Influence of microbial activity on polar xenobiotic degradation in activated sludge systems
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ABSTRACT
The influence of activated sludge quality on the co-metabolic biodegradation of three aminopolycarboxyl acids was investigated for a variety of Luxembourg sewage treatment plants. A combination of biodegradation experiments and respirometric techniques are presented as a reliable approach for the estimation of biokinetics and biological xenobiotic degradation rates that allow for identification of governing parameters such as microbial activity and active biomass. Results showed that biokinetics and degradation rates vary greatly between different plants. The fraction of active biomass on the total suspended solids ranged between 16.9 and 53.7%. Xenobiotic biodegradation rates correlated with microbial activity suggesting a relationship with WWTP performance for carbon and nutrient removal. The biokinetic information can be used to increase the prediction accuracy of xenobiotics removal by individual WWTPs.

Key words | activated sludge, active biomass, co-metabolism, respirometry, xenobiotics

INTRODUCTION
The fate of polar xenobiotics in the course of wastewater treatment has turned into a major issue of water research during the last decade. These micropollutants often pass wastewater treatment processes without being completely degraded. Their main degradation pathway was reported to be biodegradation during biological treatment (Thomas & Foster 2004).

In order to assess the fate of micropolllutants, biodegradation rates are usually obtained in lab-scale studies. However, their applicability remains limited to the investigated sludge and test conditions. The transfer of those biodegradation rates to other wastewater treatment plants (WWTPs) is not accurate. Moreover, biodegradation rates observed in standardized tests such as e.g. OECD procedures do not adequately account for prevailing conditions at full scale treatment like co-substrates, hydraulic and solids retention times. Additionally no biokinetic information about activated sludge such as growth rates, active biomass or respiration is considered.

The influent composition, hydraulic load and operational parameters as well as the design of WWTPs select for microorganisms best adapted to reproduce (Comeau 2008). As a consequence, activated sludge characteristics are expected to vary to large extends between different sites. This complicates to transfer biodegradation rates between WWTPs and the need to refine xenobiotic degradation experiments by considering biokinetics is apparent.

Activated sludge has recently been characterized with respirometric tests in a cost-effective and adequate manner (Young & Cowan 2004; Plattes et al. 2007). It enables the monitoring of the oxygen uptake rate (OUR) which is a measure for the microbial activity in oxidative substrate removal. Supporting this hypothesis, the maximum OUR was found to correlate with catalase activity in municipal activated sludges (Guwy et al. 1998). Modeling simulations of the OUR generated by defined substrate additions allow for the estimation of the most relevant biokinetic parameters (Koch et al. 2000).
Simultaneously, the biodegradation of three well known polar xenobiotics from the class of aminopolycarboxylic acids (APCAs) was tracked. They were chosen in this study because of their differences in biodegradability, broadly available data on biodegradation and widely spread occurrence in wastewater (Knepper 2003; Schmidt et al. 2004) They are extensively used in industrial applications and detergents. Nitrilotriacetic acid (NTA) is easily biodegradable under aerobic conditions. Diethylenetriaminepentaacetic acid (DTPA) is less biodegradable while ethylenediaminetetraacetic acid (EDTA) is known for its persistence and is barely eliminated during biological treatment (Bucheli-Witschel & Egli 2001). NTA, EDTA and DTPA strongly bind metal ions and are subsequently present as complexes. Their fate can significantly depend on the type of metal (Sillanpää et al. 2001). APCAs are non-volatile, very hydrophilic and not expected to adsorb on sludge solids (Alder et al. 1990).

The present study investigates the link between classical substrate degradation processes and the removal of APCAs using a combination of respirometry and biodegradation experiments. Biokinetic parameters of six Luxembourg WWTPs were investigated yielding reliable xenobiotic predictor qualities.

**METHODS**

**Sludge sampling**

Activated sludge grab samples were taken from aerated biological basins of six WWTPs and decanted into the respirometer vessel the same day. The sludge was brought to endogenous respiration over night but not longer than 14 h. Triplicate measurements of the total suspended solids (TSS) were measured with each sampling.

**Respirometer**

Respirometric experiments were conducted in a custom made 3 L closed bioreactor with a surrounding chamber for temperature control purchased from Ochs GmbH. The beaker was filled with 2.4 L of activated sludge sample. Agitation was provided by a magnetic stirrer and the liquid was assumed to be completely mixed. Temperature was maintained at 20 ± 1°C by means of a RM6 Lauda thermostat. A Metrohm GPD 751 Titrino served as pH control at 7.5 ± 0.2 during the experiment by addition of hydrochloric acid or sodium hydroxide. The sample was aerated using a Schego M2K3 air pump and two aeration stones. Dissolved oxygen (DO) limits were set to 3 and 6 mg O₂ L⁻¹, respectively. The DO concentration was measured with an oxygen sensor from Hach Lange LDO. The OUR was calculated from the depleting DO concentration in the non-aeration phase by moving window 10-point linear regressions. Automatic aeration and data acquisition were accomplished by a program written in LabVIEW (National Instruments). To inhibit nitrification, N-allylthiourea was added to a concentration of 10 mg L⁻¹ (Dircks et al. 1999). The input of atmospheric oxygen via the liquid surface was considered: K_La = 5.01 × 10⁻⁴ min⁻¹.

**Biokinetic parameter estimation**

Four parameters were chosen to be derived from experimental OURs: the maximum heterotrophic specific growth rate μ_max,h, the readily biodegradable substrate half-saturation coefficient K_S, the heterotrophic yield Y_H and the active heterotrophic biomass X_bh. The decay rate was set to default value and assumed to be constant in the course of the tests. Defined volumes (2–4.5 ml) of a 26 g L⁻¹ NaCH₃COO 3H₂O solution corresponding to 50–130 mg BOD L⁻¹ were injected before and after every biodegradation experiment into the respirometer to account for possible biokinetic changes during the experiment. The obtained OUR responses were simulated with Activated Sludge Model No.1 (ASM1) (Vanrolleghem et al. 1999) that is implemented in the wastewater treatment modeling software GPS-X from Hydromantis. The target parameters were estimated by minimizing the absolute difference between measured and simulated data (cf. Plattes et al. 2007). Heterotrophic yields could be manually calculated from the ratio of theoretical and experimental biological oxygen demand (BOD) by the following equation:

\[ Y_H = \frac{\text{BOD}_{\text{theo}} - \int \text{OUR}_{\text{ex}}}{\text{BOD}_{\text{theo}}} \]  

(1)
where $Y_H$ is the heterotrophic yield (g COD g COD$^{-1}$), BOD$_{theo}$ is the theoretical biological oxygen demand (mg L$^{-1}$) and OUR$_{ex}$ is the exogenous oxygen uptake rate (mgO$_2$ L$^{-1}$h$^{-1}$).

**Biodegradation experiments**

A mixture of EDTA, NTA and DTPA was added to the respirometer resulting in a concentration of 1 mg L$^{-1}$ for each substance. Sodium acetate, ammonium chloride and sodium dihydrogen phosphate monohydrate served as co-substrate with a C:N:P ratio of 100:5:1 as it is typical for municipal WWTPs (Janke et al. 2002). The ratio of xenobiotic to co-substrate was 1:550. The same (co-)substrates were used for all experiments. This provides the basis for a reliable comparability between the different sludges. The OUR was kept at its maximum during the period of the experiment by supplementary additions of co-substrate. Every 30 min a sample of 5 ml was drawn ($n = 11$), filtered and directly analyzed.

**APCA analysis**

NTA, EDTA and DTPA were analyzed using a Dionex HPLC System, consisting of an autosampler AS 40, an IP 20 isocratic pump and a Shimadzu SPD-10AV UV/Vis detector. A C$_{18}$ column (Hypersil 150 x 4; 5 µ HyPurity Elite C$_{18}$) was used. Acidic water (HNO$_3$) containing 7.4 mmol L$^{-1}$ of tetrabutylammoniumhydrogensulfate and 2.6 mmol L$^{-1}$ of tetrabutylammoniumhydroxide as ion pair reagents served as eluent with a flow of 0.9 ml min$^{-1}$. A chromatographic separation of the three compounds could be achieved with an isocratic eluent program. A precolumn derivatization was done by adding 40 µl of a 37 mmol L$^{-1}$ Fe(III)/130 mmol L$^{-1}$ TBAOH solution to 0.5 ml of sample volume to convert all APCAs into their Fe(III)-complexes. Samples were heated for 20 min at 60°C in a water bath and afterwards detected at 260 nm.

**RESULTS AND DISCUSSION**

**Variation of biokinetics in activated sludge**

The metabolic activities of the investigated sludges showed clear differences. Fast oxidative substrate removal resulted in a high maximum OUR, whereas less active microorganisms had a slower oxygen consumption (Figure 1). In all experiments the OUR could be reliably modeled using the ASM 1. Simulations were only accepted if their percentage of explained variation exceeded 85%.

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**Figure 1** | OUR response of two respirometry experiments with activated sludge from WWTPs Schifflange (left) and Mamer (right); generated by addition of sodium acetate.
The constantly decreasing phase at the end of the exogenous phase (Figure 1, right) is probably due to the production and transformation of storage polymers (Dircks et al. 1999) which is not considered in ASM 1.

Results showed that the fraction of active heterotrophic biomass $X_{bh}$ of TSS varied largely between the six investigated sludges (Table 1). It ranged from 16.9 to 53.7% where higher fractions of $X_{bh}$ promote low solids retention times (SRT). The very low fractions in WWTP Martelange and WWTP Boevange may be caused by their low BOD sludge loads (e.g. Boevange is operating at 20% capacity). No significant differences in half saturation coefficients and yields were visible. Heterotrophic yields corresponded to default values given in ASM 1 ($Y_H = 0.67$) and in the literature ($Y_H = 0.71$, Dircks et al. 1999). The maximum growth rates $m_{max,h}$ were found to range from 0.95 to 1.78 d$^{-1}$ and were located below defaults (3–6 d$^{-1}$). Nevertheless, they increased, even though only slightly, with increasing $X_{bh}$ and OUR.

In this context, the maximum OUR as an expression of microbial activity can be only regarded as a relative measure since it is substrate-specific. The absence of, for example, ammonium or phosphorus in preliminary tests resulted in a significantly lower maximum OUR than with a C:N:P mixed substrate. The OUR$\max$ can be used as an indicator for the rate of substrate uptake whereby the overall BOD removal remains constant.

In addition, activated sludge with high concentrations of active biomass seemed to promote also increased levels of activity per g $X_{bh}$. The enhanced metabolic activity may be caused by enzyme production, specialization or transport processes and access to energy sources. Since microorganisms are present as clustered flocs in WWTPs, the location of the active cells may also be decisive, especially in the presence of large amounts of inert solids.

### Xenobiotic biodegradation kinetics

Two model approaches, first-order (2) and pseudo first-order (3) reactions, were applied to fit data from biodegradation experiments (Table 2). APCAs are assumed to be degraded as secondary substrate and do not result in any significant microbial growth.

First-order kinetics are independent of the start concentration and biokinetics. They are used in comparison of activated sludge parameters and degradation rate constants in order to avoid auto-correlation effects with $X_{bh}$ (cf. Figure 3). In pseudo first-order kinetics, the transformation potential for xenobiotics can be attributed to the microorganisms present that are usually expressed in parameters of volatile or total suspended solids (VSS, TSS). The amount of active biomass can be used for a more adequate determination of the degradation rate constant $k_{biol}$ by substituting TSS or VSS in (3). However, this approach is limited to the assumptions that $X_{bh}$ as the main governing parameter remains constant in the course of the batch test. Variable levels of activity cannot be considered.

$$\text{firstorder: } \frac{\Delta C}{\Delta t} = -k_{biol} \cdot C_s$$

$$\text{pseudo – first order: } \frac{\Delta C}{\Delta t} = -k_{biol} \cdot X_{bh} \cdot C_s$$

where $C$ is the total compound concentration (mg L$^{-1}$), $t$ is the time (h), $k_{biol}$ is the degradation rate constant (h$^{-1}$ and L·g$X_{bh}^{-1}$·h$^{-1}$), $X_{bh}$ is the amount of active biomass (g L$^{-1}$).
and $C_s$ is the soluble compound concentration (mg L$^{-1}$). Naturally, the TSS is bigger than the amount of active biomass which, as a consequence, underestimates the actual degradation rate constants.

### Variation of APCAs biodegradation

NTA and DTPA show substantial variation within their $k_{biol}$. The pseudo first-order $k_{biol}$ of NTA in WWTP Mamer was more than one order of magnitude higher than in WWTP Reckange. The rate constants of DTPA were accordingly lower, but within the same dimension of variability. EDTA was of minor interest due to its persistence. Its $k_{biol}$ remained steadily less than 0.009 L g$^{-1}$ X$^{-1}$ h$^{-1}$ which is, however, more likely to be traced back to photolysis of Fe(III)EDTA. Pseudo first-order $k_{biols}$ obtained in respirometric batch tests were chosen to compare the absolute xenobiotics removal capacity of the six WWTPs (Figure 2). Half-lives ranged from 0.5 to 9 h for NTA and from 1.5 to 16 h for DTPA.

The complexing agents were added as sodium salts. It can be expected that Na$^+$ is substituted by Fe$^{3+}$ due to its high complexation constant and speciation kinetics. Fe(III)-complexes are likely to dominate since all investigated WWTPs use FeCl$_3$ for phosphorus precipitation upstream of biological treatment. As a matter of fact, it is improbable that significant variation of biodegradation is a result of differences in ligand speciation.

### Linking xenobiotic elimination to activated sludge activity

The principal hypothesis was that the biological removal capacity for APCAs is influenced or even governed by microbial activity. Starting from a critical specific OUR$_{max}$ $>70$ mgO$_2$ L$^{-1}$ h$^{-1}$, differences measured in microbial activity correlated with variable $k_{biols}$ of NTA and DTPA resulting in a positive relationship (Figure 3). Increased microbial activity seemed to promote faster xenobiotics removal. The level of enzyme production, which can be expected to increase with increasing activity, may be responsible for enhanced APCAs degradation. However, below this OUR no significant changes in $k_{biol}$ were observed, although NTA alone can be used as sole carbon source (Egli et al. 1990). EDTA (not shown) is not influenced at all.

Moreover, the influence of the activity appears to be substance specific. NTA is more strongly affected by changes in $k_{biol}$ compared to DTPA.

### Table 2

<table>
<thead>
<tr>
<th>WWTP</th>
<th>NTA Pseudo first-order</th>
<th>First-order</th>
<th>DTPA Pseudo first-order</th>
<th>First-order</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{biol}$</td>
<td>± error</td>
<td>$k_{biol}$</td>
<td>± error</td>
</tr>
<tr>
<td>Schifflange</td>
<td>0.197</td>
<td>0.032</td>
<td>0.185</td>
<td>0.030</td>
</tr>
<tr>
<td>Reckange</td>
<td>0.049</td>
<td>0.018</td>
<td>0.047</td>
<td>0.029</td>
</tr>
<tr>
<td>Petange</td>
<td>0.849</td>
<td>0.095</td>
<td>1.036</td>
<td>0.117</td>
</tr>
<tr>
<td>Martelange</td>
<td>0.084</td>
<td>0.017</td>
<td>0.106</td>
<td>0.014</td>
</tr>
<tr>
<td>Boevange</td>
<td>0.211</td>
<td>0.027</td>
<td>0.115</td>
<td>0.014</td>
</tr>
<tr>
<td>Mamer</td>
<td>1.238</td>
<td>0.179</td>
<td>2.050</td>
<td>0.293</td>
</tr>
</tbody>
</table>

$r^2 > 0.8$ for all fits.
in OUR\textsubscript{max} than DTPA. Also the ratios of $k_{\text{biol}}$ NTA/DTPA (calculated from Table 2) were observed to vary from 2.8 to 8.5. The value of WWTP Reckange (0.68) seems unrealistic, since a ratio $<1$ reveals that NTA is faster degraded than DTPA. Nonetheless, the $k_{\text{biol}}$ of both substances seem to increase after reaching the same critical OUR.

Results can be seen as a strong indicator for biodegradation of APCAs via co-metabolism as a governing process. The latter provides no significant advantage for microorganisms in terms of energy or catabolism (Rittmann 1992), but can be related to the presence of non-specific enzymes. Key enzymes involved in activation reactions of NTA breakdown, e.g. oxygenases, are very likely to occur in fairly high numbers of WWTPs (Bucheli-Witschel & Egli 2001; Yuan & VanBriesen 2008). However, investigating these requires their quantification and identification which cannot be achieved by means of this approach.

Hence, it is assumed that no occurrence of specialists in the microbial consortium is required for co-metabolic biodegradation. NTA and DTPA were clearly more effectively removed at low SRTs that are usually related to high microbial activities. In contrast, other authors observed higher biodegradation of xenobiotics, such as pharmaceuticals, at high SRTs (Clara et al. 2005). In this context, estimations about xenobiotic biodegradation and microbial behavior and consortium are difficult to derive from the SRT since it is only a hydraulic parameter.

Eventually, APCA degradation rates must be seen in the context of activated sludge quality. Here, the OUR\textsubscript{max} as a possible predictor variable is suitable to xenobiotics that follow co-metabolic degradation pathways. For the latter, the microbial activity is a central parameter and can be used to benchmark their degradation rates for individual WWTPs. It is suggested that xenobiotic elimination capacity has to be assessed individually for each plant according to its performance. At this point, results provided an approach for a model that is transferable between WWTPs with known biokinetics. Nevertheless, a broader statistical basis is required as well as the need to clarify under which conditions co-metabolic processes start to act. Moreover, the transfer of the biodegradation rates from batch tests to full scale WWTPs is still limited as long as no further investigations are made on co-metabolic processes especially in regard of inhibiting substances and available biodegradable co-substrates in real wastewaters.

**CONCLUSIONS**

Xenobiotic biodegradation experiments combined with respirometry could be forwarded as a reliable methodology for the estimation of biokinetics and APCAs degradation rates. Both were found to vary to a large extent between the six investigated different WWTPs. The variability of degradation rates could be explained by differences in microbial activity for the most part where the link to elimination of carbon and nutrients and thus co-metabolism is suggested. Increased activity resulted in faster acetate and also in enhanced xenobiotics removal. Activated sludge biokinetics allow for extrapolating the variable fate of APCAs to individual WWTPs of different design and performance.

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