Status quo report on wastewater treatment plant, receiving water’s biocoenosis and quality as basis for evaluation of large-scale ozonation process

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ABSTRACT

The project DemO3AC (demonstration of large-scale wastewater ozonation at the Aachen-Soers wastewater treatment plant, Germany) of the Eifel-Rur Waterboard contains the construction of a large-scale ozonation plant for advanced treatment of the entire 25 million m³/yr of wastewater passing through its largest wastewater treatment plant (WWTP). In dry periods, up to 70% of the receiving water consists of treated wastewater. Thus, it is expected that effects of ozonation on downstream water biocoenosis will become observable. Extensive monitoring of receiving water and the WWTP shows a severe pollution with micropollutants (already prior to WWTP inlet). (Eco-)Toxicological investigations showed increased toxicity at the inlet of the WWTP for all assays. However, endocrine-disrupting potential was also present at other sampling points at the WWTP and in the river and could not be eliminated sufficiently by the WWTP. Total cell counts at the WWTP are slightly below average. Investigations of antibiotic resistances show no increase after the WWTP outlet in the river. However, cells carrying antibiotic-resistant genes seem to be more stress resistant in general. Comparing investigations after implementation of ozonation should lead to an approximation of the correlation between micropollutants and water quality/biocoenosis and the effects that ozonation has on this matter.

Key words | advanced wastewater treatment, antibiotic resistances, ecotoxicity, EU Water Framework Directive, integrated approach, micropollutants

INTRODUCTION

Micropollutants have generated special attention over recent years and the investigation into elimination methods can be expected due to the required good ecological status for flowing waters according to EU Water Framework Directive (EU-WFD). Micropollutants are ubiquitous in our environment (Ternes 1998; Tixier et al. 2003) and of anthropogenic origin. They enter surface waters in a significant amount through wastewater treatment plants (WWTP). Proven techniques to eliminate micropollutants are ozonation and activated carbon treatment (Hollender et al. 2013; Margot et al. 2013; Altmann et al. 2014). Bioanalytical tools are highly recommended to evaluate the efficacy of the advanced treatment methods because adverse biological effects of wastewater compounds and metabolites can be identified using such methods (Maletz et al. 2013; Müller et al. 2016).

The Eifel-Rur Waterboard is building a large-scale ozonation plant for the elimination of micropollutants at its largest WWTP, the Aachen-Soers plant in the city of Aachen, Germany. The plant is designed to accommodate the combined sewage inflow, so that the entire 25 million m³/yr of wastewater will be treated by ozonation. This scale of wastewater treated via ozonation is unprecedented in Germany. To evaluate the effect that ozonation has on the receiving water’s biocoenosis, extensive monitoring of the plant and its receiving water, the River Wurm, is...
performed before and after implementation of ozonation. This paper gives an overview of the most important results of a status quo investigation before ozonation (phase 1) and includes analyses of wastewater related parameters, micropollutants, acute/chronic/ecotoxicity, total cell counts and antibiotic resistances as an integrated approach, comparable to Papa et al. (2016) and Schindler Wildhaber et al. (2015). Comparative investigations will be conducted subsequently (in phase 2) – after the installation of ozonation as an advanced treatment step. Final results could have significant impact on future investment and/or regulatory decisions and will be of international interest, if this treatment step becomes mandatory.

**MATERIAL AND METHODS**

The WWTP Aachen-Soers treats up to 25 million m³/yr of wastewater (458,300 inhabitant equivalents). It is equipped with activated sludge treatment and additional clearwater filtration. The receiving water is highly influenced by treated wastewater, especially since the Aachen-Soers plant is the largest plant discharging into the River Wurm. The River Wurm consists of up to 70% treated wastewater during dry periods. Samples were collected from July 2015 to July 2016, with a total of six samples collected during dry weather and nine samples during rainy weather conditions. A sampling event consisted of a parallel sampling of the WWTP over 24 hours and random samples from the receiving water. Sampling points are listed in Table 1.

Wastewater related parameters were analysed according to the respective analytical method shown in Table 2.

Micropollutants were analysed via high performance liquid chromatography/gas chromatography (HPLC/GC). For a detailed description, see Gebhardt & Schroeder (2007).

**Table 1** | Overview of sampling points within the project DemO₂AC

<table>
<thead>
<tr>
<th>Sampling point</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>WWTP inlet</td>
<td>Inlet of the WWTP Aachen-Soers</td>
</tr>
<tr>
<td>Outlet 2nd</td>
<td>Outlet of secondary clarifier = future inlet of ozonation</td>
</tr>
<tr>
<td>WWTP outlet</td>
<td>Outlet of the WWTP Aachen-Soers</td>
</tr>
<tr>
<td>W2 (Wurm 2)</td>
<td>River before WWTP and before stormwater tank</td>
</tr>
<tr>
<td>W3 (Wurm 3)</td>
<td>River before WWTP and after stormwater tank</td>
</tr>
<tr>
<td>W5 (Wurm 5)</td>
<td>River approx. 2.5 km downstream of WWTP outlet</td>
</tr>
</tbody>
</table>

**Table 2** | Wastewater related parameter analysis method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Analysis method</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD</td>
<td>DIN EN 1484</td>
</tr>
<tr>
<td>P, ortho-P</td>
<td>DIN EN ISO 6878</td>
</tr>
<tr>
<td>TN</td>
<td>DIN EN 12260</td>
</tr>
<tr>
<td>NH₄-N</td>
<td>DIN 38406-5-1</td>
</tr>
<tr>
<td>NO₃-N</td>
<td>Dr Lange cuvette test LCK 339 and 340</td>
</tr>
<tr>
<td>NO₂-N</td>
<td>DIN EN 26777 (ISO 6777)</td>
</tr>
<tr>
<td>FS</td>
<td>DIN 38409</td>
</tr>
</tbody>
</table>

Acute ecotoxicity testing was conducted via determination of the inhibition of mobility using *Daphnia magna* according to DIN EN ISO 6341 (2012) and through the determination of the inhibitory effect on the light emission of *Alivibrio fischeri* according to DIN EN ISO 11348-1 (2008) (Luminescent bacteria test). Chronic toxicity of *A. fischeri* was investigated in a cell multiplication inhibition test according to DIN 38412-L57 (1999) and fresh water algae growth inhibition was investigated with *Desmodesmus subspicatus* according to DIN EN ISO 8692 (2012). All samples were tested by dilution steps (G-levels) and data are reported as the most concentrated level (reciprocal value of the volume fraction of wastewater in the test mixture) without effect.

Ecotoxicity testing also includes fish embryo toxicity tests (FET) with *Danio rerio*, *in vitro* cytotoxicity tests, Lyticase-assisted Yeast Estrogenic Screen (L-YES) assays as well as ERα® CALUX assays, Ames fluctuation assays, the micronucleus (MN) assays, and MTT assays. Additionally, *in situ* feeding tests with *Gammarus pulex* were conducted in the River Wurm. FET tests (ISO 15088) and MN assays (ISO 21477-2) were conducted according to standard guidelines to assess the aquatic (eco-) toxicity of the samples. L-YES assays and ERα® CALUX assays were also performed in accordance to ISO draft guidelines and followed methods detailed in Maletz et al. (2013). Samples were tested with native water samples and/or with extracted samples. Results are given in percentage (%) or relative enrichment factor (REF), respectively. Feeding evaluations with gammarids (*G. pulex*) were conducted following methods outlined by Maltby et al. (2002) with minor modifications. Modifications to the *G. pulex* feeding tests included different exposure cages, longer incubation period (plus one day) and less individuals (20 compared to 30 gammarids).

Total colony counts in the water samples were determined with the most-probable-number (MPN) method according to DIN regulations for *Escherichia coli* (DIN...
EN ISO 9508-3 1999) and Enterococcus (DIN EN ISO 7899-1 1999) as selected indicator microbial groups. For the execution of the MPN method, different ready-to-use media-filled 96-well plates were used for E. coli and Enterococcus, MUG/EC well plates for E. coli and MUD/SF well plates for Enterococcus (both Bio-Rad, Germany). All individual samples were analysed in triplicates.

Further, the microbial indicator groups Enterococcus and Staphylococcus in the water samples were tested for antibiotic resistances to ampicillin, gentamycin, clarithromycin and vancomycin, while E. coli were also tested for antibiotic resistances to the first three antibiotics, and a sulfamethoxazole/trimethoprim combination instead of vancomycin (all antibiotics at 20 μg/ml concentration). For a quantitative estimation of resistances, different selective nutrient agar plates were used to select for the different microbial groups. For Enterococcus, Slanetz-Bartley agar (Carl Roth, Germany) was prepared, for E. coli the RAPID E. coli 2 agar (Bio-Rad, Germany) was used, and for Staphylococcus mannitol salt agar (Carl Roth, Germany) was used.

To obtain the percentage of antibiotic-resistant colonies of each microbial group, samples were microfiltered (VWR membrane filter, diameter 47 mm, pore size 0.45 μm) and plated with the spread plate method on the respective selective agar plates with and without antibiotics. After incubation at 37 °C (E. coli and Staphylococcus 12–18 h, Enterococcus 24–36 h), colonies were counted with an electronic colony counter pen and colony numbers of plates with antibiotic were normalized to colony numbers of plates without antibiotic.

RESULTS AND DISCUSSION

Wastewater characteristics at the Aachen-Soers WWTP showed chemical oxygen demand (COD) concentrations at the WWTP inlet of 638.6 ± 235 mg/l (arithmetic mean with standard deviation; n = 330). COD elimination from inlet to outlet amounts to ~99% (concentration WWTP outlet 2.91 ± 3.1 mg/l; n = 362). Dissolved organic carbon (DOC) concentrations at the WWTP inlet had a mean 107 ± 46.7 mg/l (n = 18) with an elimination of 95% (concentration WWTP outlet 5.9 ± 1.3 mg/l; n = 362). Filterable substances (FS) were held back in the filtration steps of the WWTP (~99.5%) leading to values <1.0 mg/l and in some cases even to a reduction in the receiving water. Nutrient removal (N_{tot} and P_{tot}) reached elimination rates of ~90%. In terms of phosphate, the WWTP revealed a positive impact on the River Wurm and even reduced the phosphate level in the river after the WWTP outlet. However, nitrogen compound concentrations in the river increased by the discharge of the WWTP. Nitrite concentrations at outlet 2nd (future inlet ozonation) were negligible. Nitrite serves as an ozone scavenger (Buffel et al. 2006) and, if present, has to be considered when calculating the ozone demand. Temperature in the river slightly rose after the WWTP outlet, which may have an impact on in situ testing. WWTP outlet has temperatures of ~19 °C during summer and ~15 °C during winter (n = 365). Temperatures in the River Wurm are shown in Table 3.

Regarding micropollutants, a wide spectrum of different substances was analysed, yet not all substances were found. Figure 1 shows all considered substances and their frequency, calculated as the quotient of the number of monitoring events and the number above limit of quantification (LOQ).

Micropollutant analyses showed mainly the presence of pharmaceuticals and plasticisers/phthalates. Individual substances of groups were present with a high frequency, e.g. bisphenol A (endocrine disrupting substances), iopamidol and iopromid (X-ray contrast media), galaxolid and tonalid (musk). However, other individual substances of the same group (e.g. muskabrette/musk, iomeprol/X-ray contrast media) differed strongly in their appearance. Of all 60 substances analysed, eight substances could not be found at all at any sampling point. Of the remaining 52 substances, 48 were found in the inlet water of the WWTP with a frequency of 25% or higher. The mean elimination of the found substances (n = 52) from the WWTP inlet to its outlet was about 61%. Mean eliminations for a selection of substances, calculated as the quotient of the mean inlet concentration and the mean outlet concentration, are shown in Figure 2. Results of elimination rates at WWTPs without an advanced treatment step for the elimination of micropol- lutants are in accordance with literature data, e.g. Carballa et al. (2004) or Verlicchi et al. (2012). Carbamazepine is excreted unmetabolized for only 1–2% and the negative eliminations in Figure 2 could be due to cleaving of the glucuronide-conjugates that may lead to an increase in the

<table>
<thead>
<tr>
<th>Average temperatures during summer [°C]</th>
<th>Average temperatures during winter [°C]</th>
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<tbody>
<tr>
<td>Before WWTP discharge, W2/W3</td>
<td>After WWTP discharge, W5</td>
</tr>
<tr>
<td>15.5</td>
<td>17.5</td>
</tr>
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</table>

Table 3 | Temperature profile of River Wurm influenced by WWTP discharge
environmental concentrations (Ternes 1998). Similar results for carbamazepine could be found in Ternes (1998) and Clara et al. (2005).

Analysing and comparing the sampling points along the River Wurm can reveal information about the WWTP’s impact on the receiving water and it also shows the river’s prior pollution. Figure 3 shows a selection of substances at the relevant sampling points W2 (before WWTP), WWTP outlet and W5 (after WWTP) as mean concentrations with maximum and minimum concentrations (whiskers). Freights of the WWTP outlet (calculated from mean concentrations) are shown as circles. An impact of the WWTP on the receiving water was present if the concentrations at W5 were higher compared to W2. From 52 substances considered (substances with concentrations always below LOQ are not shown), 28 substances appeared in higher mean concentrations after the WWTP than before (W5 > W2), highlighting the influence of the WWTP on the River Wurm. In contrast, for 19 substances with W2 > W5, prior pollution of the River Wurm became apparent, e.g. for ibuprofen, bisphenol A, different phthalates and in some samples tramadol and valsartan. The remaining five substances were present at the WWTP only and were fully eliminated during treatment. For some substances, the detected concentrations in the river were higher than predicted no effect concentration (PNEC) values, e.g. diclofenac, ibuprofen, in some samples also carbamazepine, clarithromycin, sotalol, sulfamethoxazole and naproxen. The prior pollution may be a result of the inlet of an upstream-located WWTP, which will be investigated in 2017.

The toxicity potential, both acute and chronic, was high in the inlet WWTP (inhibition mobility with *D. magna*, Luminescence bacteria with *A. fischeri* and algal growth with
D. subspicatus); however, toxicity was already reduced at sample point outlet 2nd. Only one sample demonstrated a slight increase (G2) in the toxicity potential through measured freshwater algal growth inhibition in the River Wurm.

Biotest results demonstrated embryo toxicity for both native samples and extracts at WWTP inlet. LC50 values were between 34% and 62% (n = 4) and 0.7 and 1.2 REF (n = 4) for native and extracted samples after 96 hpf (hours post fertilization), respectively. All other sampling sites in the WWTP and the River Wurm (native and extract) revealed no embryo toxic effects. The MTT assay did not result in any cytotoxicity except for the inlet of the WWTP, in which a slight reduction of the cell viability to a minimum viability of 80% was determined.

The MN assay indicated no genotoxic impact by the WWTP or the River Wurm. The results obtained have shown that all measured MN rates were less than 3%. This percentage is set as the threshold for micronuclei formation as it is the highest percentage that can be measured in the negative control according to ISO 21427-2 (2006). No mutagenic effects were observed in any sample of the River Wurm (NOEC ≥ 8.3 REF) as indicated by the Ames fluctuation assay. However, significant mutagenicity in the WWTP outlet (LOEC: 8.3 REF) was detected during the first sampling campaign using the strain TA 98 without metabolic activation. No mutagenicity could be detected downstream of the WWTP through either mutagenicity test.

The endocrine evaluations via L-YES and ERα® CALUX assays demonstrated endocrine-disrupting activity in inlet WWTP samples and, to a lesser extent, in outlet 2nd and outlet samples. Endocrine-disrupting activity was detected in the River Wurm through the ERα® CALUX assay, carried out with 2.5-fold concentrated samples, as well as in the L-YES assay, tested with 17-fold concentrated samples. The different enrichment factors were used due to the different sensitivities of the applied bioassays. With the test design, a worst-case scenario was investigated using the extracts for analyses. However, native samples will also be investigated within the project to compare the results to the extract data.

Feeding experiments with G. pulex showed no significant difference in feeding rate between the sampling sites upstream and downstream of the WWTP. Hence, the discharged wastewater had no influence on the feeding activity during the periods of deployment and observed variations. This is in contrast to former studies carried out in Switzerland and Germany (Hollender et al. 2009; Bundschuh & Schulz 2011; Bundschuh et al. 2011; Englert et al. 2015). Statistical analyses indicate temperature as a main influence on feeding rates, which will be investigated further by attaching temperature loggers to the setup for all future experiments, as this impact was demonstrated in former studies (Nilsson 1974).

In terms of total colony counts, data from three independent dry and rain weather screenings, respectively, showed
no significant influence of the WWTP effluent on the River Wurm regarding the counts of *E. coli* and *Enterococcus* (Figure 4). In addition, there is no significant difference in cell counts for one sampling site between the dry and the rain weather screening data. For *E. coli*, values at sampling points W2, W3 and W5 were between $5.0 \times 10^2$ and $1.35 \times 10^3$ cfu/ml. Values at the inlet of the WWTP (inlet WWTP) reached up to $1.90 \times 10^7$ cfu/ml. During the treatment stages, a steady decrease of cell counts was apparent, with the outlet of the secondary clarifier (outlet 2nd) showing up to $1.62 \times 10^5$ cfu/ml and the final outlet (outlet WWTP) only up to $9.33 \times 10^3$ cfu/ml. For *Enterococcus*, values at sampling points W2, W3 and W5 were between $2.82 \times 10^1$ and $4.90 \times 10^2$ cfu/ml. Values at the inlet WWTP were up to $8.32 \times 10^6$ cfu/ml. There is also a steady decrease of colony counts during the different treatment stages, with the outlet 2nd up to $5.50 \times 10^3$ cfu/ml and at the outlet WWTP up to $5.50 \times 10^2$ cfu/ml. A literature review from studies with similar river sampling points to our sites W2 and W5 (i.e. river analysis before and after WWTP influent) showed colony counts for *E. coli* of $1.0 \times 10^5$–$1.0 \times 10^3$ cfu/ml and for *Enterococcus* of $1.0 \times 10^6$–$5.0 \times 10^3$ cfu/ml (Vilanova et al. 2002; Abegglen et al. 2009; Gasse 2009). The cell counts before and after the influent of the WWTP Aachen-Soers effluent therefore lie in the lower range of similar environmental situations.

Antibiotic resistances to clarithromycin, ampicillin, gentamycin and sulfamethoxazole/trimethoprim were analysed for *Staphylococci*, *Enterococci* and *E. coli* at the different sampling points of this study, for rain and dry weather conditions, respectively. Except for *Staphylococcus*, no differences in resistance frequencies were detected for the rain or dry weather samples. For *Staphylococcus*, we observed increased resistances to gentamycin during dry weather sampling in spring with 20–50% resistant colony counts in the River Wurm and ~10% resistances in the WWTP. Since gentamycin is a major prescribed antibiotic in cattle farming, this might be explained by manure spreading activities and cattle pasture grazing on surrounding agricultural land areas especially in the spring. Other than that, *Staphylococci* did not show any resistances to the other tested antibiotics.

Figure 5 summarizes the results of the dry weather screening for the river is sampling before and after the WWTP (W2 vs. W5) and the WWTP inlet and outlet for *Enterococcus* and *E. coli*. Resistance data for ampicillin are not shown explicitly, but agree well with known natural resistance levels to this antibiotic (with <10% resistances in the river and ~10% resistances in the WWTP for *Enterococcus* and 10–40% resistances across all sampling points for *E. coli*). It is known that the natural sensitivity of *E. coli* to ampicillin is lower than 60% (Solbach 2013) and that about 50% of the *E. coli* strains carry β-lactamase TEM-1, which can cleave the β-lactam ring of the antibiotic (Sanders & Sanders 1992). There was no significant difference between the River Wurm and the WWTP samples for the other antibiotics (Figure 5). Previously reported data showed that the sensitivity of *Enterococcus* to gentamycin is generally lower than 60% (Solbach 2013). Accordingly, 55% of the *E. faecalis* strains isolated in the USA are described as highly resistant to gentamycin (Rüden & Edmond 1998). Our results confirm this widespread natural resistance of *Enterococcus* to gentamycin, while *E. coli* showed no resistance to this antibiotic. Observed resistances to clarithromycin on the other hand were higher for *E. coli* than for *Enterococcus*. Here, it is known that the mode of action of clarithromycin is not specific for coliform bacteria and limited activity of clarithromycin against *E. coli* was
therefore expected (Jacks et al. 2003). *E. coli* resistances to sulfamethoxazole/trimethoprim (5:1) were around or below 40%, while *Enterococcus* resistances to vancomycin were mostly below 5%.

For several antibiotics, a trend for a slight increase in resistance levels (albeit not statistically significant with the given sample number) from the WWTP inlet to the outlet was observed (Figure 5), although the total cell counts drastically decreased during the treatment process (Figure 4). This result might reflect the fact that stress-tolerant organisms, which sustain the nutrient-deprived later stages of the WWTP, are also more likely to carry resistances.

**CONCLUSIONS**

The Aachen-Soers WWTP eliminated nutrients, organics and solids to a high degree, while micropollutants were eliminated to an average-to-moderate degree. The loads of micropollutants in the wastewater and also in the River Wurm were significant. However, no significant potential for acute or chronic toxic effects were detected at sampling points other than at the WWTP inlet. Similarly, the results among the ecotoxicity assays were consistent, but effects were partially present after outlet 2nd (endocrine-disrupting potential). One sample also showed mutagenic potential effects for the outlet of the WWTP. Feeding tests will be investigated according to their correlation to temperature. An influence of the WWTP could not be demonstrated for the feeding test with *G. pulex*. Total colony counts at the WWTP were, compared to other investigations, slightly below average and the WWTP had no significant influence on the total colony count of the River Wurm. Antibiotic resistances were mostly in the range of expected natural resistances, with the exception of *Staphylococcus* resistance to gentamycin in the spring. This might indicate seasonal agricultural pollution in the River Wurm. The trend for a higher fraction of antibiotic-resistant *Enterococcus* after outlet 2nd compared to inlet WWTP might reflect the fact that stress-tolerant organisms, which sustain the nutrient-deprived later stages of the WWTP, are also more likely to carry resistances. However, overall, a successful reduction of microbial contaminants carrying antibiotic resistances occurred in the WWTP. Phase 2 of the DemO3AC project (approx. 2018–2019) will contain comparative studies investigating the situation after implementation of full-scale ozonation. This may lead to an approximation of the correlation between micropollutants and water quality/biocoenosis and clarify the impact that ozonation has on this matter.
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First received 20 March 2017; accepted in revised form 13 October 2017. Available online 27 October 2017.