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DEPARTMENT OF INDUSTRIAL ENGINEERING

MASTER’S THESIS:

STAGED AND UNSTAGED MOVING BED BIOFILM REACTORS FOR THE REMOVAL OF CONVENTIONAL POLLUTANTS AND XENOBIOTIC TRACE CHEMICALS

- EXPERIMENTAL ASSESSMENT

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Preface

This 30 ECTS point thesis was completed at the department of Environmental Engineering at the Technical University of Denmark in fulfillment of the requirements for acquiring an M.Sc in Environmental Engineering. This master’s thesis was carried out from November 2013 to April 2014 under the supervision of Professor Benédek G. Plósz, Professor Barth F. Smeths, Candidate Scient. Fabio Polesel from the Department of Environmental Engineering of Technical University of Denmark and Professor Luca Palmeri from the Department of Industrial Engineering of University of Padua.

April 3rd 2013

Luca Loreggian
Acknowledgements
Abstract
This master thesis explored the potential of staging moving bed biofilm reactor (MBBR) to achieve increased removal efficiencies of conventional and xenobiotic trace pollutants, as compared to single-staged systems. The project investigated the operation of a laboratory-scale anoxic staged MBBR system in continuous and in batch operation modes. Through comparison with a unstaged MBBR system, operating in parallel to the staged one, we aimed at understand whether the rate of heterotrophic denitrification increased in the staged system and what caused higher performances. Special attention was given to pharmaceuticals as xenobiotic organic pollutants, since much is still unknown about their fate and their hazardous effects on the environment. Several investigations confirmed the presence in the aquatic environment of different active compounds and among others pharmaceuticals and their metabolites. Most of the pharmaceuticals in the environment are not readily degradable and prone to bio-accumulation. However for the majority of them, the fate and the environmental effects are still largely unknown. Where little evidence exists, medications have been demonstrated to influence gradual or indirect changes in the aquatic ecosystems. Nevertheless they are not yet targeted as prior pollutants by international environmental protective agencies.

Presently, the improper disposal and the excretion by urines and faeces are the most significant causes of environmental contamination. With regard to the latter, traditional biological WWTPs are unable to efficiently remove pharmaceuticals from wastewater, thereby representing the most relevant source of pollution for the receiving water and soil bodies. So far the most effective strategy to address the problem is the further optimization of wastewater treatment processes. Degradation of pharmaceuticals in WWTPs depends primarily on the biological treatment step. The applied technologies and the design of bioreactors are the factors affecting the most the overall removal performance.

In this study we investigated denitrification on moving bed biofilm reactors (MBBR). Biofilm reactors show very similar performances to the traditional activated sludge systems, but it also display additional advantages (i.e. no final sludge separation, biomass further specialization and less space demanding plants). High denitrification rates in MBBR have already been shown, but more effective configurations still have to be investigated. Regarding to this, reactor staging of activate sludge system have been demonstrated as effective solution. The purpose of this project is to assess the potential of MBBR staging for the removal of conventional (i.e. nitrate) and trace organic pollutants (i.e. pharmaceuticals) from municipal wastewater. In the staged configuration bacteria adapt to the specific conditions in each reactor and primarily to the available substrate. In this way, XOCs may potentially be a relevant carbon/energy source in the last reactor and bacteria may specialize to use them as substrate. This adaptation is supposed as the most probable reason for potential higher performances.

By a continuous flow experiment and four batch experiments after an adaptation period, we aim to assess and compare a 3-staged MBBR to an unstaged MBBR using
real municipal wastewater as feed. Despite of conventional pollutants (COD, nitrates and nitrites) we assess and compare the removal of eight representative pharmaceuticals (atenolol, propranolol, metoprolol, diclofenac, carbamazepine, thrimetoprim, sulphametoxazole and venlafaxine).
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1 Introduction

The occurrence of active xenobiotic organic compounds (XOCs) in aquatic systems is an emerging issue. Several studies already detected the presence of active compounds and among others pharmaceuticals and their metabolites (Heberer T., 2002). The term pharmaceutical refers to prescription human drugs, non-prescription human drugs, illegal drugs and drugs metabolites. Together with personal care products, these compounds are collectively known as PPCPs (Pharmaceuticals and Personal Care Products).

PPCPs are persistent compounds, this property affects the human metabolic pathway and ensure substances to express their curing effect (Halling-Sørensen B., 1997) (Sterner O., 2010). After administration, pharmaceuticals are fully or partially metabolized, being excreted in the form of Phase I, Phase II metabolites (i.e., conjugates) or in parent form (Sterner O., 2010). Many pharmaceuticals in the environment are not readily degradable (Halling-Sørensen B., 1997) and prone to bio-accumulation (Sterner O., 2010). The fate and the effects on the environments and human health are still largely unknown (Halling-Sørensen B., 1997) (Dana W. Kolpin, 2004). Where little evidence exists, medications have been demonstrated to influence gradual or indirect changes in the aquatic ecosystems (Kaplan S., 2013); Despite of being potentially harmful for the environment, they are not yet targeted as prior pollutants by international environmental protective agencies.

Presently, the improper disposal and the excretion by urines and faeces are the most significant causes of water contamination. With regard to the latter, traditional biological WWTPs are unable to efficiently remove pharmaceuticals from wastewater, thereby representing the most relevant source of potential harmful impact on the environment (Kaplan S., 2013) (Ternes T.A. and Joss A., 2006) (Plosz et al., 2009). As results, pharmaceuticals tend to accumulate in environmental compartments, particularly receiving water and soil bodies (Daughton, 1999) (Heberer T., 2002).

In the absence of scientific consensus, the “precautionary approach” suggests that any possible strategy to prevent environmental contamination and to remediate contamination should be enforced. Several options have been so far suggested to address this problem. Considering that pharmaceuticals consumption and discharge in the environment will not decrease in the next years, the most consistent and effective strategy is the optimization of wastewater treatment processes (Kaplan S., 2013).

Degradation of pharmaceuticals in WWTPs depends primarily on the biological treatment step (Zorita S. et al., 2009). Applied treatment technologies and design of bioreactors are the factors affecting the most the overall removal performance (Falas P. et al., 2012). Parallel investigations on biofilm reactors and on the activated sludge processes showed comparable removal of a number of pharmaceuticals. Furthermore, biofilm processes display a number of additional advantages, such as: no final sludge separation, biomass further specialization and less space demanding plants (Gobel A., 2007) (Falas P. et al., 2012) (Odengaard H., 1994).
Based on this, the interest in biofilm reactors increased considerably in the last decade, particularly in moving bed biofilm reactor (MBBR). MBBR has established itself as a robust, compact and flexible reactor (Odenaard, 2006). Even if it was developed for the nitrification process (Rusten B., 2005), today it is applied also for denitrification and high denitrification rates have been shown (Odenaard, 2006).

On the other hand the processes themselves can also be improved significantly. For example the potential for further optimization of biological kinetics in activated sludge reactors has been demonstrated by means of bioreactor staging (Grady Jr. et al., Biological wastewater treatment, 1999) (Plosz B. et al., 2003). In staged systems, the bacterial population further specialize to the specific conditions in each “subreactor”, mainly the substrates availability, and eventually the consumption of more recalcitrant compounds becomes possible (Sean E. Scuras et al., 2001). There are direct evidences that high-biodiversity communities take greater advantages of the multiple niche opportunities and diverse systems capture a greater proportion of biologically available resources (Bradley J. Cardinale, 2011).

1.1 Denitrification

Complete denitrification is the bio-reduction of nitrogen oxidized species to nitrogen gas ($\text{N}_2$). In absence of oxygen, nitrate ($\text{NO}_3^-$) and nitrite ($\text{NO}_2^-$) are the most common electron acceptors (Knowles, R., 1982) (Madigan M. T. and Martinko J.M., 1970) and by complete denitrification they are reduced to nitric oxide (NO), to nitrous oxide ($\text{N}_2\text{O}$) and finally to $\text{N}_2$.

$$\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO}(g) \rightarrow \text{N}_2\text{O}(g) \rightarrow \text{N}_2(g)$$

Organisms capable of denitrification include fungi, archaea and bacteria (M.B.Allen and C.B. van Niel, 1952) (W.G. Zumft, 1997), the latter being the most common and widespread (Knowles, R., 1982). Denitrifying bacteria are biochemically and taxonomically very diverse (Knowles, R., 1982) In an anoxic reactor on a pre-denitrification configuration, considering that no dosing of external carbon (methanol, ethanol) occurs, the only organic substrate available is the biodegradable fraction naturally occurring in the wastewater. U.S. EPA (1993) estimated the average composition of the biodegradable fraction in wastewater and the following stoichiometric relation describes the complete reductive process:

$$\text{C}_{10}\text{H}_{19}\text{O}_3\text{N} + 10\text{NO}_3^- \rightarrow 5\text{N}_2 + 10\text{CO}_2 + 3\text{H}_2\text{O} + \text{NH}_3 + 100\text{H}^-$$

$$2\text{NO}_3^- + 12\text{H}^+ + 10\text{e}^- \rightarrow \text{N}_2 + 6\text{H}_2\text{O}.$$  

According to the previous half reaction, considering the heterotrophic yield ($Y_H$), i.e. the amount of biomass grown per amount of substrate degraded, and the grams of COD per electron equivalent (8 gCOD) the following ratio estimates the amount of COD to reduce completely 1 mg of NO$_3$-N by heterotrophic denitrification:

$$\frac{10 \text{ e}^-}{2 \text{ moles of NO}_3^-} \times \frac{1}{Y_H} = \frac{80 \text{ gCOD}}{2 \times 14 \text{ gNO}_3^- - N} \times \frac{1}{Y_H} = \frac{2.86 \text{ gCOD}}{(1-Y_H) \text{ gNO}_3^- - N}$$
The electrons from the degraded biodegradable fraction are transferred through the respiration chain and each step is catalyzed by a specific enzyme. Thus the synthesis and the expression of these enzymes regulate the entire process. The synthesis of denitrification enzymes depends mostly on the environmental conditions affecting the ecological niche (i.e. dissolved oxygen—DO, pH, COD/NO₃-N). The more these conditions deviate from the optimum the more the complete denitrification is inhibited and the inhibition eventually results into the accumulation of intermediates (Y.C. Chiu, M.S. Chung, 2003).

The first step in the respiration chain is catalyzed by the membrane-bound nitrate reductase (Nar). Some bacteria (i.e. *Escherichia Coli*) are capable to reduce nitrate to nitrite but not to complete the whole denitrification (M.B. Allen and C.B. van Niel, 1952) and are not considered as denitrifiers. Nitrite reductase (Nir) catalyzes the reduction of nitrite to the first gaseous products, i.e. nitric oxide (NO). Since all the bacteria involved in the N-cycle carry out the reduction of nitrite to nitric oxide, this is the most important step. Consequently Nir is a key enzyme in the process (W.G. Zumft, 1997) (F. Schreiber, PP. Wundelin, K.M. Udert and G. F. Wells, 2012) and furthermore it is a good biomarker to investigate the community of denitrifiers (Boll M., 2004). There are two types of nitrite reductase, which differ in terms of structure and metal content (W.G. Zumft, 1997). The first one (NirS) contains hemoproteins while the second copper (nirK) and their distributions have been studied by the detection of the genes and (W.G. Zumft, 1997). Normally nitrite is reduced to nitric oxide. The reduction of nitric oxide to nitrous oxide is catalyzed by nitric oxide reductase (Nor) (W.G. Zumft, 1997) Most of denitrifying bacteria possess the gene encoding for this enzyme (F. Schreiber, PP. Wundelin, K.M. Udert and G. F. Wells, 2012). The last step is the nitrous oxide respiration (N₂O), it is mediated by nitrous oxide reductase (N₂Or) (W.G. Zumft, 1997). The majority of denitrifiers have the genes to encode all the enzymes but some of them lack the N₂Or (W.G. Zumft, 1997). Since this enzyme can be easily repressed in case of inhibiting environmental conditions, the process may result in the accumulation of nitrous oxide as final product. Since the enzymes involved in the last steps are the most sensitive, “incomplete” denitrification may result in significant emissions of nitric oxide and nitrous oxide. Importantly, nitrous oxide is a very strong greenhouse (GWP = 300) gas and recently it became reason of concern in wastewater treatment plant design and management (W.G. Zumft, 1997). The number of studies investigating the factors influencing nitrous oxide emissions has significantly increased in the last 5 years (M.J. Kampschreur, H. Temmink, R. Kleerebezem, M.S.M. Jetten and M. C.M. Loosdrecht, 2009).

Factors influencing denitrification and nitrous oxide emission


- DO concentration;
- pH;
• COD/ NO$_3$-N ratio.

The presence of oxygen represses differently the action and the synthesis of the enzymes in the complete denitrification (Knowles, R., 1982) (K.L.Thomas, D. Lloyd and L. Boddy, 1994). The sensitivity to oxygen concentration is also variable among the denitrifying species (K.L.Thomas, D. Lloyd and L. Boddy, 1994). On the other hand some bacteria (Azospirillum brasilense, Pseudomonas denitrificans, Thauera denitrificans) synthetize Nar, but not Nir, also at low concentration of oxygen (vit Mateju et al., 1992). Denitrification in presence of oxygen is termed “aerobic denitrification” or “co-respiration” of O$_2$ and NO$_3^-$, but this is rarely completed to the final electron acceptor, i.e. nitrogen gas. When oxygen is removed the reductases are de-repressed. A study showed that Nar required a period of 40 min to 3 h (Knowles, R., 1982) (vit Mateju et al., 1992), while Nir requires longer (ore than 4 hours) for de-repression because of stronger repression by oxygen (Knowles, R., 1982). N$_2$O seems to be the most sensitive enzyme to oxygen (M.J. Kampschreur, H. Temmink, R. Kleerebezem, m.S.M. Jetten and M. C.M. Loosdrecht, 2009). Consequently in case of low oxygen concentration, denitrification results into high nitrous oxide emissions.

During denitrification nitrate is reduced to nitrogen gas and protons are consumed. The process produces 3.57 g CaCO3 equivalents per 1 g of NO$_3$-N reduced (G. Tchobanoglous, F. L. Burton and H.D. Stensen, 2003), with consequent increasing pH tendency. In traditional WWTPs, denitrification is coupled with nitrification. Since nitrification consumes alkalinity, the two processes have opposite requirements and the pH is partially balanced (Henze M. et al., Biological Wastewater, 2008). The optimum pH range for denitrification is 7.0–8.0 (Knowles, R., 1982), but denitrification was found to occur at lower rates also at pH up to 11 (Knowles, R., 1982). Experimental assessment on pure cultures showed that the emissions of N$_2$O increased when pH was not in the optimum range, being maximum at pH 8.5 and 6 (M.J. Kampschreur, H. Temmink, R. Kleerebezem, m.S.M. Jetten and M. C.M. Loosdrecht, 2009).

Finally, the availability of biodegradable organic carbon also limits denitrification. Carbon source availability depends both on quantity (mgCOD/L in pre-clarified influent) and on quality (readily biodegradable versus slowly biodegradable COD). The requirement can be expressed as COD/N-NO$_3$, which can be highly variable depending on the quantity and quality of influent COD. Stoichiometrically, 2.86/(1-Y_H) mgCOD are required to completely reduce 1 mg of NO$_3$-N. Due to significance of electron availability of denitrification, expressed by the COD/NO$_3$-N ratio, this factor was reported to affect the production of nitrous oxide more than other factors (Y.C.Chiu, M.S.Chung, 2003).

1.2 Fate of pharmaceuticals in conventional wastewater treatment

Since the efficiency of human metabolism is variable (20%-50% of the administered dose may be excreted as the parent compound, (Sterner O., 2010)), pharmaceuticals can reach WWTPs in the form of parent compounds and metabolites (including Phase II conjugates). As mentioned before, WWTP effluents are the most relevant sources of
emission of pharmaceuticals to the aquatic environments (Kaplan S., 2013), mostly as a result of the fact that WWTPs have been historically designed to remove exclusively conventional pollutants (COD and nutrients).

Biological treatment is generally the step where most of the removal influent loads of pharmaceuticals occurs. The fate processes identified include

- biotransformation of parent by microbial communities
- sorption-desorption
- biological retransformation of metabolites and/or conjugates to the parent compound.

The observed removal efficiency for pharmaceuticals and contrast media is used to screen substance according to their persistence in biological wastewater treatment. This parameter can be defined as:

$$\eta_{obs} = \frac{(C_{in} - C_{out})}{C_{in}} \times 100$$

In general, removal efficiencies can vary significantly among WWTPs and depend on the applied treatment technology. Up to now, the only effective treatments are the sorption onto sludge, and the biological transformation (Ternes T.A. and Joss A., 2006).
<table>
<thead>
<tr>
<th>compound</th>
<th>plant</th>
<th>influent concentration [ng/L]</th>
<th>effluent concentration [ng/L]</th>
<th>apparent removal [%]</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac</td>
<td>Källby, Sweden</td>
<td>160</td>
<td>120</td>
<td>22</td>
<td>(J.L. Zhou et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Scaynes Hill, UK</td>
<td>397</td>
<td>119</td>
<td>70,1</td>
<td>(J.L. Zhou et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Manor Farm Road, UK</td>
<td>782</td>
<td>176</td>
<td>77,5</td>
<td>(J.L. Zhou et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Basingstoke, UK</td>
<td>981</td>
<td>78</td>
<td>92</td>
<td>(J.L. Zhou et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Greifensee, CH</td>
<td>1185</td>
<td>620</td>
<td>47,7</td>
<td>(Buser H.R. Poiger T. and Muller M.D., 1998)</td>
</tr>
<tr>
<td></td>
<td>**5WWTPs, FIN</td>
<td>350</td>
<td>170</td>
<td>51,4</td>
<td>(Lindqvist N., 2005)</td>
</tr>
<tr>
<td></td>
<td>Howdon, UK</td>
<td>1000</td>
<td>290</td>
<td>71,0</td>
<td>(Roberts P.H. and Thomas K.V., 2005)</td>
</tr>
<tr>
<td></td>
<td>**4WWTPs Sevilla, Spain</td>
<td>720</td>
<td>530</td>
<td>26,4</td>
<td>(J. Martin, D. Camacho-Muñoz, J.L. Santos, I. Aparicio, E. Alonso, 2012)</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td></td>
<td>**57,3</td>
<td></td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Källby, Sweden</td>
<td>1680</td>
<td>1180</td>
<td>30</td>
<td>(J.L. Zhou et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Sheffield Park, England</td>
<td>1786,5</td>
<td>525,5</td>
<td>70,6</td>
<td>(J.L. Zhou et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Scaynes Hill, UK</td>
<td>1662</td>
<td>950</td>
<td>42,8</td>
<td>(J.L. Zhou et al., 2009)</td>
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<td></td>
<td>Manor Farm Road, UK</td>
<td>1237</td>
<td>637</td>
<td>48,5</td>
<td>(J.L. Zhou et al., 2009)</td>
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<tr>
<td></td>
<td>Basingstoke, UK</td>
<td>1833</td>
<td>837</td>
<td>54,3</td>
<td>(J.L. Zhou et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>**4WWTPs Sevilla, Spain</td>
<td>1690</td>
<td>1000</td>
<td>40,8</td>
<td>(J. Martin, D. Camacho-Muñoz, J.L. Santos, I. Aparicio, E. Alonso, 2012)</td>
</tr>
<tr>
<td>average</td>
<td></td>
<td></td>
<td></td>
<td>**48</td>
<td></td>
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<tr>
<td></td>
<td>Manor Farm Road, UK</td>
<td>690</td>
<td>134</td>
<td>80,4</td>
<td>(J. Martin, D. Camacho-Muñoz, J.L. Santos, I. Aparicio, E. Alonso, 2012)</td>
</tr>
<tr>
<td></td>
<td>**4WWTPs Sevilla, Spain</td>
<td>390</td>
<td>340</td>
<td>12,8</td>
<td>(J. Martin, D. Camacho-Muñoz, J.L. Santos, I. Aparicio, E. Alonso, 2012)</td>
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<tr>
<td>average</td>
<td></td>
<td></td>
<td></td>
<td>**62,34</td>
<td></td>
</tr>
<tr>
<td>X-ray contrast media</td>
<td>average</td>
<td>3840</td>
<td>2100</td>
<td>**45,3</td>
<td>(K. Fent et al., 2006)</td>
</tr>
</tbody>
</table>

Biological processes are capable of degrading pharmaceuticals, evidences have been provided both for lab scale systems (Carballa M. et al. O. F., 2007) (Carballa M. et al. O. F., 2006) and for industrial plants, tab. 2.2. Other studies show that the applied technology and the reactor-design are the most relevant factors to optimize the overall performance of the biological treatments (Falas P. et al., 2012) (Plosz B., 2007). The “intensification” of biological processes is one of the feasible solutions (Grady Jr. et al., 2012).
Biological wastewater treatment, 1999) (Plosz B. et al., 2003) and “reactor staging” is a promising technic to achieve high reaction rates (Plosz B., 2007).
1.2.1 Reactor staging as process optimization

A number of studies on the activated sludge show that by reactor staging it is possible to achieve high reaction rates and an optimized design. Higher performances rely mainly on the specialization of the bacterial community to the specific conditions in each stage. This hypothesis has been so far tested for denitrification with activated sludge (Sean E. Scuras, A. Jobbagy and C.P. Leslie Grady Jr, 2001). A staged configuration requires shorter sludge retention time and less amount of biomass to achieve comparable performance of unstaged configuration. Furthermore if the reactor operates at low biomass concentration, less biomass flux into the sedimentation and the required settler area is reduced (Sean E. Scuras, A. Jobbagy and C.P. Leslie Grady Jr, 2001). An optimum of three reactors in series for the staged configuration has been indentified (Plósz, 2007), providing for overall higher conversion rates as compared to unstaged configuration and increased flexibility. It has been also hypothesized that, due to the uptake of readily and slowly biodegradable COD in the first two stages, more recalcitrant organic chemicals could be degraded in the last stage configuration. (Scuras et al., 2001; Plósz, 2007).

Further compared assessment of staged and unstaged configurations for denitrification of landfill leachate has been performed using biofilm systems (REFERENCE).

Conceptual approach of reactor staging

The conceptual approach for the staging of an anoxic reactor is already discussed by Scuras et al. (2001)and Plósz (2007) and Here we briefly present the most relevant steps in order to understand how optimize the dimension of stages in the staged configuration.

The heterotrophic denitrifying bacteria in the biofilm oxidize nitrogen species as electron acceptors and use organic compounds as electron donor. The rate of denitrification depends also on the availability of biodegradable substrates, and it is optimum if the electron donors are not limiting. The availability of biodegradable compounds depends both on quantity and quality of the substrate. Considering the Activated Sludge Model No.1 structure and nomenclature (Henze M. et al., Biological Wastewater, 2008), the ready biodegradable fraction \(S_s\) is immediately available, while the particulate fraction \(X_p\) need to be first hydrolysed to \(S_s\). Thus if \(S_s\) is not available, the hydrolysis of \(X_p\) is the limiting step. Biomass decay is also providing new particulate biodegradable fraction; it liberates inert biomass debris \(X_d\) and \(X_p\), which is further hydrolysed to \(S_s\) (Grady Jr. et al., Biological wastewater treatment, 1999) (Plosz B. Gy., 2007) (Madigan M. T. and Martinko J.M., 1970).

The amount of COD theoretically required for the complete oxidation has been estimated by stoichiometric calculations. Assuming that the heterotrophic yield is 0,71, the following equation estimated the ratio COD/ \(\text{NO}_3^-\text{N}\) to denitrify 1 mg of \(\text{NO}_3^-\text{N}\) denitrified to \(\text{N}_2\) (Henze M. et al., Biological Wastewater, 2008);

\[
\frac{2,86}{\text{mgNO}_3^- \text{N}} \times \sqrt{(1 - Y_H)} = 8,6\text{mgCOD}
\]
The amount of denitrified nitrates is approximately the difference between the incoming and the outgoing flux (Plosz B. Gy., 2007), by the previous COD/NO$_3$-N ratio we estimate the amount of COD theoretically reduced;

\[
(S_{N,0} - S_{N,e}) \frac{2.86}{1 - Y_H} = (S_{S,0} + S_{S,XS,0} + S_{S,XH})
\]

And if $X_H$ is the active biomass in the continuously stirred reactor, then:

\[
\frac{(X_H V)_{CSTR}}{F} = \frac{S_{S,0} - S_{S,e}}{q_S} = \frac{(S_{S,0} + S_{S,XS,0} + S_{S,XH})}{q_S} \frac{2.86}{1 - Y_H}
\]

where $q_S$ (mgNO$_3$/mgCOD*h), the specific substrate utilization rate and it is the ratio of the specific growth rate, $\mu_H$ (h$^{-1}$), and the observed growth yield, $Y_H$ (mgCOD/mgCOD). For an hypothetical unstaged configuration, the previous equation becomes:

\[
\frac{V_{CSTR}}{F} = (S_{N,0} - S_{N,e}) \frac{2.86 Y_H 1}{1 - Y_H X_H Y_H}
\]

Whereas for a series of CSTRs, where the effluent of tank $n-1$ is the influent of tank $n$:

\[
\sum_{i=1}^{n} \frac{V_{CSTR,i}}{F} = \sum_{i=1}^{n} (S_{N,0} - S_{N,i}) \frac{2.86 Y_{Hi}}{1 - Y_{Hi} X_{Hi} Y_{Hi}} 1
\]

Assuming that $X_H$ is constant in the reactors, the followings are also valid (Plosz B. Gy., 2007):

\[
\frac{V_{CSTR}}{F} = (S_{N,0} - S_{N,e}) \frac{1}{-r_d}
\]

\[
\sum_{i=1}^{n} \frac{V_{CSTR,i}}{F} = \sum_{i=1}^{n} (S_{N,0} - S_{N,i}) \frac{1}{-r_{di}}
\]

Knowing the specific denitrification rates $r_d$ (e.g. through batch experiments), we can estimate the required hydraulic retention time to achieve that removal. The optimum design of the stages based on the rate of denitrification, this depends on the substrate available in each reactor. This kinetic-control design ensure the best exploitation of the volume available in each reactor.

### 1.2.2 Biofilm and MBBR

As opposed to suspended biomass systems, biological wastewater treatment in biofilm systems is performed by microorganisms attached to physical surfaces. The biofilm structure consists of bacteria imbibed in a matrix of extracellular polymeric substances (EPS). The EPS is made of free nucleic acids and water, it acts as glue and holds the biofilm onto the surface of attachment (also known as “substratum”).

Biofilm exhibits structural, chemical and biological heterogeneity. The whole structure is ideally divided in three compartments: the bulk liquid, the boundary layer, the biofilm itself and the substratum, which supports the biofilm (Grady Jr. et al., Biological wastewater treatment, 1999) (Henze M. et al., Biological Wastewater, 2008). The
metabolic activities within the biofilm layer and the diffusion process generate concentration gradients of nutrients, signalling compounds and bacterial waste (Stewart P.S. and Franklin M.J., 2008). These processes drive the mass transport of substrates through the entire biofilm layer and diffusion may be the limiting process, hindering substrate degradation (Odengaard H., Rusten B. and Westrum T., 1994) (Odenaard, 2006). The substrates availability, together with the bacteria location, also affects the microbial competition. Bacteria respond to gradients and adapt to the local chemical and physical conditions and differentiation occurs within the biofilm layer. The organisms on the surface access easily to the substrate but they are more frequently washed out, vice versa the bacteria living deeply in the layer are protected from detachment. These conditions may eventually change and biofilm evolves over time (Stewart P.S. and Franklin M.J., 2008).

The most significant advantage of biofilm systems, as opposed to activated sludge, relies in the fact that biofilm reactors generally do not require a following biomass separation (e.g., a settler) process (Henze M. et al., Biological Wastewater, 2008) and biomass recirculation (Grady Jr. et al., Biological wastewater treatment, 1999), resulting in less space demanding treatment plants.

Three types of biofilm reactors are generally distinguished (Henze M. et al., Biological Wastewater, 2008): non submerged system, submerged fixed bed biofilm reactors and different kinds of fluidized bed reactors.

With respect of XOCs, examinations on biofilm systems and on traditional activated sludge reactors, the latter operating at longer hydraulic retention time, showed comparable removal of several pharmaceuticals (Falas P. et al., Suspended biofilm carrier and activated sludge removal of acidic pharmaceuticals, 2012) (Gobel A., 2007). Higher removal rates per unit of biomass were also shown for biofilm systems (Falas P. et al., 2012).

Biofilm have performances similar to the activate sludge system, but many more advantages have been experienced by using biofilm. The main difference is that in the biofilm microorganisms are immobilized on a carrier, while for the activate sludge they are free to move in the reactor. The biofilm structure consists of bacteria imbibed in a matrix of extracellular polymeric substances (EPS). The EPS is made of free nucleic acids and water, it acts as glue and it holds the biofilm on the carrier which is a non-permeable solid support (James P. McQuarrie and Joshua P. Boltz, 2011). The carriers differ on material, shape and mobility; we distinguish three types of biofilm reactors (Henze M. et al., 2008): non submerged system, submerge fixed bed biofilm reactors and different kinds of fluidized bed reactors.

Biofilm exhibits structural, chemical and biological heterogeneity. The whole structure is ideally divided in three compartments: the bulk liquid, the boundary layer, the biofilm itself and the carrier, which supports the biofilm (Grady Jr. et al., Biological wastewater treatment, 1999) (Henze M. et al., 2008). The metabolic activities within the biofilm layer and the diffusion process generate concentration gradients of nutrients, signalling compounds and bacterial waste (Stewart P.S. and Franklin M.J.,
2008). These processes drive the mass transport of substrates through the entire biofilm layer and diffusion is the limiting process. Indeed it is very slow and it limits the availability of substrates and consequently the biodegradation of these chemicals (Odengaard H., 1994) (Odenaard, 2006). The substrates availability, together with the bacteria location, also affects the microbial competition. Bacteria respond to gradients and adapt to the local chemical and physical conditions and differentiation occurs within the biofilm layer. The organisms on the surface access easily to the substrate but they are more frequently washed out, vice versa the bacteria living deeply in the layer are protected from detachment. These conditions may eventually change and biofilm evolves over time (Stewart P.S. and Franklin M.J., 2008).

Examinations on biofilm and on traditional activated sludge reactors show comparable removal of several pharmaceuticals, but longer hydraulic retention time in the activated sludge process (Falas P. et al., 2012) (Gobel A., 2007). Indeed the biofilm has higher removal rates per unit of biomass (Falas P. et al., 2012). Furthermore the following advantages have also been demonstrated:

- As soon as the biomass is active, the biofilm reactor does not require settler (Henze M. et al., 2008) and no recirculation of the biomass, as in the activated sludge systems (Grady Jr. et al., Biological wastewater treatment, 1999); these result in less space demanding plants;
- The biomass separation is 10 times lower (Odenaard, 2006) and final sludge separation is not necessary.

Based on these considerations the interest for biofilm reactors increased in the last decade and recently a new technology has been developed; it is known as moving bed biofilm reactor (MBBR). The MBBR adopts the best of the activated sludge technology and it is an intermediate technology between the activate sludge and the biofilm. The innovative feature of MBBR is that the carriers are free to move within the reactor and the whole tank is available for the biomass growth. The MBBR optimizes the use of the volume available in the reactor and it achieves a high homogenization level. The MBBR is also very flexible and it operates on different conditions (Rusten B., 2005). Even if it was originally developed for the nitrification (Rusten B., 2005), today MBBR is used also for the organic removal, the phosphorus removal and de-nitrification (Odenaard, 2006) (Henze M. et al., 2008).

The carriers of the MBBR are designed to provide a large protected surface area and optimal conditions for the bacteria culture. They are usually made on high density polyethylene (0.95 g/cm³). The density of polyethylene is close to water density and this facilitates the suspension even with low mixing energy. There are many types of carrier; they differ on dimension, thickness and geometry. Depending on the selected carriers, the producer suggests the optimal filling ratio, which is the most important parameter to design MBBR. The filling ratio is defined as the volume occupied by the carriers in the empty reactor (Odengaard H., 1994), the maximum filling ratio used is about 70% (Rusten B., 2005). The standard filling ratio also influences the “carrier suspension” and the proper ratio ensures that the mixing is easily established.
Regarding to this, the turbulence is also important. It ensures the optimal diffusion of compounds through the biofilm layer.

Since the type of carrier and the filling ratio affect the effective specific area available for the growth of biofilm, they are the most important reactor parameters.

The MBBRs have been investigated on different configurations; different operational conditions and different substrates consumption have been monitored. Recently also the pharmaceutical removal in Swedish WWTPs has been documented (Falas P. et al., 2012). It is proven that MBBR has superior removal capacity than the activated sludge, but the full potential is still to be explored and further investigations are required.

1.2.3 Fate of XOCs in biological wastewater treatment systems

In biological wastewater treatment systems, XOCs, and among them pharmaceuticals (including metabolites), undergo a number of physico-chemical and biological fate processes. The most significant processes have been identified (Ternes and Joss, 2006, Plósz et al., 2010)

- Biotransformation (i.e. one-step transformation) of dissolved parent compound forms,
- Sorption to suspended particles and sludge particles
- Retransformation of other dissolved fraction, e.g. conjugated metabolites, to the dissolved parent compound fraction

Among other factors, the rate of biotransformation can depend also on the operating conditions that affect the degradation of the primary substrate. Indeed these conditions define the niche where the microorganisms live, grow and replicate. With regard to denitrification, the most relevant factors include the species of denitrifying bacteria occurring in the reactor, concentrations of biodegradable COD and of nitrate, DO concentration in the water, pH and the temperature (Ternes T.A. and Joss A., 2006) (Rittmann B.E., 1992) (Knowles, R., 1982). Within their niche the microorganisms consume growth substrates that supply carbon and energy. A distinction between essential substrates which are primary for growth and secondary and/or co-metabolic substrates is usually considered. Pharmaceuticals and XOCs generally belong to the second group due to their persistence and their low concentration, which is not able to support biomass growth (Rittmann B.E., 1992).

Rittmann (1992) referred to biological transformation and degradation processes as “detoxification”, for which two possible mechanisms were described. In the first mechanism the microorganisms utilize the primary substrate as electron and energy sources but they consume also a fraction of the secondary substrates (Rittmann B.E., 1992). In this case, XOCs are referred to as secondary substrate, being consumed when primary substrate concentrations are not sufficient. In case enzymes catalysing the consumption of primary substrates are active also for XOCs, co-metabolism occurs (Rittmann B.E., 1992). In the second mechanism the XOCs availability is high enough to trigger the specialization of microorganisms to the consumption of XOCs as unique substrate. In this case XOCs become primary substrates and higher removal of
Under batch experiments conditions (with, e.g., activated sludge), XOC biotransformation can be described using \textit{pseudo first order} kinetic (Ternes et al., 2006)

\[
\frac{dC}{dt} = \frac{C_{t+dt} - C_t}{dt} = -k_{bio} \cdot X_{SS} \cdot C
\]

where \( C \) is the dissolved XOCs concentration [\( \mu g*L^{-1} \)] or [ng \( L^{-1} \)], \( X_{ss} \) the suspended solids concentration in the reactor [\( gSS*L^{-1} \)] and \( k_{bio} \) the biotransformation rate coefficient [\( L*gSS^{-1}*d^{-1} \)]. Similar batch experiments with MBBR carriers (Falås et al., 2012) showed that the biotransformation of pharmaceuticals could be described using the same kinetic equation.

The biotransformation rate is proportional to the substance concentration \( C \), where pseudo first order refers to the concentration of biomass (\( X_{ss} \)), assumed to be constant in the period of a batch experiment (Ternes T.A. and Joss A., 2006) (Plosz B. Gy., 2007).

1.2.4 Pharmaceuticals and contrast media: sources, fate and toxicity

The term pharmaceutical refers to prescription human drugs, non-prescription human drugs and their metabolites.

The release of pharmaceuticals in the environment has been recognized as one of the emerging issue in the environmental chemistry (Heberer T., 2002), and it was also targeted as a research priority in the European Union 5th framework Programme for Research (Ternes T.A. and Joss A., 2006). The concern on pharmaceuticals, rather than on their acute toxic effects, focuses on the chronic toxicity.

Little is known about how pharmaceuticals behave and their effects on the environmental ecosystems where they enter. Since pharmaceuticals are biologically active compounds, even very small quantities (fig.1) may have harmful effects (Jones O.A.H. et al., 2011). Recently the aquatic organisms have been indicated as an important target (K. Fent et al., 2006). Indeed even if medications are designed to target specific metabolic pathways in the human body and in domestic animals (in case of veterinary uses), they may explicit similar function on non-targeted organisms and cause direct or in-direct changes on the whole ecosystems (Halling-Sørensen B., 1997) (Wilson B.A. et al., 2003) (Fair P.A., 2009) (Guler Y. and Ford A. T., 2010). Moreover, nowadays we ignore the modes of action of each compound on the many potential receptors and it is hard to predict direct and in-direct changes on the ecosystems (Daughton, 1999). Since in the aquatic environments pharmaceuticals have been detected at low concentrations, they are supposed to pose low risk for acute toxicity (K. Fent et al., 2006). On the other hand the situation is different for the potential chronic effects and more is to be investigated. The complete understanding of the environmental risk should also consider that organisms are exposed to a highly variable “cocktail” of medications and this condition is very difficult to be investigated.

Nowadays the global consumption of pharmaceuticals is estimated to be around 100,000 t a\(^{-1}\) per year (Ternes T.A. and Joss A., 2006). Consumption in industrialized
countries represents the main fraction of the total global use and it varies between 50 and 150 g cap$^{-1}$ a$^{-1}$ (Ternes T.A., 1998). In table 1.1, we reported the consumption of the most widespread medications in some European countries.

Thus the reason of environmental concern is mostly because WWTPs fail in the treatment of XOCs and that we ignore the potentially serious effects on the environment (Heberer T., 2002) (Ternes T.A. and Joss A., 2006) (Ternes T.A., 1998) (J.L. Zhou et al., 2009).

Two approaches to the problem have been suggested and discussed so far. The first strategy relies on “minimization and prevention”. According to this the less pharmaceuticals are produced and consumed the less compounds eventually reach the environment (Kaplan S., 2013). The second approach aims to tackle the discharge of chemicals in the short term period by the optimization of traditional WWTPs. Regarding to this; biological treatments and innovations in the more design of the reactors have very big potential to face the problem (Plosz B., 2007).

Probably a more accurate design of the compounds on the market is also a key factor. Indeed an highly refined and efficient medication may require a lower dosage and the lower is the consumption of medications, the less will be the amount of pollutants reaching the environment.

### 1.2.5 Routes of pharmaceuticals exposure

There are many potential routes by which pharmaceuticals reach the environment (Fig. 1.2). Here we shortly present the most important ones. First of all, the manufacture and the disposal of pharmaceutical waste have been strictly regulated and severe thresholds currently apply. Therefore, water contamination from waste disposal by manufacturers, wholesalers, retailers and hospitals can be considered a remote event (Jones O.A.H. et al., 2011).

On the other hand, emission pathways related to human consumption are significant mainly because of excretion with urines and faeces. Pharmaceuticals are designed to explicit a curing effect and their performance varies according to chemical and physical properties, defined e.g., by the chemical composition and the molecular geometry (Sterner O., 2010). After the uptake pharmaceuticals are retained in the organisms until the curing effect is accomplished (Sterner O., 2010) (Ternes T.A. and Joss A., 2006). Substances are usually metabolized in the liver and excreted through urines or faeces. Excreted forms include the unchanged parent substance and the metabolites, which include conjugates. Metabolites in water can be as persistent as the parent compounds (Halling-Sørensen B., 1997). By this route a wide range of pharmaceuticals and their metabolites are released into the sewage system and reach municipal wastewater treatment plants (WWTPs).

Since traditional WWTPs are not able to achieve complete removal of pharmaceuticals their effluents have been detected as the most notable pathway by which pharmaceuticals reach surface waters and ground waters (Kaplan S., 2013) (Daughton
In this thesis, this has been considered the main route of emission/exposure and is highlighted in red in Fig. 1.1.

Pharmaceuticals regularly disposed as solid waste are usually incinerated or landfilled. While in the first case they are completely oxidised, if landfilled they may eventually reach the ground waters by leaking through failures in the landfill liners (Jones O.A.H. et al., 2011).

Pharmaceuticals and contrast media have been detected in a wide variety of aquatic environments including surface waters, groundwater and drinking water (REFERENCE). Concentrations in treated wastewater effluents range from the low ng L^{-1} to low µg L^{-1}, while in the aquatic environment concentrations are generally lower (up to 1µg*L^{-1}) (Jia-Qian Jiang et. al, 2013) (J.L. Zhou et. al, 2009) The highest environmental concentrations have been detected next to point sources, such as in proximity of WWTP discharges (Ternes T.A. and Joss A., 2006).

A number of studies have highlighted and investigated the problem of environmental release of pharmaceuticals in different countries (Halling-Sørensen B., 1997) (Kaplan S., 2013) (Daughton, 1999) (Jia-Qian Jiang et. al, 2013) (J.L. Zhou et. al, 2009). The highest number of compounds detected in the environment is 3000 pharmaceuticals (Ternes T.A. and Joss A., 2006), among these 80-100 pharmaceuticals and metabolites are commonly detected in different water bodies (Ternes T.A. and Joss A., 2006). Fig. 1.1 shows the concentrations of the most ubiquitous pharmaceuticals in treated sewage and surface water reported until year 2006 (K. Fent et al., 2006).

Figure 1-1 Concentration of pharmaceuticals in treated sewage (a) and surface water (b) in U.K. (K. Fent et al., 2006)
Figure 1-2 Exposure routes of pharmaceuticals into water bodies; the red line represents the focus of this thesis which is the most relevant route of exposure to the aquatic environment (Heberer T., 2002) (Ternes T.A. and Joss A., 2006) (Jia-Qian Jiang et al., 2013)
1.3 Thesis aims and research hypothesis

The overall purpose of this project is to investigate the performance of lab-scale unstaged and staged anoxic MBBR as innovative technology for the removal of conventional (i.e. nitrate) and trace organic pollutants (i.e. pharmaceuticals) from municipal wastewater. More specifically, the objectives of the thesis are:

- To assess and compare the denitrification performance of 3-staged and unstaged MBBR reactor configuration under continuous flow conditions, using real municipal wastewater as feed;
- To assess and compare the removal of eight representative pharmaceuticals (atenolol, propanol, metoprolol, trimethoprim, carbamazepine, diclofenac, sulphamethoxazole, and venlafaxine) in the staged and unstaged MBBR under continuous flow operation;
- After a sufficiently long period of continuous operation, assuming that steady state is reached in terms of biomass characteristics, to assess and compare biological kinetics of denitrification in staged and unstaged MBBR reactors by means of targeted batch experiments;

The assessment of the experimental set-up implied the verification of the following hypotheses:

- In the staged configuration, bacteria adapt and specialize to the substrate available in each reactor. In this way, XOCs may potentially be the only available carbon/energy source in the last reactor and bacteria may specialize to use them as substrate. The specialization of the bacterial community could be expressed by different metabolic kinetics, different composition in the bacterial community and/or different enzymes expression.
- The evolution of the bacterial community in the staged configuration during the adaptation period is responsible for higher removal rates of conventional and xenobiotic pollutants.
- Considering the results of the batch experiments, the optimization of the design of the staged configuration could result in the achievement of higher performances with the same overall volume.
2 Methods

In this chapter we provide the descriptions of the set-ups for the continuous flow experiment and for the batch experiments. We also describe the analytical methods by which we monitor the performances and the active biomass in the biofilm of each reactor. The protocols for the all analysis are in the appendices.

We were running the continuous flow reactors since the beginning of December 2013 until the end of March 2014. In the first two month of operation we tested different operational solutions, consequently we experienced fluctuations in the performances and data from the first two months are not reliable to discuss the research hypotheses. In the end of January we started to control the pH level in the storage tank and this was the last change in the operating conditions. Apart from the quality of the feeding medium, since the 29 of January the system operated at constant operating conditions for 55 day in a row. By monitoring the removal of conventional pollutants and XOCs in this period we discuss the performances of the two configurations. In the beginning of March we stopped the continuous flow reactors in order to run the batch experiments. The batch experiments have been performed on the same operating conditions of the continuous experiment; the filling ration and the amount of biomass have been also constant. By the observations on the batches we aim to estimate the rates of denitrification first and later to optimize the staged reactor design according to the procedure by Plosz (2007) for the activate sludge.

2.1 The continuous experimental set-up

The set-up of the continuous flow experiment consists of two anoxic moving bed biofilm reactors on a laboratory scale employing K1 carriers from AnoxKaldnes, figure 2-1. The set-up is similar to that one of Plosz (2007). It consists of a staged and an unstaged line disposed in parallel. The staged line is composed of three sub-reactors in series, while the unstaged line is made up of one single reactor. The two lines are running on the same operating conditions (volume, hydraulic retention time and feeding medium) and by comparing them we evaluate the reactor staging as optimization tool.

![Figure 2-1 cultivated K1 Anoxkaldnes](image)

In the end of December we measured the biomass growth in the reactors and we observed that it differs among the reactors; on this fact we assumed that somehow the
bacteria communities in the reactor are responding differently to the specific operating conditions and to the availability of substrate among others parameters. In the end of January we also started to control the pH level in the storage tank and this was the last change in the operating conditions. Despite of the quality of the feeding medium we assumed that the operating conditions did not change since the 29 of January. The continuous experiment operates for 55 days in a row from the 29 of January to the end of March.

By this set-up, we investigate the hypothesis by which the diverse substrate available in the reactors along the staged line drives the specialization of the bacterial communities. With regarding to this we assumed that:

- the ready biodegradable fraction \(S_1\) is almost entirely consumed in the first reactor (S1);
- In the second reactor (S2) bacteria oxidize the last \(S_1\) from S1 and new \(S_1\) from the hydrolysis of the particulate biodegradable fraction \(X_s\);
- In the third reactor (S3) the more persistent biodegradable fraction is still available for biodegradation and bacteria are supposed to evolve and specialize in order to consume this hardly degradable substrate.

By measuring the removal of pharmaceuticals and by microbiological analyses in the two lines, we want to understand if any specialization is occurring in the staged configuration. Eventually this specialization results into a different bio-diversity in the reactors and a higher biodiversity in the staged line than in the unstaged line. We expected that the staged line has higher biodiversity and consequently higher
performances of nitrates reduction, of sCOD consumption and pharmaceuticals degradation.

The staged line consists of three cylindrical “sub-reactors” in series. Previous studies on staged activated sludge reactors indicate that no more than three tanks are required (Sean E. Scuras, A. Jobbagy and C.P. Leslie Grady Jr, 2001) (Plosz B., 2007). The first two reactors (S1 and S2) have same sizes; they are transparent PVC cylinders, 1 cm thick, 60 cm high and 7 cm in external diameter. The third one (S3) is again a transparent PVC cylinder, 60 cm high and 10 cm in external diameter. The reactors have respectively 1.5 L and 3 L volume capacity and the total volume capacity is 6L. Each reactor is connected to the next one by approximately 50 cm long PVC pipes of 0.5 cm diameter. The un-staged line consists of one cylindrical reactor of 1 cm thick PVC with 6 L volume capacity, it is 80 cm high and 12 cm in diameter.
Figure 2.3 scheme of the experimental set-up

CSTR 2-3 °C
Wastewater storage tank (200 L)

1.2 L/d

NO₂
N₂

S1
V=1.5 L/d

S2
V=1.5 L/d

S3
V=3 L/d

30 L/d

V=6 L/d

Effluent collection
We estimated the required dimensions of the three reactors in the staged line by the optimizing procedure from Plosz (2007). The rates of removal have been estimated by simulating a batch experiment with WEST. The reactor specifications are summarized in the table below.

**Table 2-1 set-up dimensions**

<table>
<thead>
<tr>
<th>Line</th>
<th>Reactor (mL/h)</th>
<th>Reactor volume [L]</th>
<th>Height [cm]</th>
<th>Outer diam. [cm]</th>
<th>Inner diam. [cm]</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNSTAGED</td>
<td>625</td>
<td>6</td>
<td>80</td>
<td>12</td>
<td>11</td>
<td>PVC</td>
</tr>
<tr>
<td>STAGED</td>
<td>S1</td>
<td>625</td>
<td>1,5</td>
<td>60</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>625</td>
<td>1,5</td>
<td>60</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>625</td>
<td>3</td>
<td>60</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

The four moving bed biofilm reactors (MBBRs) are filled with plastic suspended carriers, Kaldnes K1. We set a filling ratio of 30% to ensure the homogeneous mixing and the circulation of the carriers. AnoxKaldnes suggest 60% for K1, but the small diameter in S1 and S2 limit the maximum filling ratio. Continuously stirred reactors and circulation of the carriers are primarily achieved by sparging nitrogen gas (N₂) with jet nozzles from the bottom of the reactors and secondarily by wastewater flowing through the system. The definitive adjustment of gas consumption accounts also for the removal of solids settling down in the reactors.

By sparging N₂, we also ensure low concentration of DO and no aerobic zones. We measured the DO concentration in the reactors on a daily basis during the sampling procedure; measurements have been performed with Dissolved Oxygen meters from WTW, the detected values are low in the scale of the instruments where the potential error might be relevant (HOW much). We experienced similar DO ranges in the four reactors; the minimum concentration was 0,34 mgO₂/L in the S2 and the maximum was 0,45 mgO₂/L in the unstaged reactor and in S1.

Studies demonstrated that such concentrations may still inhibit the enzymatic activity and consequently the whole denitrification (vit Mateju et al., 1992). We provided the reactors with polystyrene lids and rubber sealings to further reduce the potential exchange of O₂ between the air and the water surface. The DO concentrations in the reactors have been measured again with from S::CAN, the measurements confirmed the absence of DO in the four reactors.

The feeding medium is pre-clarified wastewater from Lundtofte WWTP (Lyngby), every batch was stored in a 200 L tank for 4-5 days at the average temperature of 2-3 °C. The tank has been continuously mixed. The two lines are fed with a peristaltic pump from the loop for the recirculation. The flow rate is 15 L/d (625 ml/h) in both the lines and the entire set-up consumes 30 L/d. The two reactors have 6 L capacity each and the average hydraulic retention time (HRT) is 9,6 h.
The COD content naturally occurring in the medium is the only organic substrate available for the denitrification and no extra source of carbon is added. Nitrates are supplied in the form of dissolved Potassium Nitrates (KNO$_3$). We supply very large amounts of nitrates in order to ensure that denitrification is not limited in any of the reactors. The solution is pumped by peristaltic pump directly into S1 and U, the flow is 25 mL/h (3.8% of the total flow) and the concentration is 10 gKNO$_3$/L.

### Table 2-2 set-up specification

<table>
<thead>
<tr>
<th>Line</th>
<th>reactor</th>
<th>flow [mL/h]</th>
<th>NO$_3$-flow [mL/h]</th>
<th>NO$_3$-dose [mg/L]</th>
<th>Reactor volume [L]</th>
<th>HRT [h$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNSTAGED</td>
<td>S1</td>
<td>625</td>
<td>25</td>
<td>20</td>
<td>6</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>625</td>
<td>25</td>
<td>20</td>
<td>1.5</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>625</td>
<td>25</td>
<td>20</td>
<td>3</td>
<td>4.8</td>
</tr>
<tr>
<td>STAGED</td>
<td>S1</td>
<td>625</td>
<td>25</td>
<td>20</td>
<td>1.5</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>625</td>
<td>25</td>
<td>20</td>
<td>1.5</td>
<td>2.4</td>
</tr>
</tbody>
</table>

**The biofilm’s origin – K1 AnoxKaldnes from Sjölunda WWTP (Malmo, SWE)**

The reactors have been filled with AnoxKaldnes K1 carriers already colonized from the post-denitrification anoxic reactor in Sjölunda WWTP (Malmo, SWE). The specific surface of K1 carriers is 530 m$^2$/m$^3$, AnoxKaldnes estimated an average “carrier density” of 1000 carrier/L. The plant is designed for 550,000 population equivalents; the biological treatment consists of high-loaded activated sludge, nitrification with trickling filters and post-denitrification with MBBR. The anoxic reactor consists of two zones in series and the carbon is supplied in the first zone in the form of methanol. The dosage is quantified in order to ensure the complete consumption; the methanol is estimated to be entirely consumed in the first zone and here the bacteria are carbon limited. Since also in predenitrification configuration no extra source of carbon is supplied, we collected the carriers from the second zone in order to shorten the adaptation period. The biomass per carrier in the second zone is 2.9 mg/carrier and the concentration is 1.4 kgSS/m$^3$. While the average concentration of classical biofilm ranges between 2 and 5 kgSS/m$^3$; such low concentration can be caused by carbon source or nitrates limitation (M.Mases, I. Dimitrova, U. Nybeg, C. Gruvberger, B. Andersson, 2011).

**The feeding medium – wastewater from Lundtofte WWTP (Lyngby, DK)**

The feeding medium is pre-clarified wastewater from Lundtofte WWTP (Lyngby, DK). Lundtofte WWTP has been designed for a population of 135,000 person equivalents, its catchment area is 32 km$^2$ and no hospitals are served. The sampling point in the plant is the sump between the primary settling tank and the aeration tank before the recirculation of the activated sludge. We were sampling around 200 L of pre-clarified wastewater every 4-5 days with dry weather conditions and mainly at 13:00 p.m., when the peak of flow occurs.
We monitor the primary and the secondary metabolism in terms of nitrate removal, sCOD removal and pharmaceutical removal:

\[ \Delta N_{O_3} - \text{reduced} = N_{O_3,\text{influent}} + N_{O_3,\text{dosed}} - N_{O_3,\text{effluent}} \]

\[ \Delta sC_{OD,\text{oxidated}} = sC_{OD,\text{influent}} - sC_{OD,\text{effluent}} \]

\[ \Delta X_{OCS,\text{oxidated}} = X_{OCS,\text{influent}} - X_{OCS,\text{effluent}} \]

The previous equation required the screening for conventional pollutants and XOCs both in the inlet and in the reactors (CSTR assumption). Thus every batch of wastewater have been characterized the conventional pollutants and the trace organics. We measured the complete fractionation according to the guidelines of Roeleveld (2002) and Weijers (1999) and the concentrations of \( NO_3^-N \), \( NH_4^-N \), TP and 19 pharmaceuticals. Furthermore the characterizations of the medium have been verified on the data of clarified wastewater provided by Forsyning A/S, the company managing the plant. The following parameters have been measured:

- \( COD_{inf,\text{tot}} \)
- \( sC_{OD,\text{infl}} \)
- \( BOD_7 \)
- \( bC_{OD} \)
- \( S_i \)
- \( NO_3^-N \)
- \( NH_4^-N \)
- Total Phosphorus
- 19 pharmaceuticals.

The analyses have been performed on 1 L grab-sample collected from the storage tank in the laboratory. The soluble non-biodegradable fraction (\( S_i \)) was measured on grab-sample after the secondary sedimentation before the discharge in the Mølleån river. The concentrations of sCOD, \( S_i \) and nutrients (N, P) are measured on samples filtered with sterilized 0,45μm filters from the Greyer. The quantification of \( COD_{inf,\text{tot}} \), \( sC_{OD,\text{infl}} \) and total phosphorus have been done with HACH-Lange test kits and a Hach-Lange DR 2800 spectrophotometer for the quantification. We used test kits from MerckMillipore and Spectroquant NOVA 60 from Merck to measure the concentration of \( NO_3^-N \), \( NO_2^-N \) and \( NH_4^-N \). We measured the BOD7 with Oxitop OC 100 from WTW, each measurement have been triplicated. The data from the Oxitop have been treated with ACHAT.exe and SIGMAPLOT.

The concentrations of pharmaceuticals have been measured with High Pressure Liquid Chromatography Mass Spectrometry/Mass Spectrometry (HPLC-MS/MS) with Ultimate 3000 from Dionex. The samples have been collected with glass pipette in glass vials to avoid the adsorption; the samples are diluted with methanol before being stored at -20°C.

The following protocols are attached in the appendices:
• wastewater complete characterization according to Roeleveld (2002) and Weijers (1999);
• BOD7 analysis and analysis;
• use of test kits from Hach-Lange and MerckMillipore;
• quantification of pharmaceuticals with HPLC-MS/MS.

The pH-control system
The complete denitrification produces 3.57 g of alkalinity as \( \text{CaCO}_3 \) per 1 g of NO\(_3\)-N reduced (G. Tchobanoglous et al., 2003). In traditional WWTP the alkalinity produced by the denitrification is consumed by the nitrification and the pH level is partially buffered because the two processes are “opposite”.

In the continuous flow experiment the denitrification is the only process occurring and the pH level increases naturally and eventually it deviates from the optimum range (i.e. 7-8). We measured the pH with portable pH meter from WTW. We experienced that the pH level in the storage tank after the sampling is usually greater than 7.4, but sometimes values around 7.6-7.8 have also been measured. In the staged reactor the pH increases along the line, the maximum level is reached in S3 and the average is close to that one in U. Furthermore, sometimes the pH in the tank increased during the period of storage.

Considering the optimum pH range for denitrification (i.e. 7-8) and that the pH in the biofilm is greater than the pH in the bulk phase, we decided to dose acid in the storage tank in order to establish more advantageous conditions in the reactors or at least to avoid the inhibition of the process as much as possible. Thus from the end of January we were controlling the pH by dosing 1M solution of HCl. We roughly estimated that 8-9 hours are required for the pH to reach high value again.

Wastewater sampling for the conventional pollutants
We were sampling for conventional pollutants since the system starts to operate. Since January 2014 we used to sampling every day in the morning. By sampling in the morning, we ensure that no perturbations occurred at least in a time period of 9 hours, which is the HRT of the system. If anything provoked the system to deviate from the ideal conditions, we delayed the sampling 9 hours later than the last perturbation. We were sampling the reactors in the staged line from downstream to upstream in order to minimize any perturbations on the system. Assuming that the reactor is completely mixed the effluents of the two lines have the same concentration as the wastewater in S3 and U. During sampling we observed the following procedure for each reactor:

• opening the reactor;
• flushing the syringe by pipetting back and forth the wastewater from the centre of the reactor;
• collecting wastewater in the 10 mL syringe;
• immersion of the pH meter;
• the first 4 mL sample from each reactor are stored in a 6 mL labelled plastic flask;
• the second 10 mL sample from each reactor are filtered with 0,45 μm sterilized filter from The Geyer;

The non-filtered sample is always been analysed just after the sampling, while the filtered sample is preferably analysed the same day of the sampling and then it is stored at 2°C in case of the analysis have to be repeated. By storing the samples in this way, we experienced that the results of the analysis with HACH-Lange test kits and Merck test kits do not change even after a period of 15 days.

Wastewater sampling for the pharmaceutical screening

In the last two weeks of operation we were sampling for the quantification of pharmaceuticals as XOCs. During the first week we sampled from the all reactors every second day after the collection of the medium, in the second week the samples have been collected daily until the end of the continuous experiment. We sampled also from each batch of water after the collection in Lundtofte. The observed removals for each chemical have been estimated by calculations on the mass balances.

The sampling procedure has been suggested by the department of Environmental Chemistry and Microbiology of Aarhus University in Roskilde. They also developed the method and the protocols for the quantification of the targeted micropollutants. The samples have been analysed in the end of the experiment, in the meanwhile the samples have been stored at -20 °C. In order to avoid any sorption of trace organics on plastic materials, we used new glass pipettes for every set of samples. The sampling procedure consists of:

• pipetting 10 mL of wastewater with glass pipette in a 14 mL glass vial;
• mixing the samples with 3,5 mL of methanol and storage at -20 °C until the analysis.

Biomass sampling for TSS and microbiological analysis

In order to minimize the depletion of biomass in the reactors within the operational period, we minimize as much as possible the sampling of biomass for the TSS measurement and for the microbiological analysis.

The measurement of TSS has been repeated three times and we collected five non deformed carriers from each reactor for each quantification. In order to keep the filling ration constant within the operating period, the empty carriers have been replaced in the reactors after the biomass collection. With regarding to the microbiological analysis we sampled two carriers per week. Again, the empty carriers have been replaced in the reactors, in this way the filling ratio did not change during the operating period. The samples have been stored as pellets at -20°C until the operating period of the continuous flow experiment finished. The procedure for sampling and storing consists of:

• brushing the carriers with disposable Gynobrush directly into a 1,5 mL Eppendorf tube;
• flushing the carrier with distilled water (the water is collected in the same Eppendorf tube as before);
• centrifuge the biomass at 10.000 rpm for 5 minutes;
• removal of the supernatant;
• storage at -20°C.

2.2 The batch experiments

After 99 days of operation, we stopped the continuous flow experiment in order to perform the batch experiments on the same four reactors. We assume that in this period the bacterial communities adapted to the specific operating conditions and eventually the reactors perform at different denitrification rate and show different elimination of pharmaceuticals as XOCs. By the batch experiments we aim to estimate the denitrification rates, the specific denitrification rates and to observe the elimination of some of the pharmaceuticals that we were screening during the continuous flow experiment.

In the batch experiment the substrates and the biomass are placed almost simultaneously, the process proceeds for 24 hours. By continuous sparging N₂ we provide homogeneous mixing and anoxic conditions.

The pH level in the batches is controlled by dosage of 1M HCL until pH 7 is reached; before the start of the experiment and whenever it reaches very high level in any reactors; the pH is continuously monitored. The temperature is monitored but not controlled (appendix G).

The four reactors operate on the same conditions, same feeding medium and 20% filling ratio (lower than in the continuous flow experiment). In order to operate at constant filling ratio during the entire experiment, after each sampling a precise number of carriers has been removed.

The feeding medium for the experiment is pre-clarified real wastewater from Lundtofte WWTP. The medium has been collected in the same day of the experiment; its quality has been characterized according to the guidelines from Roeleveld (2002) and Weijers (1999), as we did for the continuous flow experiment (appendix B). The medium has been screened for 19 pharmaceuticals via HPLC-MS/MS with Ultimate 3000 from Dionex. The method for the quantification of micropollutants has been developed by the department of Environmental Chemistry and Microbiology of Aarhus University in Roskilde, (appendix F).

2.2.1 Estimation of the biological kinetics

On the results from the batch experiment, through the method presented by Kujawa and Klapwijk (1998) the following parameters have been estimated for the fours reactors:

• Denitrification rates \( r_1, r_2 \) and \( r_3 \);
• Specific denitrification rate \( K_1, K_2, K_3 \);
• Growth yield \( Y_h \);
- Maximum growth rate ($\mu_h$).

In WWTP denitrification occurs with wastewater as electron donor and three rates are identified depending on the COD fraction utilized, (Henze M. et al., 2008) (K.kujawa and B.Klapwijk, 1998). These are the soluble biodegradable ($S_s$), the particulate biodegradable ($X_s$) and the particulate biodegradable by decay of biomass ($X_{s,BH}$). Three different linear phases describe the reduction of nitrates, (K.kujawa and B.Klapwijk, 1998); they occur simultaneous until the substrates are consumed. The denitrification on $S_s$ is the fastest and this is the first fraction to be consumed, $X_s$ has lower denitrification rate and $X_{s,BH}$ in the lowest. The process gets in the endogenous phase when $X_{s,BH}$ is the only available substrate (K.kujawa and B.Klapwijk, 1998). Since denitrification occurs exclusively on $S_s$, the utilization of $X_s$ and $X_{s,BH}$ is slower because driven the hydrolysis is required.

The production/accumulation of NO$_2$-N is not negligible and we account for it in the calculations; the nitrate utilisation curve is built on NO$_3$-N+0.6*NO$_2$-N as suggested by Kujawa and Klapwijk (1998). Where the correction factor is estimated by stoichiometry considering that 1 mg of NO2-N required as many electrons as 0.6 mg of NO$_3$-N to be completely reduced to N$_2$ (Henze M. et al., 2008).

The slope in each phase is estimated by the following equation:

$$r_D = \frac{\Delta(NO_3-N + 0.6NO_2-N)}{\Delta t}$$

The quality of the consumed substrate affects the rate of denitrification, the slope in the utilisation curve changes according to the available substrate. Three slopes are observed:

1. $r_{D1} = r_{D,s} + r_{D,x} + r_{D,\text{end}}$
2. $r_{D1} = r_{D,x} + r_{D,\text{end}}$
3. $r_{D1} = r_{D,\text{end}}$

By the quantification of the biomass in each reactor, we estimate the specific denitrification rate in each reactor ($k_{Di}$) by the following:

$$k_D = \frac{r_D}{X_{BH}}$$

As discussed by Kujawa (1998) and Ekama (2008), in the second phase the $X_s$ fraction is consumed. Considering this, we assume that the whole bCOD fraction in the feeding medium is oxidized to reduce the $\Delta NO_{3,2}$-N; this is estimated by the intercept of the linear regression that approximates the consumption of nitrates and nitrites over time, see the general case in the figure below.
The methodology to estimate the denitrification rates has been discussed in par. 3.2.1. We approximate the utilization of nitrates and nitrites together \((\text{NO}_{3,2}^-\text{N})\) to a first order kinetic;

\[
r_{D,1} = \frac{\text{dNO}_{3,2}^-\text{N}}{\text{dt}} = k_i \times \text{XBH}
\]

Where \(k_i\) represents the specific denitrification rate normalized per amount of biomass (mgNO\(_3\)-N/h) and XBH the concentration of biomass in the reactors during the experiments, expressed as gCOD/L.

Then the curve describing the utilization is a linear regression;

\[
y = y_0 + a \times x
\]

where \(a\) is the rate of utilization and \(y_0\) the initial concentration. The interval time has been defined to have the highest \(R^2\) as possible. The initial concentration (100 mg NO\(_3,2\)-N) is the only constrain in the regression.

### 2.2.2 Estimation of growth yield and maximum growth rate

The growth yield, \(Y_H\), has been estimated by the following equation from the stoichiometry of the process (par. 2.3.1):

\[
Y_H = 1 - 2.86 \frac{\text{bCOD}}{\Delta(\text{NO}_3^- - \text{N} + 0.6\text{NO}_2^- - \text{N})}
\]

Where 2.86 mgCOD/mgNO\(_3\) is valid for the complete denitrification to nitrogen gas. Since we ignore if the process is complete and where in the chain it stops, we estimate the \(Y_H\) for three possible scenarios:

- Denitrification to nitric oxide, correction factor is 3/5;
- Denitrification to nitrous oxide, correction factor is 4/5;
- Complete denitrification.
The possible accumulation of nitrites has been excluded because the concentration of nitrites has been monitored both in the continuous flow experiment and in the batch experiment.

In order to estimate the maximum growth rate ($\mu_H$) we neglect the role of substrate diffusion through the biofilm. By this simplification the case of biofilm system is reduced to the activate sludge system. The specific denitrification rate in phase one ($k_1$) is given by:

$$k_1 = \frac{(1 - Y_H) \cdot \mu_H}{2.86 \cdot Y_H} \cdot \frac{S_s}{k_s + S_s}$$

Assuming that in the first phase the following is valid:

$$S_s \gg k_s ; \frac{S_s}{k_s + S_s} = 1$$

From the previous one, we extract $\mu_H$ for each reactor in the three scenarios.
2.3 The analytical methods
In the following paragraph we describe the methods for the quantification of conventional pollutants, for pharmaceuticals (as XOCs) and for the biomass on the carrier.

2.3.1 The analysis of conventional pollutants
We measured the concentration of total COD, soluble COD (sCOD), NO$_3$-N and NO$_2$-N since the beginning of the operational period. In the last 40 days of operation we measured also NH$_4$-N and total phosphorus (TP) concentration. COD fractions and nitrogen species are measured spectrophotometrically.

The total COD and total phosphorus are measured on the unfiltered samples, while the others pollutants are measured on filtered samples with cellulose sterilized disposable 45 μm filters by The Geyer.

We measured the total COD, the soluble COD and the total phosphorus photometrically with HACH-Lange test kits (LCK 514, LCK 314, LCK 348 and LCK 350) and HACH-Lange spectroquant DR 2800 for quantification. Nitrates, Nitrites and Ammonium have been also measured photometrically; we used MerckMillipore test kits and spectroquant NOVA 60 from Merck for quantification.

We assumed that the measurements of nitrates, nitrites and ammonium have normal distribution. We choose a confidence interval of 95% and the selected error on the measurements is two time the standard deviation. HACH-Lange itself calculated the standard deviation for every cuvette test on an international inter-laboratories test. LCK 514 has been evaluated on 358 measurements, LCK 314 on 274 measurements, LCK 348 and LCK 350 on 424 measurements. The standard deviation, $\sigma$, for each test is available in the quality certificates produced by HACH-Lange itself; the values are listed below:

- $\sigma_{\text{LCK514}}=2,9$
- $\sigma_{\text{LCK314}}=0,5$
- $\sigma_{\text{LCK348}}=0,091$
- $\sigma_{\text{LCK314}}=0,018$

With regarding to MerckMillipore test-kits, we repeated the measurements of every parameters 6 times on the same sample. We assume that the 6 values distribute on a Gaussian curve and we select the same confidence interval (95%). Again the error is twice the standard deviation for each series. We assumed also that the estimated error does is constant and valid fr every measurement of the same parameter. Since for the ammonium test kit the sample is not diluted, its standard deviation is significantly smaller than the one of the other test kits. Indeed for the quantification of nitrates and nitrites we dilute the sample five times and the error on the measurement is 5 times the twice of the standard deviation on the nitrate test (?). The nitrates test kit has bigger error because the execution is more difficult. The values of standard deviation and the errors for the nitrates test kits is $\sigma_{\text{NO}_3\text{-N}}=2,93$ mgNO$_3$-N/L.
2.3.2 Screening for xenobiotics

We monitored the concentration of 30 pharmaceuticals in the last 21 days of operation on continuous flow configurations. The quantification consists of High pressure liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS). The method has been developed by the department of Environmental Chemistry of Arhus University in Roskilde.

The HPLC-MS/MS is a dual low-pressure mixing ternary-gradient system Ultimate 3000 from Dionex. The system is equipped with a pump of the 3000 series (DGP-3600 M), a 3000 TSL autosampler (WPS 3000 TSL) and a column oven and degasser also from the Dionex 3000 series. The HPLC operates with two eight-port Valco valves. The mass spectrometer is an API 4000 (ABSciex, Framingham, MA, USA). The API 4000 operates in ESI in positive mode at 400°C with a capillary voltage of 5500 V. The HPLC operates following gradient elution of methanol and Millipore water, both containing 0.2% formic acid (v/v) on a Synergi-Polar column (Phenomenex, Torrance, California, USA). All the assessment is based on the peak areas. A multilevel calibration is performed with four repeats each.

2.3.3 TSS measurements

The applied procedure for the TSS measurement is similar to Dupla et al. (2006) and Falas et al. (2012). We estimated the TSS on the carriers three times during the entire period. Indeed the first data in the series is from M. Mases (2011). We did the analysis after 44, 74 days of running and at the end of the continuous experiment, immediately before the batch experiments. Every time we sample five carriers from each reactor, the amount of TSS is estimated as the average amount of biomass on each carrier. In order to not reduce the filling ratio too much, we repeat the analysis three times. The protocol for the TSS analysis is presented in details in the appendix C; it mainly consists of three steps:

- Dry the biomass on the carriers by heating them at 105°C for 1,5 h and weigh them;
- Remove the biofilm from the carrier by flushing with sulphuric acid (H₂SO₄) 4 M, brushing and flushing again with water;
- Dry the empty carriers by heating them at 105°C for 15 minutes and weigh them.

The amount of biomass on the carriers is given the difference between the weight of the colonized carrier with dried biomass and the empty carrier after the biofilm removal. Assuming that the measurements have a normal distribution, we select a confidence interval of 95% and the error on the measurements is two time the standard deviation.

Considering the estimations from Anoxkaldnes about the carriers concentration per litre and on the knowing the amount of biomass per carrier, by the following equation we estimated the total biomass ($M_{\text{total biomass}}$) and the biomass concentration ($m_{\text{biomass}}$) in each reactor:
\[ M_{\text{total biomass}} = 1000 \times f \times V_{\text{reactor}} \times M_{\text{carrier}} \]
\[ m_{\text{biomass}} = M_{\text{total biomass}} / V_{\text{reactor}} = 1000 \times f \times M_{\text{carrier}} \]

Where \( f \) is the filling ration and \( V_{\text{reactor}} \) is the volume of considered reactor.
3 The continuous experiment - Results and discussion

3.1 Denitrification

3.1.1 Characterization of the feeding medium
The feed for the continuous flow experiment was pre-clarified wastewater from the Lundtofte WWTP waste water treatment plant in Lundtofte (Lyngby, DK). From 120 L up to 200 L of medium were collected twice per week and stored in a tank at temperature 1-3 °C. Every wastewater feed was characterized in terms of COD fractionation, NO$_3$-N, NH$_4$ and total phosphorus (TP) content. As shown in Fig. 3-1, we experienced significant fluctuations in the quality of the feed. Since the system required a certain time for adaptation to the medium, we believe these fluctuations affected the observed efficiencies during the continuous flow experiment.

![Graphs showing COD fractionation and nitrogen and phosphorus species over time](image)

Figure 3-1 Characterization of pre-clarified wastewater from Lundtofte WWTP (Lyngby, DK) in terms of COD fractionation (a, b) and nitrogen and phosphorus species (c).
3.1.2 Consumption of primary substrates

Here we describe and discuss the primary metabolism of heterotrophic bacteria in the staged and in the unstaged system. The intensity of the process is investigated in terms of consumption of primary substrates: the sCOD as electron donor and the NO$_3$-N as electron acceptor. The observed period is limited to a time period of 50 days, from day 43 (beginning of the pH-controlled period) until day 88 (7 days before the batch experiment) of the entire operation period. During this period of time, we collected 9 different wastewater inocula. Due to variations of the amount of wastewater collected in each inoculum the period during which the system was running with the same medium changed. With regard to the heterotrophic denitrification, the availability of COD as electron donor is expressed in terms of biodegradable COD and on its quality (particulate or dissolved). Since nitrate was supplied with constant dosage and in excess (100 mg/L), the system was operated under COD-limiting conditions. As indicators of denitrification, we investigated in each reactor nitrate removal ($\Delta$NO$_3$-N) and soluble COD consumption ($\Delta$sCOD), where sCOD includes the fraction of readily biodegradable COD available for denitrification in the instant of sampling ($S_s$) plus the amount of inert COD ($S_I$), assumed to be constant during for each feeding period.

The electron donor - $\Delta$sCOD

The plots in Fig. 3-2a show the rate of consumption of sCOD ($\Delta$sCOD) over time in the unstaged and staged configurations (a) and in S1, S2 and S3 of the staged MBBR (b). We observed significant differences in $\Delta$sCOD for every wastewater inoculum. The largest variations were registered in case of large fluctuations in the content of $S_s$ (day 57, 63, 70, 73). It could be observed that variations of $\Delta$sCOD in both configurations with a change of medium were rather fast and $\Delta$sCOD reached and a steady state was reached one day after the change of the feeding medium (feeds 5-9).

At first we observed that the total sCOD was found to be the highest when when $S_s$ concentration in the medium was also high (feed number 5), comparably low minimum when influent $S_s$ was reduced (feeds 3, 6, 8). Fluctuations of $\Delta$sCOD in S1 ($\Delta$sCOD$_{S1}$) over time were significant if compared with ones in S2 ($\Delta$sCOD$_{S2}$) and S3 ($\Delta$sCOD$_{S3}$). $\Delta$sCOD in S2 and S3 was rather constant and ranging between 0 mgCOD/L and 15 mgCOD/L.

$\Delta$sCOD$_{S1}$ was also found to increase at higher influent $S_s$ concentrations.

<table>
<thead>
<tr>
<th>Num. batch</th>
<th>$\Delta$sCOD$_{S1}$ (mgCOD/L)</th>
<th>$S_s$ (mgCOD/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64.7</td>
<td>72.7</td>
</tr>
<tr>
<td>2</td>
<td>67.95</td>
<td>76.7</td>
</tr>
<tr>
<td>5</td>
<td>103.12</td>
<td>103.84</td>
</tr>
<tr>
<td>6</td>
<td>27.95</td>
<td>32.54</td>
</tr>
</tbody>
</table>

This confirms the hypothesis by which the influent readily biodegradable fraction is mainly degraded in S1 and its bacterial community seems to have adapted to the use
of this substrate. Whenever influent $S_s$ is less abundant (low quality of the medium) the process in S1 was less intense and a lower removal of sCOD was observed.

As most of the utilization of $S_s$ occurred in S1, the COD fraction available in S2 and S3 for denitrification was likely to derive from the hydrolysis of the slowly biodegradable COD, $X_S$ and from endogenous respiration of decayed biomass ($X_H$). Therefore, hydrolysis drove the denitrification in S2 and S3. A negative removal of sCOD (i.e. increased concentration from S1 to S2 or from S2 to S3) was observed in S3 and more rarely in S2, possibly indicating that extensive hydrolysis was occurring (e.g., as a result of detached biomass) and/or at higher rates than the utilization of $S_s$ for denitrification.
Figure 3-2 (a) $\Delta sCOD$ in the staged and in the unstaged system; (b) $\Delta sCOD$ in S1, S2 and S3 in the last 44 days of operativity, the red dashed lines represent the change in the quality of the feeding medium.
The electron acceptor - $\Delta \text{NO}_3^-$N

Nitrate removal ($\Delta \text{NO}_3^-$N) trends over time are reported in Fig. 3-3. The variation of $\Delta \text{NO}_3^-$N was found to be more evident than in the case of $\Delta \text{sCOD}$ and no steady state removal was reached for nearly each feed. Within day 70 and 73 we experienced the lowest $\Delta \text{NO}_3^-$N, corresponding to a minimum in $\Delta \text{sCOD}$.

We also observed that the staged and the unstaged reactors performed differently. In Fig. 3-4, an explicit comparison of the performance of staged and unstaged reactors in terms of $\Delta \text{NO}_3^-$N removal is shown. The observations are based on averages within the time of running with the same feeding medium (9 different inocula are considered). The plot shows that the two reactor configurations had similar removal in batches 1, 3, 5 and 8. A higher average $\Delta \text{NO}_3^-$N$_{\text{staged}}$ was registered for 7 out of 9 feeds in the operating period. The largest difference was observed for feed 6 (days 71, 72, 73) where the ratio $\Delta \text{NO}_3^-$N$_{\text{staged}}$/$\Delta \text{NO}_3^-$N$_{\text{unstaged}}$ was found to be higher than 2. The peculiarity of this feed was the minimum concentration of $s\text{COD}$, registered during the monitoring period.
This observation may suggest that the staged configuration could perform well in critical conditions, e.g. with a diluted feed. Furthermore we suppose that the higher performance in the unstaged configuration is due to the robustness of S2 and S3. This is confirmed on the observed average $\Delta NO_3-N_{S1}$, $\Delta NO_3-N_{S2}$ and $\Delta NO_3-N_{S3}$ in the 50 days of monitoring and in within day 71 and day 72.

Table 3-2 Average removal of NO$_3$-N in the 50 days of operation and during days 71, 72 and 73

<table>
<thead>
<tr>
<th>Reactor</th>
<th>$\Delta NO_3-N_{mean,50DAYS}$</th>
<th>$\Delta NO_3-N_{mean,71,72,73}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstaged</td>
<td>37.7</td>
<td>13.5</td>
</tr>
<tr>
<td>S1</td>
<td>35.8</td>
<td>10.2</td>
</tr>
<tr>
<td>S2</td>
<td>6.0</td>
<td>8.5</td>
</tr>
<tr>
<td>S3</td>
<td>3.7</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Table 3-2 shows that the average removal in the entire period is higher overall for the staged configuration (45.5 mgNO3-N/L) than for the unstaged (37.8 mgNO3-N/L). In the staged reactor, most of the denitrification occurred in the reactor S1, being consistent with observations about $\Delta sCOD$. 

Figure 3-4 Comparison of average $\Delta NO_3$-N removal in staged and unstaged reactors
The average COD/NO$_3$-N ratio ranges between 1.2 and 2 in 6 batches out of 9 (batches num. 1, 2, 4, 6, 7, 9), table 7. It is maximum in batch num. 5 both for the staged and the unstaged, in this batch 5 we also observed the highest values $\Delta$sCOD (106.9 mg/L), low $\Delta$NO$_3$-N and the highest concentration of $S_5$ in the feeding medium. The lowest COD/NO$_3$-N ratio values are detected in batches 3 and 8. In these we observed low concentration of sCOD in the influent (fig. 5b), very low $\Delta$sCOD (fig. 5a) and $\Delta$NO$_3$-N close to the average.

We observe, fig. 3-2 and fig. 3-5, that the most of the NO$_3$-N utilization occur in the first reactor. Because of that we expect that the denitrification rates are the highest in the system. Furthermore the staged system is more flexible to the fluctuations of the quality of the medium, we supposed that this is because of $S_2$ and $S_3$. The constant operation of $S_2$ and $S_3$ with regards to the variation of $S_5$ in the feeding medium was probably due to constant carbon limited conditions. This confirms that somehow the bacterial community specialized to the specific conditions in $S_1$, $S_2$ and $S_3$. In particular, $S_2$ and $S_3$ ensured an almost constant rate of degradation, which actually increased when minimum influent sCOD in the feed where observed. This affected positively the efficiency of the whole staged reactor and guaranteed higher performances than the unstaged configuration.
Figure 3-5 Cumulative removal of NO3-N in S1, S2 and S3 of the staged configuration

Figure 3-6 Concentrations of NO3-N in S1, S2 and S3 in the last 16 days of operativity (4 batches)
3.2 Removal of pharmaceuticals

In this paragraph, we present the occurrence of 22 pharmaceuticals in the feeding medium and the removal of 10 pharmaceuticals in the MBBR configurations over the 28 investigated chemicals by the quantification method with HPLC-MS/MS. The measured concentrations corresponded to overall 6 samplings over 20 days of screening.

In this paragraph we present the observed removal efficiency of selected pharmaceuticals in staged and unstaged configurations, based on concentrations measured in the feeding medium and in the different reactors.

Removal efficiencies are presented and discussed for 10 pharmaceuticals. This selection was based on the possibility of detecting and quantifying concentrations in the dissolved phase of MBBR reactors. Within these 10 chemicals, we observed different behaviours: gradual degradation along S1, S2 and S3, low and high rate of elimination and retransformation.

Most of the pharmaceuticals undergo diverse physical-chemical and biological processes simultaneously; for this reason the discussion on the continuous flow experiment is limited to the comparison between the two configurations which operate under the same conditions. The comparison between the stages is mainly limited to the results from the batch experiment.

3.2.1 Concentrations in the feeding medium

The feeding medium is pre-clarified wastewater from Lundtofte WWTP. We detected 22 pharmaceuticals in three different wastewater inocula collected within a time period of 20 days. Reported concentrations (Fig. 3-) are the average averaged of concentrations measured in the last three wastewater feeds (7, 8, 9). The most abundant compound is Triclosan (19 µg/L) this result agree with the wide use of this chemical. Ciprofloxacin and Ibuprofen are also quantified in high concentration, respectively 13 µg/L and 4.4 µg/L. The less abundant are Phenozenone, Sulphadiazine and its metabolite Ac-Sulphadiazine.
**Figure 3-7** pharmaceuticals concentrations in clarified wastewater from Lundtofte WWTP, Lyngby DK

**Table 3-4** concentrations of detected pharmaceuticals from Lundofte WWTP, Lyngby DK

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lundtofte WWTP Influent (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metoprolol (MET)</td>
<td>1441</td>
</tr>
<tr>
<td>Ac-sulphadiazine (AC-SLF)</td>
<td>11</td>
</tr>
<tr>
<td>Diclofenac (DCF)</td>
<td>293</td>
</tr>
<tr>
<td>Sulphametoxazole (SMX)</td>
<td>141</td>
</tr>
<tr>
<td>Atenolol (ATE)</td>
<td>361</td>
</tr>
<tr>
<td>Carbamazepine (CBZ)</td>
<td>144</td>
</tr>
<tr>
<td>Ibuprofen (IBF)</td>
<td>4369</td>
</tr>
<tr>
<td>Propanolol (PROP)</td>
<td>83</td>
</tr>
<tr>
<td>Sulfadiazine (SLF)</td>
<td>24</td>
</tr>
<tr>
<td>Thrimethoprim (TMP)</td>
<td>177</td>
</tr>
<tr>
<td>Phenozenone (PHN)</td>
<td>21</td>
</tr>
<tr>
<td>Tramadolol (TRM)</td>
<td>986</td>
</tr>
<tr>
<td>Iomeprol</td>
<td>3211</td>
</tr>
<tr>
<td>Iopamidol</td>
<td>2484</td>
</tr>
<tr>
<td>Citaloprom (CIT)</td>
<td>329</td>
</tr>
<tr>
<td>Clarithromicin (CLR)</td>
<td>176</td>
</tr>
<tr>
<td>Sulphametizole (SLM)</td>
<td>2543</td>
</tr>
<tr>
<td>Venlafaxine (VEN)</td>
<td>331</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>12554</td>
</tr>
<tr>
<td>Clindamicin (CLN)</td>
<td>19</td>
</tr>
<tr>
<td>Erithromicin (ERT)</td>
<td>176</td>
</tr>
<tr>
<td>Triclosan (TRC)</td>
<td>18739</td>
</tr>
</tbody>
</table>
3.2.2 The removal of pharmaceuticals as XOCs
The observed removal efficiencies of pharmaceuticals were estimated using influent and reactor concentrations as described in the introductory chapter.

Assuming that perfect mixing conditions are established, the concentrations in S3 and U are equal to the concentrations in the effluent of staged and unstaged configurations. Whenever it was possible the results of the calculations are averaged on the 6 days of sampling. For pharmaceuticals with concentrations below LOQ and/or LOD, a range of removal efficiencies could be estimated. Concentration of ATE and PROP are not quantified. The efficiency for DCF, VEN, CBZ and SMX was calculated on 4, 3, 2 and 1 set of samples, respectively, due to low accuracy in the quantification procedure.

Table 3-7 shows maximum, minimum and mean concentrations in the influent and in the two final effluents. The applied error in the measurement is the standard deviation. With regard to SLF, error is the sensibility of the instrument (10%) because we considered only one set of sample. Observed removal efficiencies reported in literature are also listed in the table 9.

We observe:

- high degradation in the cases of ATE e PROP;
- average degradation for TMP e CIT;
- no degradation for DCF;
- negative degradation for SMX and SFM.

A negative removal was shown for SMX possibly because of the intense retransformation of its major metabolite N4-Acetyl-sulfamethoxazole (N4-Ac-SMX) to SSMX. N4-Ac-SMX is the major conjugated metabolite excreted after SMX administration, being detected at approximately 3-fold higher concentrations in urine as compared to parent SMX. The conjugated acetyl group is readily cleaved and the removal of N4-Ac-SMX is usually complete (Ternes and Joss, 2006).

Based on the results found for beta-blockers (MTP, PROP, ATE), a specific discussion was considered for these substances.
Table 3-5 Concentrations of pharmaceutical in the feeding medium and in the effluents (ng/L); and observed removal efficiency (%);  

<table>
<thead>
<tr>
<th>Compound</th>
<th>Influent (ng/L)</th>
<th>Effluent (ng/L)</th>
<th>Removal efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum</td>
<td>Minimum</td>
<td>Average / St.Dev.</td>
</tr>
<tr>
<td>Atenolol (ATE)</td>
<td>395.95</td>
<td>340.54</td>
<td>360.81 / 30.54</td>
</tr>
<tr>
<td>Propanolol (PROP)</td>
<td>88.38</td>
<td>79.19</td>
<td>83.04 / 4.77</td>
</tr>
<tr>
<td>Metoprolol (MTP)</td>
<td>1526.35</td>
<td>1365.54</td>
<td>1441.21 / 81.82</td>
</tr>
<tr>
<td>Sulfamethoxazole (SMX)</td>
<td>173.92</td>
<td>98.58</td>
<td>141.10 / 38.59</td>
</tr>
<tr>
<td>Trimethoprim (TMP)</td>
<td>205.68</td>
<td>142.23</td>
<td>176.69 / 32.07</td>
</tr>
<tr>
<td>Citalopram (CIT)</td>
<td>340.54</td>
<td>308.78</td>
<td>329.28 / 17.77</td>
</tr>
<tr>
<td>Carbamazepine (CBZ)</td>
<td>173</td>
<td>128</td>
<td>143.56 / 25.73</td>
</tr>
<tr>
<td>Sulfamethizole (SLM)</td>
<td>2861</td>
<td>2257</td>
<td>2542.79 / 303.29</td>
</tr>
<tr>
<td>Venlafaxine (VEN)</td>
<td>356</td>
<td>312</td>
<td>331.53 / 22.41</td>
</tr>
</tbody>
</table>
Fig.12 compares the observed removal in the system and data from the literature for 7 over 10 investigated chemicals. The removals of CBZ and SLF in the staged reactor are not discussed because only one set of sample was available. SMX has negative removal and it does not appear in the plot, it is discussed separately.

We observed that CIT and TMP are highly removed in both configurations of the system, more than twice the removal from the literature. The observed removal of DCF, CBZ and MTP are consistent with data from the literature. While VEN in the system is less removed; the ratio is almost 0.75 for the unstaged and 0.5 for the staged.

Fig.12 shows also that the removal rates for VEN, DCF, CBZ and MTP range from less 6% to 20%, while only TMP and CTL have high removal rate (>30% and ≈60%).

![Figure 3-8 ΔXOCs (%) observed and from the literature;](image)

**The case of B-blockers**

The concentration of ATE was found to be lower than LOQ in the effluent from the unstaged and it is lower than LOD in the staged reactor. The concentrations of PROP in the effluents are also lower than the LOQ. Assuming the respective LOD and LOQ as final concentrations from the systems and considering the average concentrations in the influents, we estimated that the unstaged and the staged configuration have removal up to 91% for the Atenolol and up to 23.5% for PROP. While for MET we observed lower removal; 7.93% in the unstaged and 4.17% in the staged.
The β-blockers have similar chemical structure and consequently they would be expected to show similar removal. Data on degradability of these chemicals are inconsistent also in the literature; the range of variation is wide, from 10% to 96% (M. Maurer, B.I. Escher, P. Richle, C. Schaffner, A.C. Alder, 2007).

ATE and PROP have concentration lower than LOQ in S2 and S3, thus discussions about the progressive elimination from S1 to S3 was not possible.

Regarding with the first two days of screening, we observed that MET is more eliminated in the staged reactors, Figure 13. The elimination occurs in S3 and minority in S2, the last two stages seems to be responsible for the higher performance. According to the research hypothesis, after an adaptation period the bacterial communities in S1, S2 and S3 somehow differentiate and specialize to the specific conditions in each reactor. The differentiation may result in different metabolism and eventually higher degradation of XOCs in S3 and S2 where the easy biodegradable substrate is less available. The observation on the first two days of screening would confirm the hypothesis.

However the measurements in the last 4 days do not agree completely. We suppose that the adaptation period is not completed yet and the different efficiencies between the reactors may not be remarkable yet.

The hypothesis of enhanced removal of pharmaceuticals has been confirmed only for a limited number of pharmaceuticals. However, it should be noted that the number of samples considered in the study.

By now the analysis on the continuous flow experiment do not confirm the hypothesis. Later in the chapter, the same is discussed considering the results of the batch experiments. Other tools, e.g. microbiological analysis, could further elucidate on whether any differentiation in the bacterial communities, with respect to metabolism of pharmaceuticals occurred for the reactor biomass.
3.3 The biomass

The cultivated carriers have been collected from the postdenitrification reactor configuration in Sjolunda WWTP in Sweden. The reactor is divided in 2 zones and methanol is supplied in the first zone. The supply of carbon is estimated to be completely utilized in the first zone, consequently the biomass in the second zone is carbon limited. We preferred to sample from the second zone in order to make the “transition” to our system as easy and fast as possible. We expected to observe an adaptation period in any case. However since the biomass is also carbon limited, this period may be shorter than in the case of biomass from the first zone where the methanol is supplied.

Fig. 14 shows the variation of biomass concentration (gTSS/L) from the beginning of the experiment until the day after the batch experiment. We observe that in this period the biomass grew differently in the reactors. Particularly we notice very high concentration in S1 where the sCOD is abundant. On the contrary in S2, S3 and U the availability of organic source sometimes has been limiting for the process; in these reactors the biomass content is lower and the observed values are similar.

![Figure 3-10 variation of the biomass concentration since the first day of operation, (gTSS/L).](image)
Figure 3-11 AnoxKaldnes K1 from Unstage reactor

Figure 3-12 AnoxKaldnes K1 from S1

Figure 3-13 AnoxKaldnes K1 from S2

Figure 3-14 AnoxKaldnes K1 from S2
4 The batch experiments – results & discussion

4.1 Characterization of the feeding medium

The fractionation of the feeding medium for the batch experiment has been quantified by the same procedure applied in the continuous flow experiment for the characterization of the medium (par. 3.1).

<table>
<thead>
<tr>
<th>fraction</th>
<th>Concentration (mgCOD/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total COD</td>
<td>540</td>
</tr>
<tr>
<td>sCOD</td>
<td>120</td>
</tr>
<tr>
<td>bCOD</td>
<td>237.2</td>
</tr>
<tr>
<td>X_s</td>
<td>139.2</td>
</tr>
<tr>
<td>X_i</td>
<td>280.8</td>
</tr>
<tr>
<td>S_i</td>
<td>21.6</td>
</tr>
</tbody>
</table>

4.2 Biomass

4.3 The primary metabolism

By monitoring the utilization of primary substrates during the batch experiments, we aimed at comparing biokinetics in each reactors and explain their behaviour during continuous operation.

In this paragraph we first discuss the utilization curves of the substrate on a qualitative base, then we estimate the growth yield (Yh) and the maximum growth rate. On these estimations we discuss and compare the metabolism of the bacterial communities in each reactor.

As introduced in par. 2.2.1, there are three different fractions of COD capable of supply the electrons required for denitrification, (Henze M. et al., 2008):

- the ready biodegradable fraction of bCOD in the medium (S_s);
- the slowly biodegradable fraction of bCOD in the medium (X_s);
- the slowly biodegradable organics generated by death and decay of the biomass (X_s,BH).

The availability and the utilization of these fractions are different and they affect the rate of denitrification (r_D) and thus the slope in the nitrate concentration curves. Two or three phases are defined by observing changes in the denitrification rates, where in the first case no distinction between utilization of X_s and X_s,BH is considered. , (Henze M. et al., 2008) (K.kujawa and B.Klapwijk, 1998). The rates (r_D) are defined as in par. 2.2.1.

Due to biomass detachment observed during the experiment, the extent hydrolysis of X_s and X_s,BH could not be quantified. Additionally, no storage of S_s in the biomass was considered.

Because of the stoichiometric proportion between the electron donor and the electron acceptor in the process, we estimated the differentiation in phases on the observed utilization of NO_3^-N.

Then considering the relative time period, we “assumed” the same phases also for the utilization of sCOD. Fig. 15 shows the separation between 1st and 2nd phase, where the S_s fraction in the feeding medium is assumed to be completely consumed.
By preliminary observations on the reduction of sCOD in the reactors we notice that the unstaged and the three stages perform differently. We also notice that in U (fig. 15a), S1 (fig.15b), S2 (fig.c) the 1st phase deducted from the NO$_3$-N curves agree with the variation of sCOD concentration over time, while in S3 (fig.15c) it does not.

![Graphs showing the concentration of sCOD over time for unstaged, S1, S2, and S3 reactors.](image)

**Figure 4-1 concentration of sCOD (mgCOD/L) over time.**

On the other hand the mass balance of sCOD (and so the sCOD observed removal) account for three different processes, these are: the hydrolysis, the storage and the consumption itself. They occur simultaneously and they are opposite. Again since we ignore the rates of each process, the observed removal of sCOD probably underestimates the actual consumption.

However the relevance of hydrolysis in the sCOD balance is evident in the case of S3, fig. 15b. As well as in the other reactors, probably denitrification is also intense in the 1st phase (1.6 hours), but we do not observe fast utilization of sCOD. Probably the hydrolysis is more intense than in the other cases; this may affect the balance and so the observed removal of sCOD is lower (the slope in the utilization curve is less steep).

Again we supposed that this is because of some adaptation occurred during the continuous flow experiment. In the three months during the continuous flow experiment the source of organics in S3 has been limiting the optimum metabolism; the available fraction in the medium consists
of $X_2$ and $X_{\text{S,BH}}$ because of the utilization in S1 and S2. Eventually the bacteria community developed some affinity to the form of the substrate and a faster hydrolysis.

Fig. 16 shows the utilization of nitrates and nitrites within the 24 hours of the batch experiment, by the dashed red line we approximately distinguish the first phase from the second one.

Figure 4-2 Concentrations of NO3-N and NO2-N over time in the four batch reactors.

In fig. 16 we also observe that nitrites concentrations increase during the first phase, this temporary accumulation is due to a “less energetically convenient” red-ox reaction. Since the reduction of nitrate provides more energy (2 electrons) than the reduction of nitrite (1 electrons), nitrates are “the favorite” substrate compared to the other nitrogen specie.
Fig. 17 shows the removal of NO$_3$-N (mg NO$_3$-N/L) normalized on the amount of biomass (gTSS/L) in each reactor. The concentration of TSS has been measured after the batch experiment; we supposed that the growth of biomass within the period of the experiment is negligible. The procedure for the quantification is the same that we applied for the continuous flow experiment (appendix C).

Within the 24 hours of the experiment, the four reactors achieve similar removal of nitrates per amount of biomass (≈20 mgNO$_3$-N/gTSS).

![Figure 4-3 removal of NO$_3$-N (mg NO$_3$-N/gTSS) over time in the unstaged and in the staged configuration](image)
4.3.1 Estimation of biokinetic parameters

The three phases

In S3 we did not observe the endogenous phase; the entire period is divided in two phases only.

By the amount of biomass in each reactor and assuming that the bacteria growth is negligible in 24 hours, we estimate the specific rates (tab. 5).

![Figure 4-4 three phases in the unstaged reactor](image)

![Figure 4-5 three phases in the S1](image)
Table 4-2 utilization rates (L/h/mgNO$_{3,-N}$)

<table>
<thead>
<tr>
<th>Reactor</th>
<th>$r_{D1}$ (mgNO$_{3,-N}$/L/h)</th>
<th>$r_{D2}$ (mgNO$_{3,-N}$/L/h)</th>
<th>$r_{D3}$ (mgNO$_{3,-N}$/L/h)</th>
<th>$r_{D4}$ (mgNO$_{3,-N}$/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstaged</td>
<td>12.47</td>
<td>1.6</td>
<td>10.87</td>
<td>0.15</td>
</tr>
<tr>
<td>S1</td>
<td>17.52</td>
<td>2.18</td>
<td>15.34</td>
<td>1.30</td>
</tr>
<tr>
<td>S2</td>
<td>18.59</td>
<td>1.89</td>
<td>16.7</td>
<td>0.95</td>
</tr>
<tr>
<td>S3</td>
<td>23.49</td>
<td>0.44</td>
<td>23.05</td>
<td></td>
</tr>
</tbody>
</table>
The four reactors have similar observed removal of NO\textsubscript{3,2}-N per gramm of biomass (fig.17) but different utilization rates (tab. 5).

With regard to the 1\textsuperscript{st} phase, \(k_1\) is maximum in the S3 and minimum in the S1. While the amount of \(\Delta\text{NO}_{3,2}\)-N is approximately 65 mgNO\textsubscript{3,2}-N/L in S1, S2 and S3 and 70 mgNO\textsubscript{3,2}-N/L. The duration of the 1\textsuperscript{st} phase is minimum in S1 and maximum in the unstaged reactor.

The specific rates in the 2\textsuperscript{nd} phase are similar in U, S1 and S2, but not in S3. S3 has the smallest \(k\), its 2nd phase last more than 22.5-23 hours and we do not observe any significant change in the slope to distinguish S2 and S3 during the time of the batch experiment.

S1, S2 and U reach the endogenous phase almost at the same time, approximately after 16 hours.

The previous observations disagree with what expected. Despite of the unstaged and S1 are feeded with media close to that one in the continuous flow experiment and they are supposed to be used to the abundance of Ss, S3 and S2 perform at higher or similar rates in the 1\textsuperscript{st} phase and lower rate in the 2\textsuperscript{nd} phase.

**Growth yield \((Y_{H}) and the maximum growth rates \((\mu_{H,\text{max}})\)**

\(Y_{H}\) and \(\mu_{H}\) are estimated according to the methodology in par. 3.2.1. Except for nitrite, we ignored if the other intermediates accumulated during the batch experiment, therefore for most of the experiments it was not possible to know whether complete denitrification actually occurred.

We estimate the growth yield \((Y_{H})\) and the maximum growth rates \((\mu_{H})\) in four different scenarios:

1. nitric oxide (NO) as terminal electron acceptor;
2. nitrous oxide(N\textsubscript{2}O) as terminal electron acceptor;
3. Nitrogen gas (N\textsubscript{2}) as terminal electron acceptor, i.e. complete denitrification.

The calculations based on the following assumptions:

- within the first and the second phase the bCOD fraction (Ss+Xs) in the feeding medium is completely utilized;
- Ss>> Ks and the Monod kinetic is further simplified.

### Table 4-3 specific utilization rates (mgNO\textsubscript{3,2}-N/mgCOD*d)

<table>
<thead>
<tr>
<th>Reactor</th>
<th>(X_{bh}) (mgCOD/L)</th>
<th>(k_1) (mgNO\textsubscript{3,2}-N/mgCOD*d)</th>
<th>(k_2) (mgNO\textsubscript{3,2}-N/mgCOD*d)</th>
<th>(k_{DS}) (mgNO\textsubscript{3,2}-N/mgCOD*d)</th>
<th>(k_3) (mgNO\textsubscript{3,2}-N/mgCOD*d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstaged</td>
<td>2393.33</td>
<td>0.125048</td>
<td>2393.33</td>
<td>0.125048</td>
<td>2393.33</td>
</tr>
<tr>
<td>S1</td>
<td>4068.89</td>
<td>0.10334</td>
<td>4068.89</td>
<td>0.10334</td>
<td>4068.89</td>
</tr>
<tr>
<td>S2</td>
<td>2414.11</td>
<td>0.184813</td>
<td>2414.11</td>
<td>0.184813</td>
<td>2414.11</td>
</tr>
<tr>
<td>S3</td>
<td>2262.22</td>
<td>0.249207</td>
<td>2262.22</td>
<td>0.249207</td>
<td>2262.22</td>
</tr>
</tbody>
</table>
Despite of that the observed substrates utilization is maximum in U and minimum in S3, S3 is the reactor with the highest values for $Y_h$ and $\mu_h$ in the four scenarios. This could be interpreted as evidence of a more efficient metabolism: even if less substrate is consumed the synthesis of new biomass per amount of sCOD oxidized is larger and faster. On the contrary both U and S1 show the lower values for $Y_h$ and $\mu_h$ in each of the scenarios.

The estimated values $\mu_{h,\text{max}}$ of referred to the 1st phase in the batch experiment, where the Ss is available. While during the continuous flow experiment the media in S2 and S3 are supposed to be partially and completely depleted of ready biodegradable substrate. Indeed the observed growth rate in S3 in the entire period is the lowest.
5 Conclusion

This study suggests the potential benefit of staging MBBR reactors as optimization of denitrification process under anoxic conditions, confirming the hypothesis made. The staged configuration ensured higher conversion of influent NO$_3$-N, using non-synthetic wastewater feed. Under equal operating conditions (i.e. the same operational volume) higher performance was achieved in the staged configuration possibly as a result of biomass adaptation to the different feeding conditions, both in terms initial influent (S1) and the influent to the further stages (S2, S3). This further suggests that reactor staging could ensure reaching comparable performances in terms of nitrate removal using a reduced overall reactor volume, as compared to the unstaged configuration.

It can be concluded that the staged reactor seemed to be more robust during the period of evaluation. S2 and S3 ensured almost constant denitrification efficiencies regardless of the quality of the medium. On the other hand, S1 and U performances were strongly affected by influent quality in terms of content of bCOD and S$_s$. The continuous operation of S2 and S3 under carbon-limiting conditions led to adaptation of biomass to denitrification using hydrolysis products of slowly biodegradable substrate. Further investigations, e.g., microbiological analysis and complete COD fractionation in the same reactors, are required to confirm this hypothesis.

Among the pharmaceuticals assessed, the highest removal efficiency (>40%) in the tested conditions was shown for the beta-blockers atenolol and propranolol, the antidepressant citalopram and the antibiotic trimethoprim. For a number of other substances ( ), removal efficiency did not exceed 10%. For sulfamethoxazole, retransformation of the major metabolite (N4-acetyl-sulfamethoxazole) lead to negative removal efficiencies (< -100%) No definitive conclusion on which MBBR configuration performed better in terms of pharmaceuticals removal was reached. Promising results, satisfying the initial hypothesis of improved removal in the staged configuration, were shown for metoprolol.

Batch experiment allowed to estimate biological kinetics for denitrification in the different MBBR reactors under the same feeding conditions. Different behaviour shown by the variations in specific denitrification rates and by the estimated biokinetic parameters (heterotrophic yield $Y_h$ and maximum heterotrophic growth rate coefficient $\mu_{H,max}$) seem to suggest an adaptation of the bacterial community during the three months of continuous operation. The specific denitrification rate per unit biomass during the 1$^{st}$ phase of denitrification (utilizing the non-limiting S$_s$ fraction in the feed) increased from reactors S1 to S3. Two possible explanations exist:

- Utilization of feeding S$_s$ for denitrification does not require any biomass adaptation;
- Hydrolysis in S2 and S3 was important under batch experimental conditions and may have affected denitrification in the 1$^{st}$ phase identified and, if performed at higher rates as compared to S1, could have provided for additional S$_s$ immediately available as electron donor for denitrification.

With regard to the utilization of pharmaceuticals, observed removal under continuous operation was consistent with data available in the literature. Preliminary observations on the results from the batch experiment agreed with this observation and seemed to describe removals obtained under continuous operation for atenolol, sulfamethoxazole, trimethoprim, carbamazepine and
Dicofenac. It can be concluded that three months of operation may have not been sufficient for adaptation to drive the evolution of the bacteria communities to the consumption of XOCs.

The different growth experienced during the continuous flow experiment and the different metabolic kinetics extracted from the batch experiment confirm that the bacterial community adapted to the specific conditions in each reactors. Eventually by further microbiological analysis we could assess more precisely how the biodiversity changes and how it evolves over time. Furthermore, if the expression of enzymes specifically involved in denitrification and in the degradation of XOCs is established, we could assess more precisely the intensity of denitrification itself and how much the bacteria evolve towards the consumption of XOCs in each reactor.

In accordance to the observations on the primary metabolism, this study confirms the validity of staging even in for MBBR. By the adaption of the biomass to the specific conditions in the stages, staged MBBR achieves better performances for the removal of NO$_3$-N. Further investigation on different configuration and on a longer monitoring period are still required to establish the convenience of this solution to remove XOCs in wastewater.
Appendix A – The Anaerobic Aromatic Metabolism

Next to carbohydrates, aromatic compounds are the second most abundant class of organics (Boll M., 2004). This category includes a large variety of different substances that are ubiquitous growth substrates (Harwood C.S., Burchhardt G., Herrmann H., and Fuchs G., 1999). Since the aromatic ring has resonance energy higher than 100 kJ/mole, the aromatics are usually inert and difficult to be biodegraded (Boll M., 2004). But it has been proven that major types of anaerobic heterotrophs bacteria, acetogenic bacteria and fungi can consume them also in absence of oxygen (Heider J. and Fuchs G., 1996) (Gibson J. and Harwood C., 2002). In anoxic environments oxygenases is repressed and the all oxidative enzymatic processes involving oxygen are replaced by alternative reductive pathways (Boll M., 2004) (Philip B. and Shink B., 2012). The strategy of anaerobic bacteria is to convert low-molecular weight aromatic substrates by peripheral pathways into few key intermediates. The most probable intermediates are: Benzoyl-CoA (BCoA), phloroglucinol, hydroxyquinone and resorcinol (Boll M., 2004). Then the intermediates are further degraded by central pathways (Fuchs G., Mohamed M. E., Altschmidt U., Koch J. Lack A., Brackmann R. Lochmeyer C. and Oswald B., 1994). The pathways in anoxic conditions rely on reductive biochemistry, and they include carboxilations, reductive dehydroxilations and addition reactions (Heider J. and Fuchs G., 1996). In anaerobic bacteria, most aromatic growth substrates are channelled into the BCoA degradation pathway (G. Fuchs, M. Boll and J. Heider, 2011) (Heider J. and Fuchs G., 1996) (Gibson J. and Harwood C., 2002) (K. Kuntze, Y. Shinoda, H. Mouttaki, M. J. McInerney, C. Vogt, H. Richnow and M. Boll, 2008).

So BCoA emerges as the most common intermediate and a broad variety of aromatic molecules enter this pathway, such as halogenated molecules, methoxylated molecules or compounds having a carbon side chains (Gibson J. and Harwood C., 2002) (Philip B. and Shink B., 2012). In this pathway the aromatic ring is reduced and cleaved to acetyl-CoA and CO₂ (Heider J. and Fuchs G., 1996) (Gibson J. and Harwood C., 2002). The complete degradation produces one mole of CO₂ and three moles of acetyl-CoA, which are further oxidised via the citric cycle, via the oxidative acetyl/carbon monoxide dehydrogenase pathway or via the reductive carboxylation to pyruvate. The BCoA pathway consists of the following four steps, fig 3 (K. Kuntze, Y. Shinoda, H. Mouttaki, M. J. McInerney, C. Vogt, H. Richnow and M. Boll, 2008) (Heider J. and Fuchs G., 1996) (Simon Wischgoll, Martin Taubert, Franziska Peters, Nico Jehmlich, Martin von Bergen and Matthias Boll, 2009):

I. Reductive dearomatization of BCoA to cyclohexa-1,5-diene-1-carbonyl-CoA catalysed by BCoA reductase (BCR);
II. Water addition to cyclohexa-1,5-diene-1-carbonyl-CoA forming 6-hydroycyclohex-1-ene-1-carbonyl-CoA catalysed by dienoyl-CoA hydratase;
III. Oxidation of the forming 6-hydroycyclohex-1-ene-1-carbonyl-CoA to 6-oxocyclohex-1-ene-1-carbonyl-CoA (6-OCH-CoA) by an alcohol hydrogenase;
IV. Hydrolitic ring cleavage and addition of water molecule yielding 6-hydroxypimelyl-CoA catalysed by ring opening hydrolase (6-OCH-CoA hydrolase).
The BCR is recognized as the central enzyme in the “aromatic metabolism”. In the past many investigations targeted it as it was supposed to picture the bacterial community degrading aromatic compounds. The synthesis of BCR has been detected in many denitrifying bacteria both from pure cultures and environmental samples (Harwood C.S., Burchhardt G., Herrmann H. and Fuchs G., 1999) (Gibson J. and Harwood C., 2002) (B. Song and B. B. Ward, 2005). So far two types of BCR have been found; bzd type from Azoarcus and bcr type from Thauera (K. Kuntze, C. Vogt, H.H. Richnow and Matthias Boll, 2011). Since the amino acid sequences are very different, they are very specific for each enzyme (K. Kuntze, Y. Shinoda, H. Mouttaki, M. J. McInerney, C. Vogt, H. Richnow and M. Boll, 2008).

Primers for both reductases have been isolated and by gene amplification we manage to: detect the different types of BCR, determine the dominant and/or the activated ones in particular conditions and also identify and differentiate bacteria within the whole community (K. Kuntze, Y. Shinoda, H. Mouttaki, M. J. McInerney, C. Vogt, H. Richnow and M. Boll, 2008) (B. Song and B. B. Ward, 2005) (Breese K., Boll M., Alt-Morbe Juliane, Schagger H. and Fuchs G., 1998).

But the divergence between the BCRs-genes makes impossible to establish the design of good degenerate primer to detect both types of enzymes and to monitor the whole bacterial community (B. Song and B. B. Ward, 2005) (K. Kuntze, Y. Shinoda, H. Mouttaki, M. J. McInerney, C. Vogt, H. Richnow and M. Boll, 2008). Furthermore some false-positive results have also been detected (Hosooda A., Kasai Y., Hamamura N., Takahata Y. and Watanabe K., 2005). In the end BCR is not reliable to investigate the whole bacterial community degrading aromatic compounds.

In contrast to the BCR which differs among the denitrifying species (Fuchs G., Mohamed M. E., Altenschmidt U., Koch J. Lack A., Brackmann R. Lochmeyer C. and Oswald B., 1994), the genes encoding for 6-OCH-CoA hydrolase isolated in Thauera, Azoarcus and Magnetospirillum show high similarity (K. Kuntze, Y. Shinoda, H. Mouttaki, M. J. McInerney, C. Vogt, H. Richnow and M. Boll, 2008). The 6-OCH-CoA hydrolase is encoded by the bamA gene and its homologs oah and bzdY (A. W. Porter and L.Y. Young, 2013). Further investigations found that the bamA is highly conserved in all anaerobic bacteria using aromatic growth substrates. Since regions in bamA are highly conserved in different organism, it is an ideal candidate as biomarker for anaerobic aromatic hydrocarbon biodegradation (A. W. Porter and L.Y. Young, 2013) (K. Kuntze, Y. Shinoda,
Appendix B – Wastewater characterization

The series of data collected from the Oxitop heads have been fitted with the following exponential equation:

\[ y = a(1 - e^{-bx}); \]

From the BOD curves we estimated the total BOD (\( \text{BOD}_{\text{tot}} \)) as the asymptotic value;

\[ \text{BOD}_{\text{tot}} = a; \]

As S.R. Weijers (1999) prescribes, in order to determine the biodegradable COD fraction (bCOD) one more conversion factor have been applied. We considered the distance between the fitted curve and the asymptote by the following equation;

\[ \text{bCOD} = \text{BOD}_{\text{tot}} \times \frac{1}{(1 - Y_{H,BOD})} \]

\( Y_{H,BOD} \) is not quantitively motivated (S. R. Weijers, 1999), it varies between 0,1 and 0,2; we used \( Y_{H,BOD} = 0,1 \).

As Roeleveld et al. (2002) prescribe and assuming that the biomass does not occur in the influent, on the previously defined analysis we determined the following carbon fractions:

\[ \text{COD}_{\text{inf}} = S_S + S_I + X_S + X_I; \]

\[ \text{sCOD}_{\text{inf}} = S_S + S_I; \]

\[ S_I = \text{sCOD}_{\text{eff}}; \]

\[ \text{bCOD} = \text{BOD}_{\text{tot}} \times \frac{1}{(1 - Y_{H,BOD})} = S_S + X_S; \]

\[ S_S = \text{sCOD}_{\text{inf}} - S_I; \]

\[ X_S = \text{bCOD} - S_S. \]
Appendix C – Protocols for the TSS measurement

The measurement of TSS on the carriers has been repeated three times in each reactor, five non deformed carriers have been collected for the quantification. The procedure consists of:

- Sampling of five non deformed carriers from each reactor;
- Drying the carriers for 1.5 h at 103 °C;
- Weighing the colonized carriers and recording the data;
- Flushing the carriers with Sulphuric Acid (H$_2$SO$_4$) 4 M;
- Shaking the carriers in solution of H$_2$SO$_4$ and water until the detachment of the biofilm starts;
- Flushing the carriers with tap water;
- Brushing the biomass out from the carriers and flushing again with tap water;
- Drying the carriers for 15 min at 103 °C or in the essicator for 4-8 hours;
- Weighing the empty and dried carriers.

The TSS content is estimated by the following equation:

\[
M_{\text{carrier}}(gTSS/L) = \left[ \sum W_{\text{dried cultivate carrier}} - \sum W_{\text{carrier}} \right]
\]

Considering the estimations from Anoxkaldnes about the carriers concentration per litre and on the knowing the amount of biomass per carrier, by the following equation we estimated the total biomass ($M_{\text{totalbiomass}}$) and the biomass concentration ($m_{\text{biomass}}$) in each reactor:

\[
M_{\text{totalbiomass}} = 1000 * f * V_{\text{reactor}} * M_{\text{carrier}}
\]

\[
m_{\text{biomass}} = \frac{M_{\text{totalbiomass}}}{V_{\text{reactor}}} = 1000 * f * M_{\text{carrier}}
\]

Where $f$ is the filling ration and $V_{\text{reactor}}$ is the volume of the reactor.
Appendix D – Protocols for the analysis of 30 pharmaceuticals (HPLC-MS/MS)

1. Sampling and storage
   - Sample: 10 mL sample with glass pipette in a 14mL glass vial.
   - Storage until reaching laboratory: 4°C and transport in a camping fridge
   - At the laboratory: add 3.5mL MeOH and store at -20°C until analysis.

2. Sample preparation
   - Leave the stored samples to reach room temperature;
   - homogenize and transfer aprox. 1.5 mL to an HPLC vial;
   - Centrifuge the 1.5 mL vials 10 minutes at 6000 rpm;
   - With a syringe take 900 µl of the aqueous phase and transfer to a new HPLC vial;
   - Spike each sample with 100 µL of internal standard using syringe;
   - Between samples, rinse with 1mL MeOH and 1mL MiliQ water;
   - For the IS use the 100µL syringe meant for that IS solution.

3. Internal Standard:
   - ac-sulfadiazine 13C6: 20 µg/mL, sulfadiazine 13C6: 14 µg/mL ibuprofen D3: 118 µg/mL in MeOH;
   - Syringes: For the samples, use the 1mL "Samples" syringe.

4. Analysis - HPLC-MS/MS
   The HPLC is a dual low-pressure mixing ternary-gradient system Ultimate 3000 from Dionex. The system is equipped with a pump of the 3000 series (DGP-3600 M), a 3000 TSL autosampler (WPS 3000 TSL) and a column oven and degasser also from the Dionex 3000 series. The HPLC operates with two eight-port Valco valves. The mass spectrometer is an API 4000 (ABSciex, Framingham, MA, USA). The API 4000 operates in ESI in positive mode at 400°C with a capillary voltage of 5500 V. The HPLC operates following gradient elution of methanol and Millipore water, both containing 0.2% formic acid (v/v) on a Synergi-Polar column (Phenomenex, Torrance, California, USA). All the assessment is based on the peak areas. A multilevel calibration is performed with four repeats each.
   Standard analysis: 10µL injection. Volumes up to 50µL can be injected. The obtained concentrations have to be corrected due to the initial MeOH addition.

5. Standard curve preparation:
   - Stock solution containing 30 compounds in MeOH (Specified in another document);
   - Two intermediate stock solutions were prepared by adding 5 µL and 250 µL of the concentrated stock solution and filled them up with MeOH to 5 mL.
   - 2 intermediate stock solutions were further diluted into the vials to the concentrations as shown in the table below with a methanol percentage of 36%.
<table>
<thead>
<tr>
<th></th>
<th>“0.1 ng/mL”</th>
<th>“1 ng/mL”</th>
<th>“2 ng/mL”</th>
<th>“5 ng/mL”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate Stock Solution added (1 µL/mL) [µL]</td>
<td>2.5</td>
<td>25</td>
<td>50</td>
<td>125</td>
</tr>
<tr>
<td>Milli-Q H₂O added [µL]</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>Methanol added [µL]</td>
<td>257.5</td>
<td>235</td>
<td>210</td>
<td>135</td>
</tr>
<tr>
<td>Internal Standard added [µL]</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>“10 ng/mL”</th>
<th>“20 ng/mL”</th>
<th>“50 ng/mL”</th>
<th>“100 ng/mL”</th>
<th>“200 ng/mL”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate Stock Solution added (50 µL/mL) [µL]</td>
<td>5</td>
<td>10</td>
<td>25</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Milli-Q H₂O added [µL]</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>Methanol added [µL]</td>
<td>255</td>
<td>250</td>
<td>235</td>
<td>210</td>
<td>160</td>
</tr>
<tr>
<td>Internal Standard added [µL]</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

6. Controls:

For every batch, prepare 6 controls at the level of 50 ng/mL and spiked with the IS.
Appendix E – Protocol for the MBBR batch experiments

After operation in continuous mode for approx. 3 months, staged and unstaged MBBR reactors will be operated in batch mode. Batch experiments with each reactor, containing MBBR carriers adapted to pre-clarified wastewater during continuous operation, are performed to assess denitrification kinetics and xenobiotics’ fate.

Details on the operation during batch experiments:

<table>
<thead>
<tr>
<th>Reactor</th>
<th>S1 (staged n. 1)</th>
<th>S2 (staged n. 2)</th>
<th>S3 (staged n. 3)</th>
<th>U (unstaged)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>1.5 L</td>
<td>1.5 L</td>
<td>3 L</td>
<td>6 L</td>
</tr>
<tr>
<td>Filling ratio</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>Temperature</td>
<td>20°C</td>
<td>20°C</td>
<td>20°C</td>
<td>20°C</td>
</tr>
<tr>
<td>Batch duration</td>
<td>24 h</td>
<td>24 h</td>
<td>24 h</td>
<td>24 h</td>
</tr>
</tbody>
</table>

Reactors preparation

- Stop continuous operation, disconnect reactors
- Empty reactors from wastewater and carriers
- Rinse/quickly wash reactors with water
- Re-insert carriers the reactors, adjusting filling ratio to 20%. Keep extra carriers in flasks with wastewater, preserve at < 4°C.
- Dose KNO₃ in each reactor, reaching an initial concentration of 100 mg/L NO₃⁻-N in all reactors.
- Fill all reactors with new wastewater inoculum.
- Restart N2 sparging.
- Fill aquarium with tap water, keep temperature around 20°C.
- Insert one pH meter in each reactor.
- Adjust pH in each reactor to 7.0 by adding HCl.

When dosing is completed, the batch experiment starts (t=0).

Operation

- Temperature is controlled around 20°C by filling aquarium with tap water;
- Temperature and pH in the reactors are continuously monitored using multimeters.
- For each sample collected (=volume of wastewater removed), carriers are removed to keep the filling ratio (and biomass concentration) constant over the whole experiment.
- Characterization of the wastewater inoculum is performed, determining initial concentrations of COD, sCOD, NH₄⁺, NO₃⁻ and xenobiotics.

Sampling and analysis

1. Initial sampling at t=0 from wastewater inoculum
   - Duplicates for COD, sCOD, NH₄⁺, NO₃⁻
- Triplicates for BOD
- Duplicates/Triplicates for xenobiotics

2. COD, sCOD, NO₃, NO₂
   - Volume of non-filtered sample to be collected = 3 mL
   - Volume of filtered sample = 10 mL
   - Total volume = 13 mL
   - For sample filtration, use 0.45 um siringe filters
   - Storage of samples at < 4° C

3. Xenobiotics
   - Collect 4 mL of wastewater using glass pipette
   - Add 1.4 mL of MeOH (99% purity)
   - Storage of samples at -20° C

4. BOD, NH₄
   - Samples to be collected from each reactor at the end of the experiments (t=24 h)

5. TSS carriers
   - 5 carriers are collected from each reactor at the end of the experiment (t=24 h)
   - Carriers in the oven at 105° C for > 1 h

Details on sampling time, analytes and carriers removed for each sampling are included in the following table:

<table>
<thead>
<tr>
<th>Sampling time (min)</th>
<th>Sampling time (h)</th>
<th>Sample volume (mL)</th>
<th>Sample collected for</th>
<th>Carriers removed (carr./reactor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>From inoculum</td>
<td>COD, sCOD, NO₃, (NO₃)</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NH₄</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BOD</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.25</td>
<td>17</td>
<td>COD, sCOD, NO₃, NO₂</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.5</td>
<td>17</td>
<td>COD, sCOD, NO₃, NO₂</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>1</td>
<td>17</td>
<td>COD, sCOD, NO₃, NO₂</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
<td>17</td>
<td>COD, sCOD, NO₃, NO₂</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>2</td>
<td>17</td>
<td>COD, sCOD, NO₃, NO₂</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>3</td>
<td>13</td>
<td>COD, sCOD, NO₃, NO₂</td>
<td>3</td>
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<td></td>
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<td></td>
<td>Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>4</td>
<td>17</td>
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<td></td>
<td></td>
<td>Xenobiotics</td>
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<td></td>
<td></td>
<td></td>
<td>COD, sCOD, NO$_3^-$, NO$_2^-$, Xenobiotics</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>------------------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>420</td>
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<tr>
<td>600</td>
<td>10</td>
<td>17</td>
<td>COD, sCOD, NO$_3^-$, NO$_2^-$, Xenobiotics</td>
<td>4</td>
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<tr>
<td>900</td>
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<td>COD, sCOD, NO$_3^-$, NO$_2^-$</td>
<td>2</td>
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<tr>
<td>1200</td>
<td>20</td>
<td>17</td>
<td>COD, sCOD, NO$_3^-$, NO$_2^-$, Xenobiotics</td>
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<tr>
<td>1440</td>
<td>24</td>
<td>13</td>
<td>COD, sCOD, NO$_3^-$, NO$_2^-$, NH$_4^+$, BOD, TSS on carriers</td>
<td>3</td>
</tr>
</tbody>
</table>
Appendix F– the Continuous experiment MONITORING

- NO$_2$ concentration in the last 50 days of the operating period
The NH₄ concentration in the last 25 days of the operating period is shown in the graph below.

- Fluctuations of pH and temperature at the sampling time in the last 25 days are also depicted in the graph.

---

**NH₄ Concentration**

- Concentration influent and effluents
- Time (day) vs influent
- Time (day) vs effluent stage
- Time (day) vs effluent unstage

**pH and Temperature**

- pH vs time (day)
- Temperature vs time (day)

---

*Figure 9: Concentration and Fluctuations of pH and Temperature in the Last 25 Days*
Appendix G – the Batch experiments DATA
Appendix H – linear regression NO$_{3,2}$-N utilization curve

Unstage

Friday, March 28, 2014, 14:27:49

Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
\( f = y_0 + ax \)

<table>
<thead>
<tr>
<th>R</th>
<th>Rsqr</th>
<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9750</td>
<td>0.9507</td>
<td>0.9342</td>
<td>3.6495</td>
</tr>
</tbody>
</table>

Coefficient | Std. Error | t     | P       |
---|------------|------|--------|
y0 | 100.0000  | 2.4481| 40.8472 | <0.0001 |
a  | -12.4722  | 1.4598| -8.5438 | 0.0034  |

Analysis of Variance:

<table>
<thead>
<tr>
<th>DF</th>
<th>SS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>35349.4953</td>
<td>17674.7476</td>
</tr>
<tr>
<td>3</td>
<td>39.9562</td>
<td>13.3187</td>
</tr>
<tr>
<td>5</td>
<td>35389.4515</td>
<td>7077.8903</td>
</tr>
</tbody>
</table>

Corrected for the mean of the observations:

<table>
<thead>
<tr>
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<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>769.9040</td>
<td>769.9040</td>
<td>57.8060</td>
<td>0.0047</td>
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<tr>
<td>3</td>
<td>39.9562</td>
<td>13.3187</td>
<td></td>
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<tr>
<td>4</td>
<td>809.8603</td>
<td>202.4651</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical Tests:

Normality Test (Shapiro-Wilk) Passed (P = 0.5482)
W Statistic 0.9228 Significance Level 0.0500

Constant Variance Test Passed (P = 0.0500)

Fit Equation Description:

[Variables]
x = col(2)
y = col(4)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2

[Automatic Initial Parameter Estimate Functions]
F(q) = ape(x,y,1,0,1)

[Parameters]
y0 = F(0)[1] "Auto ([previous: 100]) ([MinRange: -12.3]) ([MaxRange: 36.9])
a = F(0)[2] "Auto ([previous: -12.4722]) ([MinRange: -4.5]) ([MaxRange: 1.5])

[Equation]
f = y0 + ax
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare

[Constraints]
linear Regression

Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
\[ f = y_0 + a \cdot x \]

<table>
<thead>
<tr>
<th>R</th>
<th>Rsqr</th>
<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9367</td>
<td>0.8775</td>
<td>0.8366</td>
<td>3.3571</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>( y_0 )</td>
<td>70.1166</td>
<td>3.1118</td>
<td>22.5323</td>
</tr>
<tr>
<td>( a )</td>
<td>-1.6027</td>
<td>0.3458</td>
<td>-4.6354</td>
</tr>
</tbody>
</table>

Analysis of Variance:

Analysis of Variance:

<table>
<thead>
<tr>
<th>DF</th>
<th>SS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>2</td>
<td>16763.0523</td>
</tr>
<tr>
<td>Residual</td>
<td>3</td>
<td>33.8098</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>16796.8621</td>
</tr>
</tbody>
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Corrected for the mean of the observations:

<table>
<thead>
<tr>
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<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
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<td>242.1507</td>
<td>242.1507</td>
<td>21.4865</td>
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<tr>
<td>Residual</td>
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<td>33.8098</td>
<td>11.2699</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>275.9605</td>
<td>68.9901</td>
<td></td>
</tr>
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</table>

Statistical Tests:

Normality Test (Shapiro-Wilk) Passed (P = 0.1384)
W Statistic = 0.8297 Significance Level = 0.0500

Constant Variance Test Passed (P = 0.0500)

Fit Equation Description:

\[ \text{Variables} \]
\[ x = \text{col}(2) \]
\[ y = \text{col}(5) \]
\[ \text{reciprocal}_y = \frac{1}{\text{abs}(y)} \]
\[ \text{reciprocal}_y^2 = \frac{1}{y^2} \]

\`Automatic Initial Parameter Estimate Functions\`
\[ F(q) = \text{ape}(x,y,1,0,1) \]

\[ \text{Parameters} \]
\[ y_0 = F(0)[1] \quad \text{Auto} \quad \{ \text{previous: 70.1166} \} \quad \{ \text{MinRange: -12.3} \} \quad \{ \text{MaxRange: 36.9} \} \]
\[ a = F(0)[2] \quad \text{Auto} \quad \{ \text{previous: -1.6027} \} \quad \{ \text{MinRange: -4.5} \} \quad \{ \text{MaxRange: 1.5} \} \]

\[ \text{Equation} \]
\[ f = y_0 + a \cdot x \]
\[ \text{fit f to y} \]
\[ \text{fit f to y with weight reciprocal}_y \]
\[ \text{fit f to y with weight reciprocal}_y^2 \]

[Constraints]
### R
<table>
<thead>
<tr>
<th>R</th>
<th>Rsqr</th>
<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3787</td>
<td>0.1434</td>
<td>0.0000</td>
<td>2.3734</td>
</tr>
</tbody>
</table>

### Coefficient Std. Error t P
| y0      | 46.3335 | 7.4517   | 6.2179 | 0.1015 |
| a       | -0.1522 | 0.3719   | -0.4091 | 0.7528 |

### Analysis of Variance:

<table>
<thead>
<tr>
<th>DF</th>
<th>SS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
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<td>5635.1430</td>
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<tr>
<td>Residual</td>
<td>1</td>
<td>5.6333</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>5640.7763</td>
</tr>
</tbody>
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Corrected for the mean of the observations:

<table>
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<tr>
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<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>0.9430</td>
<td>0.9430</td>
<td>0.1674</td>
</tr>
<tr>
<td>Residual</td>
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<td>5.6333</td>
<td>5.6333</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>6.5763</td>
<td>3.2881</td>
<td></td>
</tr>
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### Statistical Tests:

#### Normality Test (Shapiro-Wilk)

Passed (P = 0.1427)

W Statistic= 0.8117 Significance Level = 0.0500

#### Constant Variance Test

Failed (P = <0.0001)

### Fit Equation Description:

**Variables**
- x = col(2)
- y = col(6)
- reciprocal_y = 1/abs(y)
- reciprocal_ysquare = 1/y^2

**'Automatic Initial Parameter Estimate Functions**
- F(q) = ape(x,y,1,0,1)

**[Parameters]**
- y0 = F(0)[1] "Auto {[previous: 46.3335]} {[MinRange: -12.3]} {[MaxRange: 36.9]}
- a = F(0)[2] "Auto {[previous: -0.152166]} {[MinRange: -4.5]} {[MaxRange: 1.5]}

**[Equation]**
- f = y0+a*x
- fit f to y
- "fit f to y with weight reciprocal_y
- "fit f to y with weight reciprocal_ysquare

**[Constraints]**
### Linear Regression

**Data Source:** NO3 in DELTA NO3 NEW  
**Equation:** Polynomial, Linear  
\[ f = y_0 + a \cdot x \]

<table>
<thead>
<tr>
<th>R</th>
<th>Rsqr</th>
<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9856</td>
<td>0.9714</td>
<td>0.9619</td>
<td>2.5966</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>( y_0 )</td>
<td>100.0000</td>
<td>2.0113</td>
<td>49.7191</td>
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<tr>
<td>( a )</td>
<td>-17.5273</td>
<td>1.6422</td>
<td>-10.6730</td>
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### Analysis of Variance:

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<tr>
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<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>2</td>
<td>34324.0556</td>
<td>17162.0278</td>
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<tr>
<td>Residual</td>
<td>3</td>
<td>20.2266</td>
<td>6.7422</td>
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<tr>
<td>Total</td>
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<td>34344.2822</td>
<td>6868.8564</td>
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Corrected for the mean of the observations:

<table>
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<th>P</th>
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<tbody>
<tr>
<td>Regression</td>
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<td>687.6536</td>
<td>687.6536</td>
<td>101.9925</td>
<td>0.0021</td>
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<tr>
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<td>20.2266</td>
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<tr>
<td>Total</td>
<td>4</td>
<td>707.8802</td>
<td>176.9701</td>
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</tbody>
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### Statistical Tests:

**Normality Test (Shapiro-Wilk)**  
Passed  
(W Statistic = 0.9296  
Significance Level = 0.0500)

**Constant Variance Test**  
Passed  
(P = 0.0500)
**linear Regression**  

**Data Source:** NO3 in DELTA NO3 NEW  
**Equation:** Polynomial, Linear  
\[ f = y_0 + a \times x \]

<table>
<thead>
<tr>
<th>R</th>
<th>Rsqr</th>
<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9618</td>
<td>0.9251</td>
<td>0.9001</td>
<td>4.6058</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>y_0</td>
<td>68.9430</td>
<td>3.4972</td>
<td>19.7139</td>
</tr>
<tr>
<td>a</td>
<td>-2.5301</td>
<td>0.4156</td>
<td>-6.0876</td>
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</table>

**Analysis of Variance:**

**Analysis of Variance:**

<table>
<thead>
<tr>
<th>DF</th>
<th>SS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>2</td>
<td>14170.2248</td>
</tr>
<tr>
<td>Residual</td>
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<td>Total</td>
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<td>14233.8637</td>
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</tbody>
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Corrected for the mean of the observations:

<table>
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<tr>
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<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
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<td>786.1216</td>
<td>786.1216</td>
<td>37.0586</td>
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<td>Residual</td>
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<td>63.6389</td>
<td>21.2130</td>
<td></td>
</tr>
<tr>
<td>Total</td>
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<td>849.7605</td>
<td>212.4401</td>
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</tbody>
</table>

**Statistical Tests:**

Normality Test (Shapiro-Wilk)  
Passed (P = 0.9326)

W Statistic= 0.9796  
Significance Level = 0.0500

Constant Variance Test  
Passed (P = 0.0500)

**Fit Equation Description:**

- **Variables**
  - \( x = \text{col}(2) \)
  - \( y = \text{col}(9) \)
  - \( \text{reciprocal}_y = 1/\text{abs}(y) \)
  - \( \text{reciprocal}_y^2 = 1/y^2 \)

- **Automatic Initial Parameter Estimate Functions**
  - \( F(q) = \text{ape}(x, y, 1, 0, 1) \)

- **Parameters**
  - \( y_0 = F(0)[1] \) "Auto \{ [previous: 68.943] \} \{ [MinRange: -12.3] \} \{ [MaxRange: 36.9] \}"
  - \( a = F(0)[2] \) "Auto \{ [previous: -2.53015] \} \{ [MinRange: -4.5] \} \{ [MaxRange: 1.5] \}"

- **Equation**
  - \( f = y_0 + a \times x \)

- **Fit f to y with weight reciprocal_y**
- **Fit f to y with weight reciprocal_y^2**

**Constraints**
linear Regression

Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
f = y0+a*x

R  Rsqr  Adj Rsqr  Standard Error of Estimate
0.8846 0.7825 0.5651 4.3800

Coefficient  Std. Error  t  P
y0 49.3515 13.7516 3.5888 0.1730
a -1.3020 0.6863 -1.8970 0.3088

Analysis of Variance:

Analysis of Variance:

<table>
<thead>
<tr>
<th>DF</th>
<th>SS</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>2</td>
<td>1755.5291</td>
</tr>
<tr>
<td>Residual</td>
<td>1</td>
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<tr>
<td>Total</td>
<td>3</td>
<td>1774.7139</td>
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Corrected for the mean of the observations:

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<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>69.0368</td>
<td>69.0368</td>
<td>3.5985</td>
</tr>
<tr>
<td>Residual</td>
<td>1</td>
<td>19.1848</td>
<td>19.1848</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>88.2216</td>
<td>44.1108</td>
<td></td>
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</table>

Statistical Tests:

Normality Test (Shapiro-Wilk) Passed (P = 0.1427)
W Statistic= 0.8117 Significance Level = 0.0500

Constant Variance Test Failed (P <0.0001)

Fit Equation Description:
[Variables]
x = col(2)
y = col(10)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2

`Automatic Initial Parameter Estimate Functions
F(q) = ape(x,y,1,0,1)

[Parameters]
y0 = F(0)[1] {[previous: 49.3515]} {[MinRange: -12.3]} {[MaxRange: 36.9]}
a = F(0)[2] {[previous: -1.30197]} {[MinRange: -4.5]} {[MaxRange: 1.5]}

[Equation]
f = y0+a*x
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare
[Constraints]
linear Regression

Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
\( f = y_0 + ax \)

<table>
<thead>
<tr>
<th>R</th>
<th>Rsqr</th>
<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8729</td>
<td>0.7619</td>
<td>0.6826</td>
<td>6.9985</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>( y_0 )</td>
<td>100.0000</td>
<td>4.5624</td>
<td>21.9181</td>
</tr>
<tr>
<td>( a )</td>
<td>-18.5976</td>
<td>4.4262</td>
<td>-4.2017</td>
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Analysis of Variance:

<table>
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<tr>
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<th>SS</th>
<th>MS</th>
<th></th>
<th>SS</th>
<th>MS</th>
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</thead>
<tbody>
<tr>
<td>Regression</td>
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<td>35301.4475</td>
<td>17650.7238</td>
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<td></td>
<td></td>
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<td>48.9784</td>
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<tr>
<td>Total</td>
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<td>35448.3826</td>
<td>7089.6765</td>
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Corrected for the mean of the observations:

<table>
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<tr>
<th></th>
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<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
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<td>470.2510</td>
<td>470.2510</td>
<td>9.6012</td>
<td>0.0534</td>
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<tr>
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<td>3</td>
<td>146.9351</td>
<td>48.9784</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
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<td>617.1861</td>
<td>154.2965</td>
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<td></td>
</tr>
</tbody>
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Statistical Tests:

Normality Test (Shapiro-Wilk) Passed \((P = 0.1467)\)

W Statistic: 0.8331 Significance Level: 0.0500

Constant Variance Test Passed \((P = 0.0500)\)

Fit Equation Description:

```
[Variables]
  x = col(2)
y = col(12)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2

[Automatic Initial Parameter Estimate Functions]
  F(q) = ape(x,y,1,0,1)

[Parameters]
y0 = F(0)[1] "Auto [{previous: 100}] [{MinRange: -12.3}] [{MaxRange: 36.9}]
a = F(0)[2] "Auto [{previous: -18.5976}] [{MinRange: -4.5}] [{MaxRange: 1.5}]

[Equation]
f = y0 + ax
fit f to y with weight reciprocal_y
```

[Constraints]
linear Regression

Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
f = y0+a*x

R | Rsqr | Adj Rsqr | Standard Error of Estimate
---|------|----------|-----------------|
0.9719 | 0.9446 | 0.9308 | 2.5384

Coefficient | Std. Error | t | P
---|------|---|---|
y0 | 66.9826 | 1.8888 | 35.4632 | <0.0001
a | -1.8897 | 0.2288 | -8.2604 | 0.0012

Analysis of Variance:

| DF | SS | MS |
---|---|---|
Regression | 2 | 17895.7134 | 8947.8567 |
Residual | 4 | 25.7731 | 6.4433 |
Total | 6 | 17921.4865 | 2986.9144 |

Corrected for the mean of the observations:

| DF | SS | MS | F | P |
---|---|---|---|---|
Regression | 1 | 439.6506 | 439.6506 | 68.2340 | 0.0012 |
Residual | 4 | 25.7731 | 6.4433 |
Total | 5 | 465.4237 | 93.0847 |

Statistical Tests:

Normality Test (Shapiro-Wilk)
Passed (P = 0.2271)

W Statistic= 0.8702 Significance Level = 0.0500

Constant Variance Test
Passed (P = 0.0600)

Fit Equation Description:

[Variables]
x = col(2)
y = col(13)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2

'Automatic Initial Parameter Estimate Functions
F(q) = ape(x,y,1,0,1)

[Parameters]
y0 = F(0)[1] ^Auto {{previous: 66.9826}} {{MinRange: -12.3}} {{MaxRange: 36.9}}
a = F(0)[2] ^Auto {{previous: -1.88971}} {{MinRange: -4.5}} {{MaxRange: 1.5}}

[Equation]
f = y0+a*x

fit f to y
*fit f to y with weight reciprocal_y
*fit f to y with weight reciprocal_ysquare

[Constraints]
Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
f = y0+a*x

<table>
<thead>
<tr>
<th>R</th>
<th>Rsqr</th>
<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9995</td>
<td>0.9991</td>
<td>0.9982</td>
<td>0.1834</td>
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<table>
<thead>
<tr>
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<th>Std. Error</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>y0</td>
<td>51.4140</td>
<td>0.5756</td>
<td>89.3154</td>
</tr>
<tr>
<td>a</td>
<td>-0.9533</td>
<td>0.0287</td>
<td>-33.1796</td>
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Analysis of Variance:

<table>
<thead>
<tr>
<th>DF</th>
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<th>MS</th>
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</thead>
<tbody>
<tr>
<td>Regression</td>
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<td>Residual</td>
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<td>0.0336</td>
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<td>3233.1512</td>
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Corrected for the mean of the observations:

<table>
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<th>F</th>
<th>P</th>
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<tbody>
<tr>
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Statistical Tests:

- Normality Test (Shapiro-Wilk): Passed (P = 0.1427)
  W Statistic= 0.8117 Significance Level = 0.0500

- Constant Variance Test: Failed (P = <0.0001)

Fit Equation Description:

- Variables:
  x = col(2)
y = col(14)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2

- Automatic Initial Parameter Estimate Functions
  F(q) = ape(x,y,1,0,1)

- Parameters
  y0 = F(0)[1] "Auto {[previous: 51.414]} {[MinRange: -12.3]} {[MaxRange: 36.9]}
a = F(0)[2] "Auto {[previous: -0.953261]} {[MinRange: -4.5]} {[MaxRange: 1.5]}

- Equation
  f = y0+a*x
  fit f to y
  "fit f to y with weight reciprocal_y
  "fit f to y with weight reciprocal_ysquare

- Constraints
Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
\[ f = y0 + a \times x \]

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<tr>
<th>R</th>
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<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
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<td>0.7770</td>
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Analysis of Variance:

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<th>MS</th>
<th>F</th>
<th>P</th>
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Corrected for the mean of the observations:

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<tr>
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Statistical Tests:

Normality Test (Shapiro-Wilk)
Passed (P = 0.7956)

W Statistic= 0.9582 Significance Level = 0.0500

Constant Variance Test
Passed (P = 0.0500)

Fit Equation Description:
[Variables]
x = col(2)
y = col(16)
reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2

'A'Automatic Initial Parameter Estimate Functions
F(q) = ape(x,y,1,0,1)

[Parameters]
y0 = F(0)[1] 'Auto {{previous: 100}} {{MinRange: -12.3}} {{MaxRange: 36.9}}}
a = F(0)[2] 'Auto {{previous: -23.4954}} {{MinRange: -4.5}} {{MaxRange: 1.5}}}

[Equation]
f = y0+a*x
fit f to y
'fit f to y with weight reciprocal_y
'fit f to y with weight reciprocal_ysquare

[Constraints]
linear Regression

S3 Data Source: NO3 in DELTA NO3 NEW
Equation: Polynomial, Linear
f = y0+ax

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<th>Adj Rsqr</th>
<th>Standard Error of Estimate</th>
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Analysis of Variance:

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Corrected for the mean of the observations:

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Statistical Tests:

Normality Test (Shapiro-Wilk)  Passed  (P = 0.6117)
W Statistic= 0.9322  Significance Level = 0.0500

Constant Variance Test  Passed  (P = 0.0500)

Fit Equation Description:

- Variables
  - x = col(2)
  - y = col(17)
  - reciprocal_y = 1/abs(y)
  - reciprocal_ysquare = 1/y^2
- Automatic Initial Parameter Estimate Functions
  - F(q) = ape(x,y,1,0,1)
- Parameters
  - y0 = F(0)[1]  Auto  
    - [previous: 65.7608]  [{MinRange: -12.3}]  [{MaxRange: 36.9}]
  - a = F(0)[2]  Auto  
    - [previous: -0.440276]  [{MinRange: -4.5}]  [{MaxRange: 1.5}]
- Equation
  - f = y0+a*x
  - fit f to y
  - "fit f to y with weight reciprocal_y"
  - "fit f to y with weight reciprocal_ysquare"
- Constraints
Appendix I – utilization rate of pharmaceutical in the batch experiment

- **Atenolol**
• Metoprolol
• Sulphamethoxazole
- Carbamazepine
- Trimethoprim

![Graphs showing the change in concentration over time for different samples.](image-url)
• Diclofenac
- Sulphametizole
Bibliography


H. Itokawa, K. Hanaki and T. Matsuo. (n.d.). NITROUS OXIDE PRODUCTION IN HIGH-LOADING BIOLOGICAL NITROGEN REMOVAL PROCESS UNDER LOW COD/N RATIO CONDITION.


