.

### 2.3. Methodology

**Sample Preparation**

Immediately after a suspended sludge sample was drawn from the Tank 6, the pH was adjusted using NaOH or sulfuric acid to a value of pH 7 ± 0.3. Then the sludge was let to settle and a fraction of the supernatant was exchanged for PBS. After this, the sludge was either placed in the incubator to be aerated overnight (18-24h) for the aerobic tests or bubbled with argon gas for 30 minutes for the anaerobic tests. Afterwards, 100ml of the suspended sludge were measured with a graduate cylinder, weighed and placed into the Oxitop® bottles along with a stirrer bar (1.5''). For the aerobic tests the bottles were then closed with the septum on the side sockets and the NaOH container and the measuring head on the top. For the anaerobic tests, one side socket and the top were closed in the same manner but the sample was bubbled

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again with argon gas for a few seconds to drive as much oxygen out of the head space as possible before that socket was also closed with a septum. The biofilm
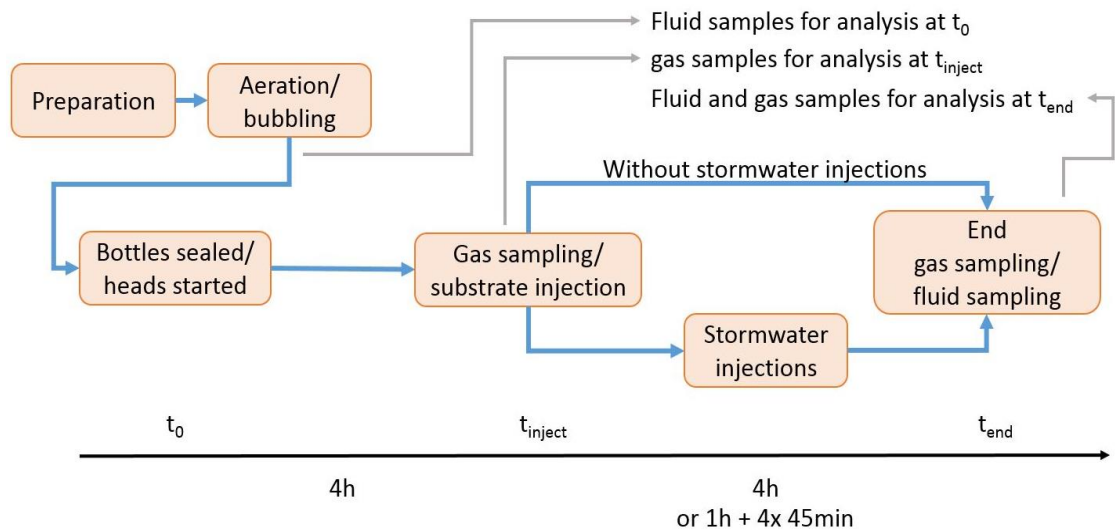


*Figure 8: Schematic of the experimental procedure; Source H. Behrmann*

support media collected from Tank 4 were placed in PBS (700ml for six bottles or 1400ml for 12 bottles) and then treated in the same way that was described in the paragraph above for the suspended sludge (aeration/bubbling with argon gas). After the aeration or bubbling, 100ml of the PBS were added into each bottle and four media per bottle and a stirrer bar (1'') were added. Then the bottles were closed in the same manner as described for the suspended sludge. The rest of the PBS (≈100ml) was retained for the analysis of the substrate concentrations. The experimental procedure can be seen in Figure 8: For all experiments the bottles were sealed, the recording of the measuring heads was started and the bottles were placed on the stirrer platforms in the Incubator. After an acclimatization period of approximately four hours gas samples were drawn from some of the bottles, depending on the experiment. In the beginning, when only six bottles were available, two (of the six) bottles were run without a substrate injection as control bottles. In these, and the anaerobic experiments, gas samples were taken from three bottles at the time of the nutrient injection. This was done to keep some samples undisturbed by the gas withdrawal in case it would impact the performance of the bacteria or the final gas composition. Later, when 12 bottles were available the tests for the aerobic

activity were run in a 3:3:3:3 array (three control bottles without substrate injection, three with a glucose solution, three with an ammonia solution and three with a nitrite solution injection). This array provided the benefit that all aerobic measurements were run on the same day on the same sludge. In the 3:3:3:3 setup only four gas samples were drawn at the time of the nutrient injection, one gas sample from one bottle of each set of bottles. An overview of the setups can be seen in Table 2. After substrate injection, the tests without stormwater injections ran for approximately four hours after before gas samples and fluid samples were drawn from all of the bottles and the experiment was ended.

For the storm water experiments the bottles, which were prepared in the same way as the others before, were run for one hour after the substrate injection and then 0.25ml of the storm water solution were injected every 45 minutes until 1ml was injected in each bottle. After the last injection, the bottles were run for another 45 minutes to 1 hour before gas and fluid samples were taken and the experiment was stopped, and pressure depletion rates were calculated in the same manner described before.

The gas samples that were drawn at the time of injection and the end of each experiment were analyzed for $N_2O$, $CH_4$ and $CO_2$ and the fluid samples that were drawn at the begin and the end of each experiment were centrifuged and analyzed for COD, $NH_4$-N, $NO_2$-N and $NO_3$-N for the aerobic tests and $NO_3$-N and TKN for the anaerobic tests.

*Table 2: Overview of the setups used for the manometric experiments*

| Total number of Bottles | 6 | | 12 | | | |
|---|---|---|---|---|---|---|
| Type measured | control | substrate | control | glucose | ammonia | nitrite |
| Number of bottles | 2 | 4 | 3 | 3 | 3 | 3 |
| Gas samples taken at $t_{inj}$ | 1 | 2 | 1 | 1 | 1 | 1 |
| Tests the setup was used for | aerobic suspended anaerobic suspended anaerobic biofilm anaerobic suspended+ SW anaerobic biofilm+SW | | aerobic biofilm aerobic suspended + SW aerobic biofilm + SW | | | |

## 3. RESULTS AND DISCUSSION

In this chapter, the results will be presented in summarizing Tables (Table 3 to Table 6) and then discussed in two parts for the aerobic and anoxic experiments.

### 3.1. Summary of the Results

Table 3 shows the results of the aerobic tests. The substrate assimilation rates in the suspended sludge were one to two orders of magnitude lower than the rates in the biofilm. Both materials showed low $N_2O$ production in the heterotrophic tests and higher production in the nitrification tests, although the highest peaks occurred in different tests, in NOB for the suspended sludge and the AOB+NOB test for the biofilm.

*Table 3: Results of the analysis of the manometric measurements of the aerobic tests*

| Aerobic | Substrate consumption | | $N_2O$ gas production | |
|---|---|---|---|---|
| | Suspended Sludge $[\frac{mg}{gTS*h}]$ | Biofilm $[\frac{mg}{gTS*h}]$ | Suspended Sludge $[\frac{ppm\ (Mol)}{gTS*h}]$ | Biofilm $[\frac{ppm\ (Mol)}{gTS*h}]$ |
| Hetero-trophic | -3.06 ± 2.46 E-02 | -0.179 ± 0.0389 | 0.0389 ± 0.2 | -0.0827 ± 0.04 |
| $NH_4$ to $NO_3$ | -2.01 ± 2.98 E-03 | -0.155 ± 0.116 | 0.8667 ± 0.12 | 4.6459 ± 0.76 |
| $NO_2$ to $NO_3$ | -1.55 ± 1.04 E-02 | -0.199 ± 0.0903 | 3.5561 ± 1.04 | 0.5899 ± 0.11 |

Table 4 shows the results of the manometric method and the gas sample analysis for the anoxic experiments. The values for the pressure change over time were not transformed into a substrate reduction rate, because the negative results do not comply with the theory on which the transformational calculations are based, after which the pressure was expected to increase due to the production of nitrogen gas. The negative results in the first line indicate a decrease in pressure but contradicting the results in the second line also show a decrease in $NO_3$-N, which should have produced an increase in pressure.

Tables 5 and 6 show the results for the experiments in which a synthetic stormwater run-off solution was gradually injected into the bottles after they had been injected with a substrate (Glucose, Ammonia, Nitrite ore Nitrate), 45 min were left between the injections. Using the data from these measurements the assimilation rates after each injection were calculated. In the Tables 5 and 6 in the first column, it is first indicated which kind of process was tested and then following, the assimilation rates after the four stormwater injections (SW inj. 1-4).

*Table 4: Results of the analysis of the manometric measurements of the anoxic tests*

| anoxic | Substrate consumption / $N_2O$ Gas Production | |
|---|---|---|
| | Suspended Sludge | Biofilm |
| $NO_3$ reduction*2 $[\frac{hPa}{gTS*h}]$ | -0.897 ± 1.85 | -2.319 ± 2.78 |
| fluid samples *3 $[\frac{mg\ NO_3-N}{gTS*h}]$ | -1.058 ± 0.59 | -1.115 ±0.13 |
| $N_2O$ gas production $[\frac{ppm\ (Mol)}{gTS*h}]$ | 3.392 ± 0.774 | 7.894 ± 0.864 |

*2 : The unit of hPa/(gTS*h) was chosen for this table because the results do not allow a further calculation with the methods compliant with the theory
*3: The values were calculated from the difference of the avg. concentrations in the end and at the beginning plus the injection

When stormwater is added, the results of manometric measurements of the aerobic and anoxic biological activity had high variability between the results from duplicate bottles and therefore reliable analysis is not possible. The calculated average $N_2O$ production rates seem to show a behavior with similar patterns to the ones seen in the experiments without stormwater.

Table 5: Results of the analysis of the manometric measurements of the aerobic tests with stormwater injections

| Aerobic | Substrate consumption | | N$_2$O gas production | |
|---|---|---|---|---|
| | Suspended Sludge $[\frac{mg}{gTS*h}]$ | Biofilm $[\frac{mg}{gTS*h}]$ | Suspended Sludge $[\frac{ppm\,(Mol)}{gTS*h}]$ | Biofilm $[\frac{ppm\,(Mol)}{gTS*h}]$ |
| **Hetero-trophic** | -0.318 ± 0.492 | 0.203 ± 0.627 | -8.6 ± 35 E-03 | -6.3 ± 2.2 E-02 |
| SW inj. 1 | -0.288 ± 0.204 | -0.192 ± 0.430 | | |
| SW inj. 2 | -0.451 ± 0.440 | -0.694 ± 0.174 | | |
| SW inj. 3 | -8.38 ± 19.5 E-02 | -0.271 ± 0.195 | | |
| SW inj. 4 | -7.51 ± 27.7 E-02 | 7.22 ± 37.6 E-02 | | |
| **NH$_4$ to NO$_3$** | -2.12 ± 19.2 E-02 | -0.152 ± 0.188 | 0.6687 ± 0.232 | 4.1737 ± 0.34 |
| SW inj. 1 | -4.75 ± 8.43 E-02 | -0.168 ± 0.328 | | |
| SW inj. 2 | -5.16 ± 23.4 E-02 | -0.217 ± 0.366 | | |
| SW inj. 3 | -3.28 ± 149 E-03 | -9.15 ± 32.5 E-02 | | |
| SW inj. 4 | 5.09 ± 15.1 E-02 | -8.89 ± 39.3 E-02 | | |
| **NO$_2$ to NO$_3$** | -0.116 ± 0.230 | -0.631 ± 0.005 | 2.2834 ± 0.212 | 0.3599 ± 0.09 |
| SW inj. 1 | -0.160 ± 0.031 | -0.821 ± 0.338 | | |
| SW inj. 2 | -4.11 ± 12.1 E-02 | -8.29 ± 1160 E-03 | | |
| SW inj. 3 | -4.52 ± 3.34 E-02 | 0.353 ± 0.419 | | |
| SW inj. 4 | 0.132 ± 0.125 | 0.181 ± 0.337 | | |

Table 6: Results of the analysis of the manometric measurements of the anoxic tests with stormwater injections

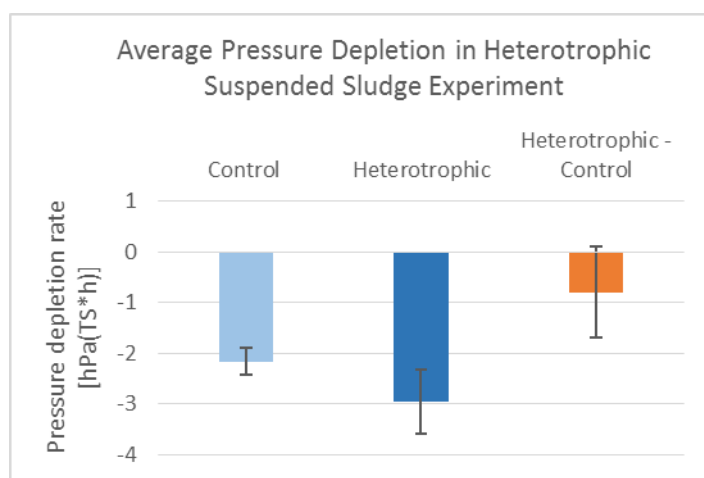| anoxic | Pressure depletion | | N$_2$O gas production | |
|---|---|---|---|---|
| | Suspended Sludge $[\frac{hPa}{gTS*h}]$ | Biofilm $[\frac{hPa}{gTS*h}]$ | Suspended Sludge $[\frac{ppm\,(Mol)}{gTS*h}]$ | Biofilm $[\frac{ppm\,(Mol)}{gTS*h}]$ |
| NO$_3$$^{*2}$ reduction | -11.14 ± 4.67 | 1.6842 ± 4.514 | 1.1522 ± 0.637 | 8.5 ± 2.1 E-02 |
| SW inj. 1 | -7.61 ± 4.18 | -8.2998 ±3.1444 | | |
| SW inj. 2 | -2.74 ± 9.71 | -7.6463 ± 3.6085 | | |
| SW inj. 3 | -1.03 ± 7.22 | -9.6069 ± 4.0682 | | |
| SW inj. 4 | -3.46 ± 2.57 | -16.6520 ±3.6125 | | |

*2: see Table 4

## 3.2. Discussion

**Aerobic tests**

The values from the aerobic suspended sludge experiments are difficult to compare, because the experiments were conducted on successive days with sludge that produced different baselines (see table 7) from the control bottles. Figure 9 illustrates how scattered the results were and making difficult its interpretation. Within the suspended sludge results the highest assimilation rates can be found in the heterotrophic experiments and one order of magnitude lower rates for the

*Table 7: Baseline pressure depletion rates from the aerobic suspended sludge experiments, sorted by date and tested substrate*

| Date/ Test | 05/22 Glucose | 05/23 Ammonia | 05/24 Nitrite |
|---|---|---|---|
| Baseline value [$\frac{hPa}{gTS*h}$] | -2.16 | -2.37 | -1.198 |



*Figure 9: Average Pressure depletion rates of the substrate injected and control Bottles from the heterotrophic suspended sludge experiment. The orange colored bar shows the difference between the two average rates; Source H. Behrmann*

ammonia oxidizing process while the nitrite oxidizing test shows about half the rate of the heterotrophic. These results qualitatively agreed with previous reports, that heterotrophic bacteria outcompete the AOB in the suspended phase [19], [20], [22]. The NOB show higher activity in the suspended phase than the AOB, which has been found before in suspended sludge, but not in ratios as high as the one found here (about one order of magnitude compared to 1:3 in other studies)[23], [33], [34]. Quantitatively the values reported in the literature are in the order of mgNO$_x$/gMLSS

[22] and mgO₂/gTVS[11], which is about two orders of magnitude larger than the results calculated from the pressure measurements.

The assimilation rates calculated from the pressure measurements in the biofilm experiments are all in the same order of magnitude ($1.55\sim1.99 * 10^{-4}\frac{mg}{gTS*h}$). The order of magnitude of the standard deviation variates, but they are in the same order of magnitude (ammonia $1.16 * 10^{-4}\frac{mg}{gTS*h}$) or one order smaller (heterotroph and nitrite, $3.8\sim9 * 10^{-5}\frac{mg}{gTS*h}$) as the one of the substrate assimilation rates. Within this close range, the nitrite oxidation rate is the highest compared to the heterotrophic and the ammonia oxidizing rates, which complies with the findings of Regmi et al.[22] and the premise that fewer heterotrophic bacteria are located in the biofilm [20].

Overall the rates found in the biofilm are one to two orders of magnitude higher than the suspended biomass phase (ratios larger than 10:1, p-values of 0.008 and 0.005 for the heterotrophic and ammonia test and 0.06 for the nitrite test). This does not agree to the ratios found by Regmi et al.[22], which are in the order of 5:1.7 for the AOB and 7.6:0.8 for the NOB between the biofilm:suspended phase, although they used MLSS instead of TS as normalization factor. The difference between the TS and the MLSS is that the TS additionally carries everything that is smaller than 45μm or dissolved in the fluid sample, which includes inorganic compounds which do not participate in the biologic processes. Therefore, the values calculated per MLSS will be higher than the ones calculated per TS. On the other hand, the results of this study agree with the results found by Plechna et al.[11] in qualitative terms (not in total values). Even though they found low OURs for biofilm compared to activated sludge, which was not the case in this study, when set in relation to the biomass, the OUR of the biofilm exceeded the activated sludge OUR by an order of magnitude, like in this study. This observation might indicate how much the results are influenced by the experimental setup and the measuring methods: Regmi et al. investigated a full-scale treatment train and determined the AOB and NOB activity by analyzing the nutrient concentration in a bench-scale reactor (volume 9L) over the course of 2h

and found activity values that were closer together[22]. Plechna et al. used a 300ml and measured the DO concentration over a short period (less than 10 minutes) of time and found a difference of the factor 10 in the activities between biofilm and suspended biomass. Plechna also used low TS concentrations (2.5 g/l) in the activated sludge, because they had found the normal concentration to lead to a too fast decline of the DO, which could mean, that in our study as well, oxygen limitation occurred, against all efforts [11]. It might be that the combination of small test volumes and the relation to the biomass leads to a qualitative overestimation of the difference in activity between suspended sludge and biofilm, which could be amplified by the difficulty of the mass determination of the biofilm.

The results of the stormwater tests were very scattered and at times showed opposite behavior between duplicate bottles, which is reflected in the high standard deviations of the data set, however some information can be drawn from the results. The calculated substrate assimilation rates from the pressure values recorded through the stormwater tests partly followed the anticipated pattern. They were expected to show the normal average assimilation rate after the substrate injection and after each injection the assimilation rate would decreasd, because of the inhibitory effect of the injected pollutants. At first, the assimilation rates increased in most cases after the substrate injection during the first and second SW injection, before the inhibitory effect could be detected, often after the third SW injection. This might have been due to the short time used, so that the bacteria were still increasing their assimilation rate because of the new food source (substrate injection) even after the first SW injection. This assumption is supported by Ren, who describes that in some toxicity studies, respirometric measurements methods took about an hour to show toxic effects [9]. Most bottles showed strong signs of inhibition after the third SW injection (equals to 27% SW, time frame from 1.5 to 2.25 h after $1^{st}$ SW injection). A strong decline in pressure took place in the bottles at high SW pollutant concentrations. The change in pressure could not be accounted for by the expected patterns or patterns from the tests without SW. The change in pressure was not

caused by the substrate assimilation, because this pressure drop was also clearly detectable in the control bottles. It is possible that the pressure decline was caused by the oxidation of the metals (Pb, Cd, Ni, Zi, Cu) in the stormwater solution or due to the increased nutrient supply caused by the dead biomass that could increase the metabolism of the active biomass. Another option could be a starting degradation of the poly aromatic hydrocarbons by bacteria, which are present as up to 1% in microbial communities and can in some instances react very fast when hydrocarbons are present [35]. It can have been contributing to this effect, that the concentration of stormwater run-off was increased successive, so that the bacteria had time to adapt, before toxic concentrations were reached.

The results for the $N_2O$ production show negligible increase or even decrease of the $N_2O$ concentration in the gas phase of both sets (suspended and biofilm) of the heterotrophic experiments, which could correspond with results found by Mannina, who found $N_2O$ consumption in the aerobic reactor [36], but opposing trends were found in the nitrogen transformation. In the suspended sludge, a lower production rate of the $N_2O$ can be seen with the ammonia oxidization and a higher production rate with the nitrite oxidization. For the biofilm, the opposite was observed. The same tendencies can be analyzed in the respective stormwater experiment, even though marginally inhibited (by 10-35%). The literature reports as causes for $N_2O$ emissions in the aerobic phase mainly low DO levels, $NO_2$ accumulation and low pH. The acidity as a cause can be ruled out because of the use of PBS to buffer changes in the pH [37]. DO could not be a cause, since there was an intensive aeration before the tests and the constant stirring. In the instances where the DO was measured at the end, it was at levels that were too high to suggest an anoxic environment in the samples (≈4mg/l), but considering the observation connected to DO by Plechna [11], it cannot be ruled out that regions of low DO in sludge flocks or the biofilm are due to possible limitations by the oxygen transfer rate in sludge flocs or biofilm [10]. The nitrite accumulation due to the direct injection of the nitrite could explain the high $N_2O$ production values in the nitrite oxidizing in the suspended sludge test. This might

not have occurred in the ammonia test because of its better equilibrium between its ammonia oxidizing and nitrite oxidizing processes, which would result in a nitrite oxidization rate high enough to avoid nitrite accumulation, that would have resulted in a negative effect (increased $N_2O$ production). The pattern in the biofilm tests was the opposite, with high $N_2O$ production in the ammonia test and lower production rates in the nitrite test. This could mean that the concentration and activity of the NOB in the biofilm is high enough to oxidize the injected concentration of nitrite without inhibitory effects. The low $N_2O$ production in the nitrite test also indicates that the high production in the ammonia test is most likely not caused by nitrite accumulation. It is likely that the high $N_2O$ production rate could be caused by a higher oxygen utilization than oxygen transfer rate, which could lead to low oxygen concentration in the biofilm even though enough oxygen is dissolved in the fluid phase [10]. These areas of low oxygen in the biofilm can cause production of $N_2O$ due to nitrifier denitrification, aerobic denitrification or intermediates of the incomplete oxidization of ammonia [17].

**Anoxic tests**

It was expected that the pressure in the anoxic experiments increased, because no oxygen was used from the gas phase and during denitrification $N_2$ and $CO_2$ should be produced. Since the produced $CO_2$ would be captured by the NaOH in the cap, the increase in pressure could fully be allocated to the production of $N_2$ and $N_2O$. When adjusted by the
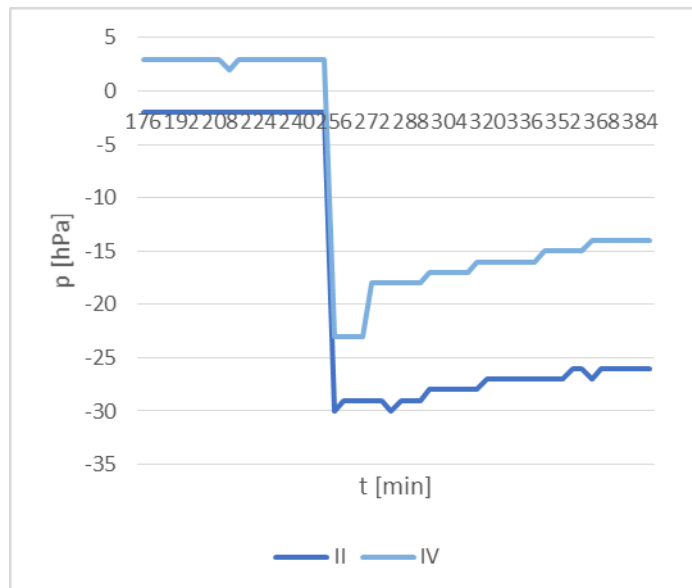


*Figure 10: Extract of two exemplary pressure graphs from which gas samples had been extracted; Source H. Behrmann*

baseline from the control bottles, all average values showed a pressure decline. This can have different reasons: first it could be that the bottles were not anoxic and a low level of aerobic activity happened in the bottles. This seems unlikely considering the bubbling and long-time of acclimatization of the bottles before the substrate and stormwater solution injections. Instead, the pressure increased in the gas phase stopped the further stripping of the gas produced in the fluid phase. Most of the $N_2O$ that is produced in the anoxic zones is released in the aerobic zones when aeration lowers the transfer resistance[27], [36]. The decline of $NO_3$ concentration in the fluid sample analysis and an increased production of $N_2O$ suggest that denitrification occurred but could not be detected by the manometric measurements. The observation that an increase in pressure could be detected in the bottles from which gas samples had been drawn at the begin of the experiment and which therefor started at low pressure levels (see Figure 10) suggests that the pressure in the bottles might have prohibited the release of the $N_2$ and $N_2O$ into the gas phase. This is contradicted by the fact that Brådskär [8] found pressure increase with a similar but larger scaled experimental setup. It is possible, that the concentrations of biomass and substrate in the experiment were too low produce an observable pressure change. It also begs the question how much $N_2O$ was dissolved in the fluid phase and therefore did not get detected in the gas sampling.

The manometric values from the anoxic stormwater experiment are different with a high deviation, coming to inconclusive results. Some of the suspended sludge bottles showed patterns that also could be seen in the aerobic tests, but then also duplicate bottles produced opposite results in different timeframes, while switching their direction (positive/negative rates) in between timeframes. In the biofilm set, the control bottles showed patterns that were expected from the bottles with the substrate, while the bottles with the substrate showed high pressure depletion that increased with the successive SW injections.

The $N_2O$ production in both the suspended sludge and the biofilm anoxic experiments was higher than in the aerobic tests, which matches the literature that

identified the anoxic zone as a main source of $N_2O$, especially, when incomplete denitrification occurs [27], [36]. These results differ from continuous reactors where the dissolved $N_2O$ is transported to the aerated sections and stripped out [36]. In this study, the $N_2O$ production could be allocated to its process of origin, due to the batch tests with the different substrates, were dissolved $N_2O$ could not be transported out of the zone where the process took place. In the suspended sludge, the production rate was as high as the highest of the aerobic tests ($NO_2$ to $NO_3$), in the biofilm it even exceeded the highest from the aerobic tests ($NH_4$ to $NO_3$) significantly (p-values 0.0008 *(DeNi vs. heterotrophic)*; 0.01799 *(DeNi vs. Ammonia to Nitrate)*; 0.0009 *(DeNi vs. Nitrite to Nitrate)*). This indicates that a reduction of $NO_3$ is happening and very likely the reduction from $N_2O$ to $N_2$ is inhibited, which can have different reasons. It is not impossible that low concentrations of DO were present in the bottles, which would inhibit the $N_2O$ reductase. On the other hand, it was likely that an accumulation of $NO_2$ inhibited the further reduction process because a decline of $NO_3$ can be observed but the further fate of the compounds is unknown. The production of $N_2O$ was lower (34% in the suspended, 2 orders of magnitude in the biofilm) in the stormwater tests, which suggests, that the denitrifying bacteria, particularly the ones in the biofilm are especially susceptible to inhibition by the stormwater pollutants.

**Limitations**

Limiting factors for this study were the small number of samples, which makes it difficult to identify outliers and larger trends. Also, the small volume of the samples, which was caused by the available equipment (bottles, stirrer plates, incubator) and easy handling, might have contributed to the high variation of the results, due to scaling effects and the normalization on the TS concentration. For the anoxic tests, it is very difficult to verify that they were actually anoxic, which could explain the negative results, even though all possible steps were conducted to produce anoxic conditions. Additionally, the results of the fluid sample analysis were not accurate, which make the verification of the manometric results impossible. Furthermore, the

choice of a normalization factor is difficult, because of the difficulty to remove the biofilm from the support media and the identification of its components. The TS was a parameter, which was possible to determine, but it also entails distortion, because the composition of the biofilm and the suspended sludge are different from each other. Finally, variation on daily operation of the model WWTP, could affect the activity and concentration of the biomass as well.

## 4.  CONCLUSION

The results of the manometric method are very variable; however, they agreed qualitatively with previous studies. The manometric method could be an option to measure aerobic activity using large sample volumes and repetitions that could produce better quality results, enable researchers to identify outliers and allow justified interpretations, but other methods like the substrate mass balancing or DO measurements would be a more efficient alternative, due to faster procedures and possibly lower sample volumes. For the stormwater test, longer time frames should be considered so that the influence of disturbance from the injection is reduced, otherwise automatized injections and gas sampling could be considered.

For the anoxic activity, the results from this study are contradictory and do not produce interpretable results.

# Appendix I:  Synthetic Stormwater Recipe

**Original Recipe**[30]**:**

| Acenaphtene | 3.2 | [mg/l] |
|---|---|---|
| Flourene | 1.9 | [mg/l] |
| Lead | 5.0 | [mg/l] |
| Cadmium | 1.2 | [mg/l] |
| Nickel | 2.5 | [mg/l] |
| Zinc | 10.0 | [mg/l] |
| Copper | 2.5 | [mg/l] |
| Nitrate | 50.0 | [mg/l] |
| Sulfate | 100.0 | [mg/l] |
| Phosphate | 10.0 | [mg/l] |

**Adaptation:** Of these Nitrate, Sulfate and Phosphorus were not used, because they are nutrients that would have interfered with the measurements.

To determine the desired target concentration in the bottles these concentrations were multiplied by 0.4, which resulted in the concentrations below (column 2). These concentrations were then multiplied by 0.09 l/Bottle to calculate the total amount of each compound per bottle, which then also equals the concentration per ml in the solution, because it had to be added to the bottle in a 1ml injection.

| Compound | Original concentration x 0.4 [mg/l] | Total amount [mg/bottle] = [mg/ml solution] | Concentration per 100 ml solution [mg/l] |
|---|---|---|---|
| Acenaphtene | 1.28 | 0.1152 | 11.5 |
| Flourene | 0.72 | 0.0648 | 6.5 |
| Lead | 2.0 | 0.18 | 18 |
| Cadmium | 0.48 | 0.0432 | 4.32 |
| Nickel | 1.0 | 0.09 | 9 |
| Zinc | 4.0 | 0.36 | 36 |
| Copper | 1 | 0.09 | 9 |

## Appendix II: Recipe for the Phosphate Buffer Solution (PBS)

Potassium Phosphate Diabasic:        5.6     g/l
Potassium Phosphate Monobasic:    2.4     g/l
EthyleneDiamineTetraaceticAcid:      0.01    g/l

Dissolved in DI-Water

# Appendix III: TS Determination of the biofilm on the support media

| No | dried [g] | cleaned [g] | Δ |
|---|---|---|---|
| 1 | 0.863 | 0.813 | 0.05 |
| 2 | 0.867 | 0.814 | 0.053 |
| 3 | 0.834 | 0.787 | 0.047 |
| 4 | 0.881 | 0.826 | 0.055 |
| 5 | 0.857 | 0.809 | 0.048 |
| 6 | 0.885 | 0.834 | 0.051 |
| 7 | 0.771 | 0.722 | 0.049 |
| 8 | 0.864 | 0.812 | 0.052 |
| 9 | 0.863 | 0.813 | 0.05 |
| 10 | 0.863 | 0.814 | 0.049 |
| 11 | 0.802 | 0.752 | 0.05 |
| 12 | 0.86 | 0.81 | 0.05 |
| 13 | 0.877 | 0.83 | 0.047 |
| 14 | 0.851 | 0.8 | 0.051 |
| 15 | 0.857 | 0.786 | 0.071 |
| 16 | 0.832 | 0.807 | 0.025 |
| 17 | 0.859 | 0.807 | 0.052 |
| 18 | 0.859 | 0.806 | 0.053 |
| 19 | 0.856 | 0.806 | 0.05 |
|  |  |  |  |
|  |  | Avg: | 0.05015789 |

$$s = \sqrt{\left(\frac{1}{n-1} * \sum_{i=1}^{n}(x_i - \bar{x})^2\right)} = \qquad 0.007754545$$

## Appendix IV: Calculations to determine the Substrate Assimilation Rates

The pressure depletion was calculated using Equation 3, where x are the specific points in time, $\bar{x}$ is the average point in time, y are the pressure values for the specific points in time and $\bar{y}$ is the average pressure value:

*Equation 3*
$$slope = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} \left[\frac{hPa}{h}\right],$$

The calculated depletion value was normalized by the TS and then used to calculate the assimilation rates of the respective substrate. An average per process (heterotrophic, ammonia oxidizing, nitrite oxidizing) was developed by arithmetic mean from the normalized pressure depletion values. These average depletion rates were then corrected by the baseline respiration rate, which was the average pressure depletion calculated from the control bottles without substrate injection.

Using the Ideal Gas Law (see eq. 4),

*Equation 4*
$$p * V = n * R * T$$

p = pressure [Pa]
V = Volume [m³]
n = amount of substance [mol]
R = universal gas constant [J/(mol*K)]
T = Total Temperature [K]

which can be transformed into Equation 5,

*Equation 5*
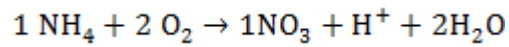$$\triangle n = \frac{\triangle p * V}{R * T}$$

the reduction of air can be calculated from the pressure depletion. Since normal air was used, the depletion of oxygen is equivalent to 20.95% [31] of the determined n value. The resulting number (n*0.2095) can then be transformed into a mass [g] as shown in Equation 6.
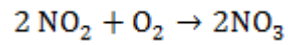
*Equation 6*
$$\triangle m = 0.2095 * \triangle n * 31.998 \frac{g \, O_2}{mol \, O_2}$$

Through stoichiometric calculations (see eq. 7, 8 and 9) the assimilation rate of nutrients can be calculated from the use of oxygen. The oxygen demands used for the calculations were: 4.57 g $O_2$/g $NH_4$ to $NO_3$, 1.14 g $O_2$/g $NO_2$ to $NO_3$ and 1.07 g $O_2$/g $C_6H_{12}O_6$ [18].

*Equation 7*

$$1 \, NH_4 + 2 \, O_2 \rightarrow 1NO_3 + H^+ + 2H_2O$$

*Equation 8*

$$2 \, NO_2 + O_2 \rightarrow 2NO_3$$

*Equation 9*

$$C_6H_{12}O_6 + 6 \, O_2 \rightarrow 6CO_2 + 6H_2O$$
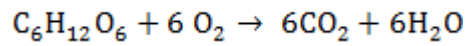
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