CWPharma

CLEAR WATERS FROM PHARMACEUTICALS

# Impact of ozonation and post-treatment on ecotoxicological endpoints, water quality, APIs and transformation products

## GoA3.3: Comparison of post-treatment options

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KALUNDBORG UTILITY

**Authors/Contributors:**
Stapf, Michael (GoA3.3-lead; KWB – Berlin Centre of Competence for Water)
Miehe, Ulf (KWB)
Knoche, Franziska (KWB)
Lukas, Marcus (UBA – German Environment Agency)
Bartz, Jennifer (UBA)
Brauer, Frank (UBA)
Gutsche, Martina (UBA)
Kullwatz, Jan (UBA)
Petkow, Christo (UBA)
Schneider, Margot (UBA)
Winckelmann, Dagmar (UBA)
Bogusz, Aleksandra (IOS, Institute of Environmental Protection – National Research Institute)
Tomczyk, Beata (IOS)
Trzcińska, Magdalena (IOS)
Dworak, Anna (IOS)
Chojniak-Gronek, Joanna (IOS)
Szumska, Marlena (IOS)
Zieliński, Marcin (IOS)
Walkowiak, Radosław (IOS)
Putna-Nimane, Ieva (LIAE – Latvian Institute of Aquatic Ecology)
Liepina-Leimane, Ineta (LIAE)
Dzintare, Laura (LIAE)
Barda, Ieva (LIAE)
Bester, Kai (AU - Aarhus University)
Kharel, Suman (AU)
Sehlén, Robert (TVAB – Tekniska verken Linköping AB)
Nilsson Josefin (TVAB)
Larsen, Sille Bendix (Kalfor – Kalundborg Utility)

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**Chapter contributions (institution):**

- Introduction (KWB)
- Site overview (KWB, TVAB, Kalfor)
- Overview on conducted ecotoxicological tests (KWB, UBA)
- Sampling and SPE procedure (KWB, UBA)
- Description/Results of Neurotoxicity (IOS)
- Description/Results of Mutagenicity (IOS, UBA)
- Description/Results of Genotoxicity (IOS, UBA)
- Description/Results of Estro-/Androgenicity (IOS, UBA)
- Description/Results of bacteria tests (IOS)
- Description/Results of chronic tests (LIAE)
- Summary and recommendations for ecotoxicological assessment (UBA, IOS, LIAE)
- Water quality parameters (KWB, TVAB, Kalfor)
- APIs and transformation products (KWB, AU)
- Summary and recommendations (KWB)

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# Table of content

# Introduction

The overall aim of the CWPharma project is to reduce the load of active pharmaceutical ingredients (APIs) going into the aquatic environment and especially the Baltic Sea. Municipal wastewater treatment plants (WWTPs) are relevant point sources of APIs, as they treat the wastewater from public households, hospitals and industry of the connected catchment area. However, conventional "state-of-the-art" WWTPs can only remove some APIs, which are either easily biodegradable and/or absorbable to activated sludge, whereas other APIs can pass the WWTP with minor to no reduction. Therefore, reduction of a broad range of APIs can only be achieved by using targeted advanced treatment techniques such as ozonation or powdered and granular activated carbon, respectively, which have already been applied on full-scale for API removal in wastewater treatment in Germany and Switzerland and proven their practical and economical suitability.

At the usual applied ozone doses, ozonation of secondary effluent does not mineralize (convert an organic substance into inorganic matter) but transforms organic compounds into smaller and (usually) more biodegradable compounds. Secondary effluent is a complex water matrix consisting of hundreds of different organic substances, and it is not feasible to determine all possible transformation products and oxidation by-products, which might be created by the ozonation process. Thus, utilities and water authorities sometimes struggle with the uncertainties of the ozonation process as they perceive difficulties to judge whether oxidation of the organic matrix is beneficial or if it is creating more problems. As chemical analysis of the water only provides quantitative data for known APIs and transformation products for which chemical standards are available, effect-based ecotoxicological test systems can be used to assess the integrated actual toxicity of the whole water matrix. Based on previous research compiled by Völker et al. (2019), ozonation has a positive impact on several toxicological endpoints. But there are also indications that ozonation can create negative effects for a few toxicological endpoints that can be reduced by a suitable post-treatment. However, only little knowledge is available regarding suitable post-treatments and which ecotoxicological test systems are appropriate to evaluate their impact. In addition, post-treatment options might also have beneficial impacts on water quality parameters, APIs and transformation products.

Thus, this report will evaluate different aspects regarding the impact of ozonation and its post-treatment options on (i) water quality parameters, (ii) APIs and transformation products (TPs) and (iii) ecotoxicological effects. The evaluation was conducted at three WWTPs in Linköping (SE), Kalundborg (DK) and Berlin (DE) and different post-treatment options such as moving bed bioreactors (MBBR), deep-bed filters, and a constructed wetland.

# Site overview

Impact of ozonation and the combination of ozonation and post-treatment, respectively, was evaluated at wastewater treatment plants in Linköping (full-scale, Sweden), Kalundborg (full-scale, Denmark), and Berlin (pilot-scale, Berlin). Within this report, only a brief description of the WWTP and ozonation system for WWTP Linköping and Kalundborg will be provided as more details can be found in the reports of GoA3.1 and GoA3.2, respectively.

**WWTP Linköping**: The full-scale ozonation plant is located at the municipal WWTP Nykvarnsverket in Linköping (Q = 40,000 $m^3/d$ at dry weather conditions). The treatment process at Nykvarnsverket consists of mechanical pre-treatment with screens, aerated grit chamber, pre-aeration and primary clarifier. Biological treatment with CAS (intermittent aeration) is followed by the ozonation plant using a MBBR with tertiary sedimentation as post-treatment.

The ozonation plant can treat up to 3,000 $m^3/h$ of secondary effluent with a maximum ozone production of 20 kg $O_3/h$. Ozone is produced by a CFV30 ozone generator (Ozonia) using liquid

oxygen and mixed into a side stream from the secondary effluent via a ventury injector system. This ozone enriched water steam is introduced into the first camber of the ozone reactor by a radial diffuser. The ozone reactor consists of a series of chambers with a total reactor volume of 524 $m^3$, maintaining a HRT between 10 and 30 min depending on the flow. At the yearly average flow of 1700 $m^3/h$ HRT is 18 min. Usually, a water flow proportional constant ozone dosage was used, e.g. 8 mg $O_3/L$ during the sampling campaigns for the ecotoxicological tests. The MBBR plant consists of three parallel lines, each with four reactors in series. The MBBR stage has a total volume of 2,220 $m^3$ with a filling ratio of 39%, resulting in about 600 million carriers contributing to a surface of 520,000 $m^2$ (70% HXF12KLL carriers and 30% K1 carriers). Since the MBBR is placed directly after the ozone reactor, the oxygen concentration is high in the first MBBR reactors. Generally, nitrification and other oxygen-consuming reactions occur in the two first reactors in each line. When the phosphate concentration in the secondary effluent is low, phosphoric acid is added in the inlet to supply phosphorus for the nitrification bacteria. Denitrification occurs in the two last reactors and ethanol is added as carbon source. Aluminum chloride is added directly after the last chambers in MBBR (before the sampling point) to precipitate remaining phosphorous and to flocculate suspended solids.

**WWTP Kalundborg**: Kalundborg municipal wastewater treatment plant is located in the far western part of Zealand, Denmark. The WWTP is placed next to a deep-water harbour, hence the area is characterized by an immense amount of heavy industries, hereunder biotech industries, power plants, oil refineries and several smaller industries, which are all influencing the composition of the wastewater entering the treatment plant. The WWTP has a dry-weather flow of approximately 16,000 $m^3/d$, corresponding to an organic loading of approximately 40,000 PE. At the WWTP, wastewater first passes the mechanical pre-treatment consisting of grids, sand and grease removal. After the primary treatment the water is directed to the biological part of the plant. The biological part consists of alternating ditches (classical BIODENIPHO plant) with a side stream hydrolysis for sludge hydrolysis. After the biological stage, iron chloride is added for simultaneous phosphorous precipitation. Effluent of the secondary clarifiers can be directed to the tertiary treatment, consisting of an ozone plant with a MBBR post-treatment stage, or led directly to the recipient.

The ozonation plant was originally designed for reduction of COD (chemical oxygen demand). The plant consists of two parallel lanes and can treat up to 1,200 $m^3/h$. Ozone is produced by two ozone generators that are coupled individually to each line. Each generator can produce between 7.2 and 90 $kgO_3/h$. Thus, ozone doses between 12 and 150 $mgO_3/L$ are possible that, when normalized for the content of DOC (dissolved organic carbon), correspond to a specific ozone dose between of 0.75 and 9.4 $mgO_3$/mg DOC. Within CWPharma, liquid oxygen (LOX, 3 x 50 $m^3$ tanks) was used as oxygen supply of the ozone generators and the ozone was injected via a ventury system. Ozone reaction took place in three 50-$m^3$ tanks operated in series (150 $m^3$ reactor volume per lane), providing a minimum hydraulic retention time of 15 minutes. Automatic process control can be used to define a setpoint (constant ozone dose) of the flow-proportional ozone dosage. The post-treatment consists of a MBBR with a total volume of 1,200 $m^3$ filled to 25 V-% with Kaldnes K1 carriers (600 $m^3/t$ surface area). Total reactor volume is divided into four zones which can be operated aerated or non-aerated. It is possible to adjust the pH and add ethanol as a COD source, however, both options were not used during CWPharma project.

**WWTP Schönerlinde (Berlin):** The pilot plant is located at a municipal WWTP Schönerlinde (Q = 105,000 $m^3/d$ at dry weather conditions), which is designed as a conventional 'state-of-the-art' WWTP with mechanical pre-treatment, aerated sand/grease trap and primary clarifier, followed by a CAS with pre-denitrification and biological/chemical P-removal. The pilot-plant is used for an advanced treatment of the secondary effluent of the full-scale WWTP and consists of an ozonation unit ($Q_{max}$ = 15 $m^3/h$) and several post-treatment options such as a constructed wetland and several deep-bed filters. The ozone generator (GSO40, Xylem Inc.) is fed with technical oxygen, provided by three oxygen concentrator units (Topaz Ultra, Chart Industries Inc.). The produced ozone is applied to a recirculation side-stream via a ventury injection system, which is then mixed with the main-stream. The ozone reaction takes place in two reactor

tanks in series (2 m³, each), which maintain a hydraulic retention time of more than 15 minutes. As the ozonation plant is within a container, reactor tanks are limited in height (h ≈ 2 m) and, thus, gas-transfer efficiency is comparable low (η ≈ 85%). Thus, applied ozone dose is calculated based on an ozone-mass balance taking into account the gas-flow and the ozone concentration in the product- and offgas, respectively. Ozone dose is adapted by a closed-loop process control, using the relative reduction of the $UVA_{254}$ ($\Delta UVA_{254}$) between the influent and effluent of the ozonation determined by $UVA_{254}$ online sensors (i::scan, s::can inc.). The operational $\Delta UVA_{254}$ setpoint was 47%, which correlates to 0.7 mgO3/mgDOC. Pilot-scale ozonation plant and the used process control are described in more detail in Stapf et al. (2016).

The deep-bed filters of the post-treatment were identically designed columns ($Ø_{inner}$ = 0.3 m) filled with different filter material: sand/anthracite filter (S/A) consisted of 0.6 m filter sand (0.7 – 1.25 mm) and 1.2 m hydro-hydroanthricite (1.4 – 2.5 mm). The sand/BAC filter (BAC = biological activated carbon) had the same layers than the S/A-filter, but used granular activated carbon (GAC, 1.4 - 2.4 mm, AquaSorb 2000, Jacobi) instead of the anthracite. The GAC-filter was operated subsequent of the S/A-filter and had only a single layer of 1.8 m AquaSorb 2000. The distinction of BAC- and GAC-filter is not based on the used filter material (as it is here the same) but in the residual adsorption capacity of the activated carbon. Each GAC-filter will eventually become a BAC filter when the activated carbon is not exchanged occasionally and biological processes dominate the treatment process. However, there is no clear criteria when a GAC-filter turns into a BAC-filter. Even though most organic compounds are not adsorbed anymore, still some reduction can be observed for well adsorbable compounds such as benzotriazole. At the Berlin pilot-plant, GAC-filter has treated about 9,800 to 17,000 bed volumes (BV) during the conducted sampling campaigns, whereas the S/A- and S/BAC-filter have treated 51,000 - 69,000 BV and 55,000 - 73,000, respectively. If not stated otherwise, S/BAC and S/A filter were operated as coagulation filters (dosage of 1.8 mg Fe/L, ferric chloride) at a filter velocity of about 5 m/h, representing an EBCT (empty bed contact time) of about 16 minutes. Due to operational boundary conditions, filter velocity of the post-GAC-filter was lower (v = 3.6 m/h) and EBCT was 30 min. Filter backwash was conducted each weekday with air (60 m/h, 2 min) and then with water (60 m/h, 8 min). The constructed wetland is in operation since May 2017 and consists of a 0.55 m sand layer above a 0.03 m layer of lava gravel. The 11 m² surface of the constructed wetland is covered by hydrophytes (starting with ½ *Phragmites australis* and ½ *Carex acutiformis*, 8 pc/m²). The wetland was always operated saturated (0.3 m supernatant) and was fed discontinuously with 400 mm/d (HRT ≈ 24 h) at the sampling campaigns. More details regarding the constructed wetland can be found in (Brunsch et al., 2019).

## Sampling points

A schematic overview of the sampling points at the three sites is presented in Figure 1. At the WWTPs Linköping and Kalundborg, samples were taken at the influent and effluent of the ozonation and the effluent of the according moving bed bioreactors (MBBR).

In Linköping, time-proportional 24-h samples were collected at the ozonation influent by an automatic sampler. At the ozonation effluent and MBBR effluent, flow-proportional 24-h samples were collected by cooled automatic samplers.

At the Kalundborg site, two types of samples were collected: at the effluent of the secondary clarifiers (ozonation influent) samples were collected with a flow-proportional sampler, whereas samples from the ozonation effluent and MBBR effluent were collected with a time-proportional sampler. The automatic samplers had a cooling system in order to chill the samples during the 24 hours sampling process.

At the Berlin site, time-proportional 24-h composite samples were taken by cooled automatic samplers at the influent and effluent of the ozonation as well as at the effluent of the constructed wetland. At the effluent of the three deep-bed filters (S/BAC-, S/A- and GAC-filter) samples were continuously taken by a multi-channel peristaltic pump and sampling containers were placed in insulated boxes.

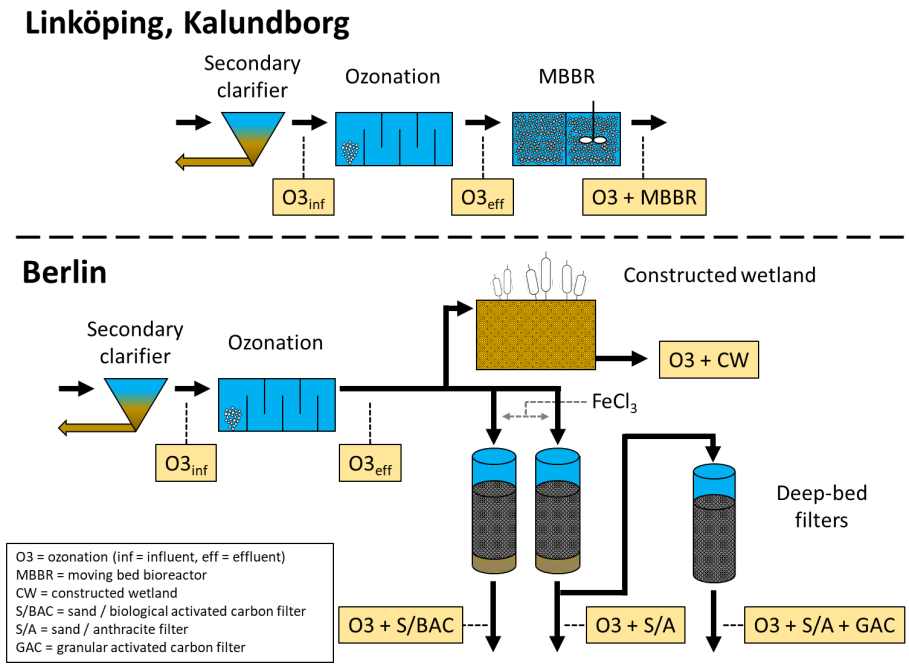


*Figure 1: Schematic overview of sampling points at WWTPs Linköping (SE), Kalundborg (DK) and Berlin (DE).*

## Water quality at the ozonation influent

A comparison of the water quality parameters at the ozonation influent reveals a broad variation at the three investigated sites (see Table 1). The level of organic substances in the water can be measured as dissolved organic carbon (DOC), whereas its corresponding aromatic character (amount of aromatic bonds of the DOC) can be estimated by normalizing the $UVA_{254}$ for DOC to determine the specific UV absorbance (SUVA). Probably due to the high share of industrial wastewater, highest level of DOC (16.7 mg/L) was detected at WWTP Kalundborg, followed by WWTP Linköping (12.1 mg/L) and WWTP Berlin (9.9 mg/L). Also, SUVA was highest for Kalundborg with an average of 3.17 L/(mg * m), whereas SUVA and, thus, aromatic character of the water, was quite similar at the other two WWTPs. Regarding the COD, WWTPs Berlin and Linköping had similar levels of 33.5 mg/L and 36.6 mg/L, respectively, whereas at Kalundborg COD level was more than 40% higher. Regarding the nitrogen species, very low levels of ammonium and nitrite were present at the ozonation influent at Berlin and Kalundborg indicating a very good nitrogen removal. In Kalundborg also nitrate levels are very low (1.7 mg-N/L). In contrast and due to the intermittent aeration at the CAS, ozonation influent of WWTP Linköping contained comparable high ammonium and nitrite levels of 6.6 mg-N/L and 0.7 mg-N/L, respectively. Both parameters show large annual variation and would usually be reduced by the nitrification/denitrification stage of the MBBR system. However, as the MBBR is used as ozonation post-treatment, present nitrite will be completely transformed to nitrate causing an additional ozone demand of 3.4 mg-$O_3$/mg-N. The very high bromide levels at WWTP Kalundborg of 2.14 mg/L pose a serious risk for bromate formation and are caused by the bromide load within the biotech industry wastewater and sea water intrusion into the municipal sewer system. In contrast, bromide levels at WWTP Berlin about 0.14 mg/L and pose a slight risk of bromate formation, whereas the LOQ of 1 mg/L prevents an estimation of the potential bromate formation as it is recommended to have a detailed look at the bromate formation if bromide levels are than 0.15 mg/L.

*Table 1: Overview on water quality parameters (average +/- standard deviation) measured at the influent of the ozonation at the WWTPs Berlin, Kalundborg and Linköping.*

| Parameter | Unit | Berlin | Kalundborg | Linköping |
|---|---|---|---|---|
| DOC | mg/L | 9.9 ± 1.0 (n = 37) | 16.7 ± 2.4 (n = 8) | 12.1 ± 1.6 (n = 35) |
| $UVA_{254}$ | 1/m | 25.3 ± 2.7 (n = 37) | 52.5 ± 9.6 (n = 10) | 30.1 ± 4.9 (n = 36) |
| SUVA | L / (mg * m) | 2.56 ± 0.24 (n = 37) | 3.17 ± 0.23 (n = 7) | 2.49 ± 0.39 (n = 35) |
| COD | mg/L | 33.5 ± 5.1 (n = 36) | 48.2 ± 5.9 (n = 18) | 36.9 ± 13.9 (n = 31) |
| Suspended solids | mg/L | 6.9 ± 3.9 (n = 22) | 6.5 ± 7 (n = 27) | 23.3 ± 12.0 (n = 18) |
| Ammonium | mg-N/L | 0.24 ± 0.32 (n = 36) | 0.39 ± 0.51 (n = 15) | 6.6 (n = 68) |
| Nitrite | mg-N/L | 0.17 ± 0.18 (n = 37) | < 0.05 (n = 14) | 0.72 ± 0.25 (n = 36) |
| Nitrate | mg-N/L | 11.9 ± 2.5 (n = 36) | 1.72 ± 0.80 (n = 21) | 10.7 ± 2.2 (n = 19) |
| Total phosphorus | mg/L | 0.73 ± 0.28 (n = 12) | 0.34 ± 0.07 (n = 11) | 0.69 (n = 44) |
| Bromide | mg/L | ≈ 0.14 (n = 35) | 2.14 ± 0.44 (n = 12) | < 1.0 (n = 9) |
| pH | - | 7.6 ± 0.2 (n = 9) | 7.8 ± 0.1 (online) | 7.3 ± 0.1 (n = 9) |
| Temperature | °C | 17.2 ± 3.8 (n = 29) | 15 - 30 (online) | 19 ± 4 (online) |

# Ecotoxicological assessment

## Overview on conducted ecotoxicological tests

In total, 17 ecotoxicological tests were performed in laboratories in Germany (UBA), Latvia (LIAE) and Poland (IOS) (Table 2). The used test systems cover a broad range of toxicological endpoints such as neurotoxicity, mutagenicity, genotoxicity, endocrine effects, growth and reproduction. While the tests at LIAE were performed with native samples, tests at UBA and IOS were performed with enriched samples based on extracts from a solid phase extraction (SPE) using dimethyl sulfoxide (DMSO) or ethanol (EtOH) as solvent.

*Table 2: Overview of evaluated ecotoxicological test systems and toxicological endpoints.*

| Sample | SPE -Solvent | Test system | Toxicological endpoint | Lab. |
|---|---|---|---|---|
| **SPE extract** | EtOH | Acetylcholinesterase inhibition | Neurotoxicity | IOS |
| | DMSO | Ames (TA1535 + TA1537, +/-S9) | Mutagenicity | |
| | DMSO | Pseudomonas putida | Growth inhibition | |
| | DMSO | SOS Chromotest | Genotoxicity | |
| | DMSO | Aliivibrio fischeri | Bioluminescence inhibition | |
| | DMSO | (anti-)YES/YAS | Estrogenicity / androgenicity | |
| | DMSO | Ames (YG7108, +/-S9) | Mutagenicity | UBA |
| | DMSO | ER-Calux | Estrogenicity | |
| | DMSO | UmuC | Genotoxicity | |
| | EtOH | YES/Anti-YES | Estrogenicity / anti-estrogenicity | |
| **native** | - | Algae growth test | Growth inhibition | LIAE |
| | - | Chronic reproduction | Reproduction | |

## Sampling and SPE procedure

In total, five sampling campaigns were conducted in Berlin (BLN), three in Linköping (LIN) and Kalundborg (KAL), respectively (see Table 3). At the first sampling campaign in Berlin (25.02.2019) a "field blank" sample (uninfluenced tap water from a suburban Berlin village) was taken instead of a sample in the effluent of the constructed wetland. The field blank served as negative control to verify that no toxic effects (e.g. substance leeching into the sample) was introduced to the sample extract by the SPE procedure. In addition, only the frozen samples taken on 24.06.2019 at WWTP Kalundborg (KAL IIa) could be processed by LIAE as the samples intended for the SPE extraction arrived too warm at UBA and had to be discarded. Therefore, sampling was repeated on 09.09.2019 (KAL II), but now samples were only evaluated at UBA and IOS and not at LIAE. Therefore, results of the sampling campaigns KAL IIa and KAL II do not correspond to each other.

For the sampling campaigns, the ozonation plants were supposed to operate at a (nitrite-corrected) specific ozone dose of 0.7 mgO3/mgDOC ($E_{DOC,corr}$). Therefore, Linköping used a constant ozone dose of 8 $mgO_3$/L, whereas in Kalundborg a constant ozone dose of 12 $mgO_3$/L was targeted. During the KAL III sampling campaign, some short-term unintended reductions of the flow at the ozonation occurred, which resulted in an overall increased average ozone dose of 25 $mgO_3$/L as ozone production capacity could not be reduced any further. In Berlin, the ozone dose was automatically adapted to achieve a stable $UVA_{254}$ reduction determined by two online sensors. As a result, ozone dose usually varied between 8 to 10 $mgO_3$/L at the sampling campaigns. Due to a disturbance of the biological process during the BLN I sampling campaign, secondary effluent (ozonation influent) had unusual high nitrite and DOC levels. Due to a loose hose of the automatic sampler at the ozonation influent, sample was taken at a spot where the water was not mixed well and was strongly influenced by the effluent of another treatment train of the WWTP. Therefore, for BLN I sampling campaign, influent ($O3_{inf}$) and effluent ($O3_{eff}$) sample do not directly correspond. Thus, nitrite-corrected specific ozone dose was estimated based on the $UVA_{254}$ reduction determined by the two online sensors. The evaluation revealed that even the very high ozone dose of 17 $mgO_3$/L resulted only in a low specific ozone dose of

0.32 $mgO_3$/mgDOC, which is in line with the low API elimination observed at this sampling campaign.

*Table 3: Overview of samples taken at the different sites. At the first sampling campaign in Berlin, a "field blank" was analyzed instead of the "O3+CW" sample. Also sample KAL IIa was only analyzed at LIAE, whereas KAL II was only analyzed at UBA and IOS.*

| Sampling day (start) | Site | # | $E_{DOC,corr}$ [$mgO_3$/mgDOC] | $O_{3inf}$ | $O_{3eff}$ | O3 + MBBR | O3 + CW | O3 + S/BAC | O3 + S/A | O3 + S/A + GAC |
|---|---|---|---|---|---|---|---|---|---|---|
| 25.02.2019 | BLN | I | 0.32 | X | X | | | X | X | X |
| 08.04.2019 | KAL | I | 0.63 | X | X | X | | | | |
| 06.05.2019 | LIN | I | 0.49 | X | X | X | | | | |
| 13.05.2019 | BLN | II | 0.83 | X | X | | X | X | X | X |
| 09.09.2019 | KAL | IIa | 0.75 | X | X | X | | | | |
| 08.07.2019 | BLN | III | 0.69 | X | X | | X | X | X | X |
| 21.07.2019 | LIN | II | 0.57 | X | X | X | | | | |
| 02.09.2019 | BLN | IV | 0.91 | X | X | | X | X | X | X |
| 09.09.2019 | KAL | II | 0.97 | X | X | X | | | | |
| 14.10.2019 | KAL | III | 0.82 | X | X | X | | | | |
| 21.10.2019 | LIN | III | 0.50 | X | X | X | | | | |
| 28.10.2019 | BLN | V | 0.85 | X | X | | X | X | X | X |
| | | | **Total** | 12 | 12 | 7 | 4 | 5 | 5 | 5 |

Potential impact of the ozonation and the combination of ozonation and post-treatment, respectively, were evaluated with samples from three wastewater treatment plants with an ozonation stage located in Linköping (full-scale, Sweden), Kalundborg (full-scale, Denmark), and Berlin (pilot-scale, Germany). In order to minimize contamination due to sample handling, each sample was collected in a single 20-liter HDPE canister. All HDPE canisters were bought centralized by KWB from the same supplier and were distributed to the other WWTP operators to avoid possible impact of different types of sample containers on the results of the ecotoxicological test. According to the CWPharma internal sampling protocol, sample containers were pre-cleaned by filling them up with deionized water. The water was left in the containers for (at least) two days in order to leach out the substances that could contaminate the sample. At the end of the sampling process, each well-mixed sample was partitioned on site into multiple sub-samples, which were sent to the according laboratories:

- 17 L for SPE-procedure at UBA
- 2.35 L (7 x 300 mL in 500 mL glass bottles, 2 x 125 mL in 250 mL glass bottles) for ecotoxicity-tests with native samples at LIAE
- 10 mL for API and TP analysis at AU
- Rest of the sample for evaluation of other water quality parameters at internal or external laboratories

Sample logistics was a crucial part of the sampling campaigns as the SPE was conducted centralized by UBA in Berlin. The SPE process was conducted centralized to avoid impacts on ecotoxicity results due to deviations in the SPE procedure (enrichment, extraction, resolution), which might have been the case when different laboratories / operators would have conducted the SPE. As a result, about 17 L per sampling point had to be shipped cooled via overnight express to be able to conduct the sample extraction within 72 h (see section SPE procedure for details). Native samples for extraction were shipped cooled (2 – 8°C) to Berlin, whereas native samples for LIAE (Latvia) were sent frozen. Extracts from SPE were stored at -18°C and shipped insulated between cooling packs at below 0°C. Figure 2 illustrates the sample logistic for each sample.

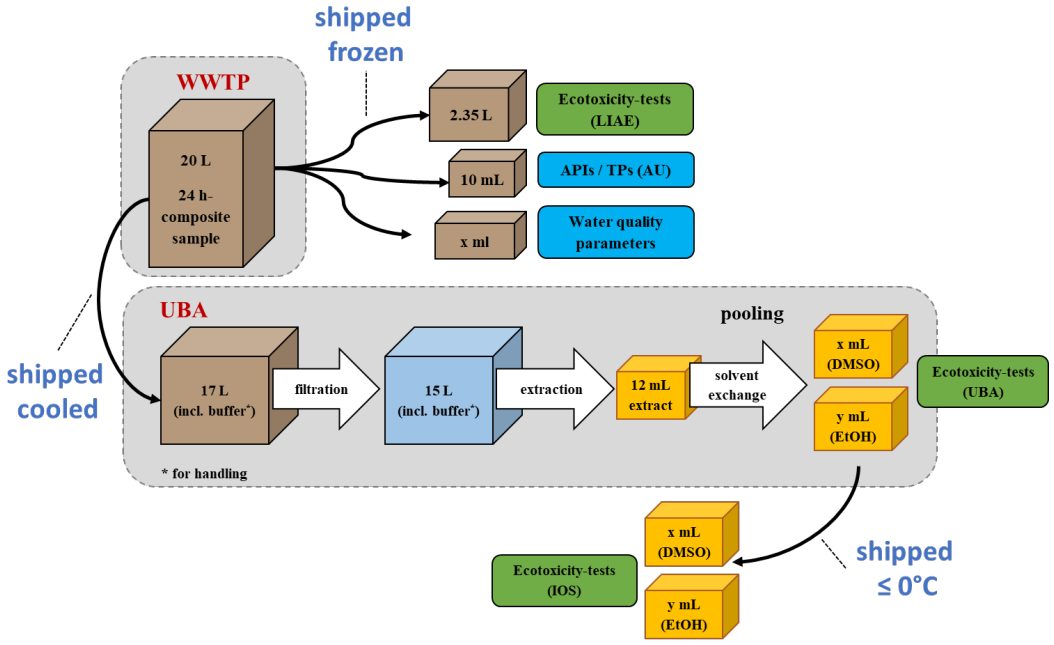


*Figure 2: Overview of sample logistics for each sample.*

### SPE procedure

In general, ecotoxicity samples should be processed within the next days (e.g. within 48 h). Due to time limitation and large sample volumes (17 L per sample), the following SPE procedure steps were carried out independently (not necessarily in the same day): (i) cartridge conditioning, (ii) sample filtration and extraction, and (iii) elution, pooling, and solvent exchange. Within CWPharma, sample logistics and handling were optimized in a way that the sample filtration and extraction step could be completed within 72 h after sampling. The general process of the SPE procedure is shown in Figure 3.

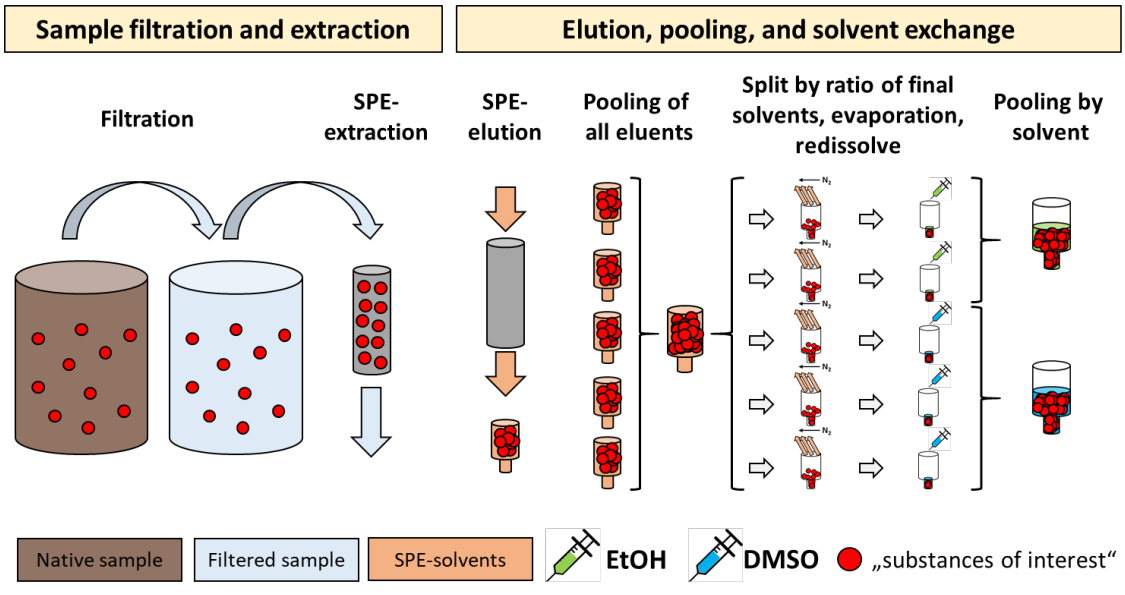


*Figure 3: Overview of SPE procedure.*

### 1. Cartridge conditioning

The SPE cartridge (Oasis HLB, 6 mL, 500 mg) was selected based on the defined goal of a broad, unselective substance extraction. The cartridge conditioning was performed by an automatic SPE-unit (AutoTrace 280, Dionex) right before extraction was performed. Each cartridge was loaded with 1 x 6 mL acetonitrile and 1 x 6 mL ultrapure water with a flow of 10 mL/min.

**2. Sample preparation and extraction**

Well-mixed native samples were filtered (0.45 µm, Ø = 110 mm, cellulose nitrate membrane filter, without binder) by a pressure filtration unit directly into a further pre-cleaned 20-L canister and then divided into 12 glass bottles each with 1.1 L sample. Extraction was performed by the AutoTrace, which could process up to six SPE-cartridges in parallel. The filtered sample was directly taken by the AutoTrace from the glass bottles of the prior step. The extraction program was as follows:

- 1000 mL sample volume per cartridge
- sample flow 10 mL/min
- final rinsing with 5% methanol (6 mL)
- drying with nitrogen gas for 30 min

The dried cartridges were sealed and stored at -21 °C until elution.

**3. Elution, pooling and solvent exchange**

The SPE cartridges were eluted automatically by the AutoTrace. Each cartridge was eluted with 1 x 10 mL methanol and 1 x 10 mL acetonitrile. The eluates (20 ml) of each sampling point were pooled to even out differences between the different cartridges, split by the ratio of the final solvents, and evaporated completely with a gentle nitrogen gas stream (TurboVap II, Biotage). The extracts were reconstituted in 1 ml of dimethyl sulfoxide (DMSO) or ethanol (EtOH). Afterwards, all extracts reconstituted in DMSO and EtOH, respectively, were pooled again by solvent. These pooled extracts were split according to the required extract volumes by UBA and IOS.

**4. Enrichment factor in ecotoxicity tests and coping with internal dilution**

The above described procedure provides extracts with an enrichment factor (EF) of 1000 (1 mL of extract from 1000 mL native sample). However, most of the ecotoxicity test systems cannot directly use the extract but require an aqueous dilution of an extract. Therefore, it was decided that within CWPharma all ecotoxicological tests have to be performed with at least 10-fold enriched samples. Thus, preparation of manageable aqueous dilution required a dilution of the extracts of 1:100 with ultrapure water resulting in 100 mL test sample (TS) with an EF of 10. However, some test systems required an additional amount of nutrients or the test organisms themselves which needed to be added by a specific volume of nutrient solution to the test sample. Accordingly, the addition of the nutrients and/or test organisms would have led to a further dilution of the TS and the enrichment factor in final test sample (fTS) would have been less than the intended enrichment factor of 10. Therefore, performing these tests directly with the TS (without further dilution) was not possible and an adaption of dilution volume was necessary.

For example in the YES test (according to ISO 19040-1) the test strain is added to the sample by adding yeast cells with culture medium (e.g. 40 µl test strain in culture medium to 80 µl sample). This leads to an internal dilution factor of 1.5 ((40 µl test strain + 80 µl sample) / 80 µl sample) within the fTS. To overcome this problem of the test internal dilution, volume of the test sample was adapted accordingly for each test system to achieve a higher EF in the test sample (less dilution of the SPE-extract). Accordingly, the same amount of extract (1 mL) was diluted by 66.7 ml ultrapure water instead of 100 ml so that the EF in the TS was 15 instead of 10. Due to the internal dilution factor of 1.5 by addition of the test strain, the final enrichment factor at the fTS was then the intended 10.

## Description of *In-Vitro* tox-tests

### Neurotoxicity

The procedure is used to determine the neurotoxicity of chemical substances or environmental samples based on the Ellman method (Ellman et al., 1961), which evaluates the kinetics reaction of the enzyme acetylcholinesterase (AChE) in the hydrolysis of the acetylcholinesterase iodide

(ATC) substrate. The reaction product forms a yellow association with 5,5' dithio-bis-2-nitrobenzoic acid (DTNB), which can be measured photometrically (E = 412 nm) and is directly proportional to the activity of AChE.

The test was carried out on 96-well plates using a test kit developed at IOS, which allows a simultaneous test of two samples. Each sample (SPE extract in EtOH) was tested in triplicate and at eight concentrations with according final enrichment factors in the test between 0.16 and 10. After preparation of the dilutions, AChE was applied to the plate and incubated for 10 minutes. After incubation, DTNB and ATC were added to start the reaction. Precise and quick application of these components is important, due to reaction kinetics. The whole test procedure took about 15 minutes. Kinetic measurements were conducted using a CLARIOstar microplate reader. The determination of neurotoxicity is relative to the standard AChE inhibitor paraoxone.

## Mutagenicity

This test is performed on specifically designed *Salmonella typhimurium* strains with point mutations in the histidine operon, making them unable to synthesize the amino acid histidine (*his*). Any chemical substance that may cause mutations at or near the histidine operon restores the *his* gene function and results in growth of the bacteria in the absence of histidine. The *Salmonella typhimurium* strains cannot only detect mutagenic potential of the substance capable of producing DNA damage, but also the mechanism which causes mutation. Bacteria, like several other rodent or human cell lines, lack or have limited metabolic activation potential. Hence, the Ames assay is almost always carried out with and without exogenous metabolic activation, to determine any mutagens in the samples which require metabolic activation (so called pro-mutagens). Usually exogenous metabolic activation is triggered by the presence of induced rat liver S9 fraction.

### Ames (TA1535 and TA1537, +/-S9)

The tests performed by IOS were conducted with the Ames MPF™ Penta 2 Microplate Format Mutagenicity Assay (Xenometrix AG, Switzerland). The used *Salmonella typhimurium* strains TA1535 and TA1537 meet the requirements of the OECD 471 Guideline for the Testing of Chemicals 'Bacterial Reverse Mutation Test' (adopted 21 July 1997). The TA1535 strain is suitable to detect base substitution mutations, whereas the TA1537 can detect frameshift mutations. The *S. typhimurium* strains have GC base pairs at their primary reversion site and the strains detects certain oxidizing mutagens, cross-linking agents and hydrazines.

All experimental steps were performed according to the protocol provided by manufacturer. Bacterial strains grew on specified growth medium for 22 - 24h, at a temperature of 37°C and on laboratory shaker with 250 rpm. After this time the optical density (OD) of the culture was checked using a spectrophotometer (E = 600 nm). After checking that OD was at least 2.0, the sample dilution series were prepared. As the test procedure requires a 25-fold enriched start concentration, 1000-fold enriched SPE extracts were diluted in sterile $H_2O$ at a ratio of 1:4 in order to achieve the targeted final enrichment factor (fEF) of 10 in the test. For each sample, six concentrations with a fEF between 0.31 and 10 were tested on a 96-well multiplate. For the highest fEF, 1:4 diluted SPE extract was put directly on the multiwell plate, while lower concentrations were spaced by the factor of 2 and diluted with DMSO. Prepared concentrations together with positive and negative control were transferred to a 24-well multiplate. Then the bacteria were added and exposed to tested concentrations for 90 min (37°C, 250 rpm) in a exposure medium (part of the test kit) that contained enough histidine to allow them approximately two cell divisions. After this, samples were diluted in a pH indicator medium which did not contain amino acid His and aliquoted into 48 wells of a 384-well plate. Then plates were incubated for 48 h under 37°C. During this period the organisms in which the reversion took place (after exposure to mutagenic substances) developed colonies and produced metabolites causing a pH decrease in the medium. Due to the pH change colour of the medium changed from violet to yellow. The number of positive wells were counted for each dose and compared to a solvent (negative) control. Each fEF was tested in triplicate to allow a statistical analysis of the data. A fEF dependent and significant increase in the number of revertant colonies

upon exposure to test sample relative to the solvent controls indicated that the sample was mutagenic in the Ames MPF™ Penta 2 assay. The sample was considered as mutagenic when two conditions were met: 1) at least 2-fold increase in number of positive wells over baseline (the baseline is obtained by adding one standard deviation to the mean number of positive wells of the solvent control) and 2) the Binomial B-value ≥ 0.99 what means that the probability that the result is due to spontaneous mutation is ≤ 1%.

#### Ames (YG 7108, +/-S9)

The Ames-test with YG 7108 (+/-S9) was conducted according to ISO 11350[1]. Bacteria from an overnight culture were exposed under defined conditions to the test sample and incubated for 100 min. Due to this exposure, genotoxic agents of the test sample may induce mutations in the marker genes of the bacterial strain, which lacks two O(6)-methylguanine-DNA methyltransferase genes, *ada* and *ogt*, and is highly sensitive to the mutagenicity of alkylating agents. Bacteria were exposed to samples with fEF of 10 and 1000. After exposure of the bacteria, reversion indicator medium, containing the pH indicator dye bromocresol purple, was added to the wells. Subsequently, the batches were distributed to 384-well plates and incubated for 48 h. Mutagenic activity of the test sample was determined by counting the number of the reverted wells where the purple colour had changed into yellow (per 48 wells of each replicate). The mean mutant induction factor (MIF) for three replicates was calculated by dividing the number of revertants in the sample treatment by the number of revertants in the control treatment (maximum 48). The higher the MIF, i.e. the number of mutated colonies of bacteria exposed to environmental pollutants, the higher the mutagenic activity in the samples.

### Genotoxicity

SOS chromotest from IOS and UmuC test from UBA were used for the detection and quantification of genotoxic activity of wastewater samples, i.e. the toxicity which specifically affects the genome of the organisms. Genetically engineered bacteria served as test organisms. Genotoxic compounds are hazardous due to their ability to react negatively with cellular DNA and induce mutations or other physical damages that alter proper protein production and cellular function. These substances can induce genetic diseases like cancer, thus, emphasizing the importance of their detection in the environment. All living cells have developed a sensitive system for detection and repair of lesions to their genetic material that involve complex signalling pathways and enzymes. In bacteria, the SOS repair system is activated to repair this kind of damage. Once a DNA lesion has been detected, a SOS promoter is induced to start transcribing genes that code for repair proteins.

#### SOS Chromotest

The bacteria used in the SOS chromotest is an engineered *Escherichia Coli* (*E. Coli*) strain developed as an indicator organism for genotoxicity testing systems and includes a gene coding for the β-galactosidase (β-gal) enzyme tethered to an SOS promoter. When DNA damage occurs, the SOS system is activated and β-gal gets transcribed proportionally to the level of SOS induction. The test is dependable and very sensitive, so that even limited repairable damage to genetic material can be detected due to the placement of the β-gal gene upstream of repair enzyme genes. Therefore, a positive response is produced regardless of cellular repair being initiated and the lesion being fixed.

Many carcinogens are known to require metabolic conversion to a reactive metabolite which then interacts with the DNA. Thus, similar to the AMES test, SOS chromotest is usually conducted with and without metabolic activation using rat liver S9 enzymes in order to detect direct and indirect genotoxins.

The SOS chromotest was conducted using a test-kit (SOS-ChromoTest™, EBPI) with and without addition of S9 liver enzymes. The test-kit allowed simultaneous testing of up to nine

[1] ISO 11350:2012: Water quality — Determination of the genotoxicity of water and waste water — Salmonella/microsome fluctuation test (Ames fluctuation test)

samples on 96-well plates. Each sample was tested with dilution factor of q = 2. Samples were prepared by 10x dilution of starting materials and added to the plates in appropriate amounts. Final enrichment factors were 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625. One day before conduction of the SOS chromotest, a solution containing the engineered *E. Coli* was prepared by dissolving the lyophilisate in growth medium and overnight incubation at 37°C. On the next day, a bacterial suspension was made from this solution based on optical density ($OD_{600}$) measurements at wavelength 600 nm. In addition, a mixture of liver homogenate from Aroclor 1254-induced Sprague Dawley rats in the presence of necessary cofactors was prepared, in accordance with SOS chromotest guideline. The SOS chromotest was prepared by first adding DMSO diluent to each well on two 96-well plates (test with and without S9 enzymes) then preparing the positive control by adding a standard genotoxic solution, containing 10 µg/mL 4-Nitroquinoline-1-oxide (4-NQO) in 10% DMSO-saline and finally adding the samples at the required dilution to each plate. At the next step, bacterial suspension and S9 enzyme solution (only on S9 plate) was added to the plates and then incubated at 37°C for 2 h. After incubation, the mixture of chromogene and alkaline phosphatase was added to both plates and absorbance was measured at 420 ±20 nm and 600 ±20 nm, respectively. Then, both plates were incubated again at 37°C for 60 - 90 minutes until a spectrum of green colour occurred, indicating that SOS repair mechanism was activated due to the β-galactosidase production. After incubation, a stop solution was added, and absorbance was measured again in order to determine viability of bacteria (420 ±20 nm) and genotoxicity (600 ±20 nm). The calculation of the induction factor (IF), which allows to observe the level of genotoxicity for the different dilutions, was done according to the test-kit guideline using an Excel spreadsheet provided by the test-kit supplier.

### UmuC

The genetically engineered bacterium *Salmonella typhimurium* (strain TA1535) serves as test organism for the umuC test to determine the genotoxicity of wastewater samples. The test is based on the capability of genotoxic agents to increase the expression of the SOS repair system associated with the umuC-gene in the *Salmonella* strain in response to genotoxic lesions in the DNA. The induction of the umuC-gene is thus a measure for the genotoxic potential of the sample. Since the umuC-gene is fused with the lacZ-gene for β-galactosidase, the induction of the umuC-gene can be easily assessed by determination of the β-galactosidase activity via absorption measurement (ISO 13829[2]).

The umu test was conducted according to ISO 13829, where the test organisms have been exposed to the test sample with and without metabolic activation system using microplates. Differently loaded microplates were prepared according the ISO standard and test organisms were added from an inoculum received from an overnight culture. For tests with metabolic activation, a mixture of enzymes prepared from the livers of male rats (S9 fraction) was added to simulate the hepatic metabolism. As the test systems requires aqueous dilutions, SPE extracts have been diluted to a final enrichment factor (fEF) in the test of fEF = 10, considering the internal dilution of samples within the test of 1:1.5. After 4 h of incubation on specific microplates, the genotox-dependent induction of the umuC-gene was compared to the spontaneous activation of the untreated control culture. Therefore, determination of absorption (E = 420 nm) as a measure for umuC-gene induction was conducted, using a photometer for microplates. The induction rate (IR) for the specific test sample was calculated. The lowest dilution level, at which IR < 1.5 was measured, was taken as results of the test.

## Estrogenicity and androgenicity

Compounds which interfere with the endocrine system of organisms are defined as endocrine disrupters. Estrogenicity is related to compounds that possess similar properties to the hormone 17β-estradiol (as main natural estrogen produced by the ovaries) and can be determined by several tests. Substances with the property of producing physiological reactions similar to those

[2] ISO 13829:2000: Water quality - Determination of the genotoxicity of water and waste water using the umu-test

produced by androgens, i.e. male sex hormone (such as testosterone) can be detected with tests for androgenicity (YAS-test).

### YES/YAS-Test

Hormonally active compounds in environmental, chemical, agrochemical, biocide, pesticide and cosmetic samples can be detected by using YES (yeast estrogen screen) and YAS (yeast androgen screen) tests, respectively. YES and YAS tests are performed by using genetically modified Baker's or Brewer's yeast (*Saccharomyces cerevisiae*). The yeast possesses stable transformation with the human estrogen hERα (YES) and androgenic hAR (YAS) receptors. In addition, the yeast cells also contain an expression plasmid carrying the reporter gene LacZ encoding the β-galactosidase enzyme and elements responding to estrogens (YES) or androgens (YAS).

The YES/YAS tests were carried out on 96-well plates using a test kit (XenoScreen XL YES YAS), which allows a simultaneously evaluation of four samples for estrogenic, anti-estrogenic, androgenic and anti-androgenic properties along with all required controls. Each sample was tested in duplicate and 8 concentration, according to a final enrichment factor in the test between 0.0032 and 10.

Before start of the test, the cultures of YES and YAS yeast cells were prepared in growth medium and incubated on an orbital shaker set at 100 rpm in temperature 31 °C for 4 days, until clearly turbid. After this time, dense yeast cultures were diluted in fresh growth medium 1:4 and incubated at 31°C for about 6–7 hours until the start of the assay. Then the optical density (OD690) of the cultures were checked using a spectrophotometer at wavelength 690 nm. This value should be at least 0.2.

Each plate contained a positive control (17-β estradiol for YES and 5α-dihydrotestosterone for YAS agonist as well 4-hydroxytamoxifen for YES and flutamide for YAS antagonist), serial dilutions of the test samples and solvent control. The calculated amount of YES/YAS cell cultures were added to the positive control wells and serial dilutions of the test samples on the YES/YAS agonist assay plates. To determine antagonist properties, a calculated amount of YES/YAS yeast cell culture with the addition of a fixed concentration of reference agonist (17-β estradiol and 5α-dihydrotestosterone) were added to the other two plates for the YES and YAS antagonist, respectively. Inhibition of the response relative to this fixed agonist concentration is a sign of antagonist activity. Then the plates were sealed with gas permeable foil and incubated in a sealed humid box for 18 hours at a temperature of 31 °C with agitation (100 rpm). After incubation, the cells were lysed in the presence of a yellow CRPG substrate, which is converted to a red product in the presence of β-galactosidase, indicating directly the hormonal activity of the test substance. The growth of yeast cells was assessed photometrical at 690 nm before adding lysis buffer. The colour change after lysis was measured at two wavelengths (570 and 690 nm) to correct diffraction caused by cells and deposits.

Colorimetric measurements were made using a CLARIOstar microplate reader. The calculation of potential endocrine activity and EEQ (estrogen) and AEQ (androgen) equivalents was done using an excel calculation spreadsheet provided by the test-kit supplier.

### YES/Anti-YES

The YES-test was conducted according to ISO 19040-1[3]. The Yeast Estrogen Screen (YES) is a reporter gene assay which was used for the measurement of the activation of the human estrogen receptor alpha (hERα) in the presence of a sample containing compounds which activate the estrogen receptor (ER). By this means the assay detects the estrogenic activity of the whole sample as an integral measure including possible additive, synergistic and antagonistic mixture-effects (ISO 19040-1).

[3] ISO 19040-1:2018: Water quality — Determination of the estrogenic potential of water and waste water — Part 1: Yeast estrogen screen (Saccharomyces cerevisiae)

The test organisms (*Saccharomyces cerevisiae*) have been exposed to the test sample with a final enrichment factor (fEF) in the test of fEF = 10, considering the internal dilution of samples within the test of 1:1.5. Differently loaded microplates were prepared according the ISO standard and test organisms were added from an inoculum received from an overnight culture followed by an incubation of 18 h on specific microplates. Estrogenic compounds of the sample which entered the yeast cell bound to the estrogen receptor protein causing its activation. This activation was measured by the induction of the reporter gene lacZ which encodes the enzyme β-galactosidase. The activity of β-galactosidase as a measure for the estrogenic potential of the sample was determined using photometric measurement (E = 580 nm) of chlorophenolred-β-D-galactopyranoside (CPRG) cleavage and compared to a reference curve with 17β estradiol. The results are expressed as equivalents of the reference compound, i.e. 17ß-estradiol equivalent (EEQ).

For the determination of anti-estrogenic effects (Anti-YES) samples have been spiked with 17 ng $L^{-1}$ 17ß-estradiol. A dose-dependent decrease in estradiol activity signaled the presence of anti-estrogens in the wastewater.

#### ER-Calux

The ER-Calux test was conducted according to ISO 19040-3[4]. A human cell line (human osteoblastic osteosarcoma U2-OS cells) containing the gene for the human estrogen receptor alpha (hERα) coupled with a reporter gene for the enzyme luciferase was used for the ER-Calux test. If an estrogenically active substance binds to the estrogen receptor in the cell, the corresponding gene and the reporter gene are activated. The latter gene encodes for an enzyme (luciferase), which oxidizes luciferin to generate light. The luminescence intensity is directly correlated to the amount of the substance bound to the receptor. The reaction was measured after 24 h exposure to the extracts where luciferase activity in cellular lysates is measured with a luminometer.

After cells have been seeded into 96 well plates, medium has been replaced by medium containing the enriched samples to be tested the next day. From the 1000-fold enriched sample dilutions of 1, 3, 10, 30 and 100 have been tested, which correspond to fEF of 1000, 333, 100, 33 and 10. After an exposure time of 24 h, the amount of luciferase was determined. The amount of luciferase produced by the samples is related to known concentrations of reference compound 17ß-estradiol and the final results are therefore also expressed as EEQ.

## Description of *In-Vivo* tests

### Bacteria tests

#### *Pseudomonas putida* inhibition growth test

The test was performed based on a European standard ISO 10712: 1995 with some modification related to the sample volume limitation. The method allows for the determination of the inhibitory effect of a sample of water (surface, underground and wastewater) and in vitro water soluble substances on the growth of *Pseudomonas putida* (*Ps. putida*) is a Gram-negative, rod-shaped, saprotrophic soil bacterium. Based on 16S analysis, *Ps. putida* was taxonomically confirmed to be a Pseudomonas species (sensu stricto) and placed, along with several other species, in the P. putida group, to which it lends its name.

The principle of the method is to determine the inhibitory effect of the sample on *Ps. putida* by measuring the optical density, proportional to the change in the number of cells exposed to the test sample (water or dissolved substance) at different concentrations compared to the number in the control culture. The optical density of the bacterial suspension in the culture (test and control) determining the growth of the culture is measured by a spectrophotometer and expressed as the absorbance value at 610 nm.

[4] ISO 19040-3:2018: Water quality — Determination of the estrogenic potential of water and waste water — Part 3: In vitro human cell-based reporter gene assay

*Pseudomonas putida* strain DSM 50026 was used as test organism, bought from the company German Collection of Microorganisms and Cell Cultures GmbH. Primary cultures of the used strain were stored on a special solid medium according to the ISO standard. 24 well plates were used, with a final volume of 2.5 ml assay mixture in each well. 1000-fold enriched SPE extracts were diluted to achieve final enrichment factors of 10, 5, 2.5, 1.25, 0.625, and 0.3125 in each well. To do so, variable volumes of sterile water and sample extract were added to constant volumes of nutrient solutions and bacterial inoculum with an optical density of 0.02 (10 FNU). Then, well plates were incubated for 16 hours at a temperature of 29 °C and the optical density was measured at 610 nm to evaluate according growth rate inhibition in relation to control.

### Aliivibrio fischeri bioluminescence inhibition test

The bacteria *Aliivibrio fischeri* serves as a test organism for the Microtox test, to determine toxicity of wastewater samples relative to the natural bioluminescence of bacteria. *Aliivibrio fischeri* produces the pigment luciferin, which emits light as a result of an oxidation reaction catalyzed by luciferase enzyme. Due to this oxidation process, a molecule (oxyluciferin) in the excited state is formed, whose transition to the ground state is associated with a green-blue light emission at 490 nm. The higher the luciferase concentration, the more light is emitted. Exposure of *Aliivibrio fischeri* to toxic substance disrupts metabolism processes and blocks the genes responsible for luciferase coding (lux operon). As a result, luciferin production decreases and so does the amount of light produced. The change in emitted light compared to the control samples is used to assess the toxicity of the test sample.

The samples were tested on basis of the Microtox®500 system (Strategic Diagnostic Ink, Newark, USA), which uses lyophilized luminescent bacteria of the *Aliivibrio fischeri* strain NRRL-B 11177. The test was conducted based on the standard manufacturer's test procedure: "81.9% Basic test with 1 sample and 5 dilution" in a temperature-controlled incubator block at a temperature of 15±0.5 $^0$C. Freeze-dried bacteria were reconstituted at a temperature of 5.5 ± 1$^0$C immediately before analysis by addition of 1 mL reconstitution solution (0.01% NaCl).

As the test procedure requires a 10-fold + 18.1% enriched start concentration, 1000-fold enriched SPE extracts were diluted in two steps with redistilled water (first extract with an enrichment factor (EF) of 20 and the second EF of 10 + 18.1%) in order to achieve the targeted final enrichment factor (fEF) of 10 in the test. The SPE extract with an EF of 10 + 18.1% was put to cuvettes and next the osmotic adjusting solution (22% NaCl) was introduced to the sample in order to adjust the osmotic pressure to the requirements of the marine bacteria. The sample prepared in this way had an EF of 10 + 9%. This sample was then diluted four times with q = 2 using a diluent (2% NaCl). The diluent was also used as control. The samples were placed in cuvettes containing bacteria received from an about half hour culture. Finally, five concentrations (fEF: 10, 5, 2.5, 1.25 and 0.625) in two replicates were tested. The test reaction of the water samples with bacteria was measured before exposition (T=0) and after 5, 15 and 30 minutes of incubation.

The analysis of the results was done using Microtox® Omni software. The results were presented as % effect (PE) of bioluminescence inhibition at 5, 15 and 30 minutes after sample introduction. In addition, EC- 50 effects and Toxicity Units (TU) were calculated. EC – 50 value determines the concentration at which the light emission is reduced by 50% and is estimated based on a linear regression of the log of each concentration level of the contaminant versus percent inhibition. Toxicity Units (TU = [1/EC-50]*100) is the value that specifies how many times the sample should be diluted to be non-toxic. A PE above 50%, an EC-50 value below 100 and a TU above 1.0 indicates toxicity of the samples.

The Microtox test is able to distinguish the source of contamination of samples: organic from inorganic[5]. Bioluminescence is generally activated by $Na^+$, $K^+$ and $Mg^{2+}$ ions and inhibited by ions of heavy metals (Carlson-Ekvall and Morrison, 1995; Dizer et al., 2002). High nutrients ions concentrations are important for the transcription of luminescence genes and can be achieved

[5] https://www.alsglobal.com/en-us/news/articles/2018/02/enviromail-120-microtox-toxicity-test, access 03.08.2020

by a high concentration of $Na^+$ through the Na/K-pump (Watanabe et al., 1991). The content of chloride and $Na^+$ ions (salinity but up to a certain level) could have a protective effect on other toxic inorganic compounds. Small concentrations of dissolved metal ions (e.g. heavy metals) are suspected to form chloro-metal complexes with chloride ions, leading to a decrease in cytotoxicity and sometimes to stimulation of the bioluminescence process (hormezis) (Dizer et al., 2002; Zgórska et al., 2019).

Higher doses of the inorganic toxicant cause already visible toxic effect, but it takes time for the cell membrane receptors to recognize whether the compounds in sample are toxic or not. Toxic ions (e.g. heavy metals) use the same pathways into the cell as nutrients (e.g. $Na^+$). Therefore, the inorganic compounds cause less toxic effects at the beginning of incubation of bacteria with samples and this effect increase after time. Significant differences in EC-50 value after 5 minutes compared to EC-50 value after 15 and 30 min testify to the contamination of samples with inorganic compounds. For organic ingredients in samples the toxic effect is faster visible. The bacterial cell could easier recognize the toxic substances and the toxic effect is visible after 5 minutes of incubation. After further incubation times (15 and 30 minutes), the EC-50 value does not change much.

Classification of toxicity to bacteria *Aliivibrio fischeri* (Table 4) was made based on the criteria proposed by Persoone et al. (2003).

*Table 4: Toxicity classification according to Persoone et al. (2003) used for result interpretation. PE = percentage inhibition effect, TU = toxicity unit.*

| Toxicity class | Toxicity level | PE | TU | $EC_{50}$ -t |
|---|---|---|---|---|
| **Class I** | No acute toxicity | ≤ 20% | <0.4 | >100% |
| **Class II** | Slight acute toxicity | 20% – 50% | 0.4 – 1.0 | 75% - 100% |
| **Class III** | Acute toxicity | 50% – 100% | 1.0 – 10.0 | 25% - 75% |
| **Class IV** | High acute toxicity | PE 100% in at least one test | 10.0 – 100.0 | <25% |
| **Class V** | Very high acute toxicity | PE 100% in all tests | >100.0 | - |

## Chronic tests

### Algal growth inhibition test with unicellular green algae

Unicellular green algae *Desmodesmus subspicatus* (SAG 86.81) is used to test fresh water algal growth inhibition. In this test monospecies algae are cultured for several generations in a defined medium containing different proportions of the test samples. *Desmodesmus subspicatus* were exposed to the water samples in microplates according to ISO 8692. Inoculum from *D. subspicatus* pre-culture was added (5 µl) to a series of sample dilutions to tests effects at five volume/volume percent concentrations – 94.3%, 47.2%, 23.6%, 11.8% and 5.9%. Each vial of microplate was also spiked (10 µl) with concentrated (10x) BG11 media to avoid supressed algae growth due to lack of nutrients in test sample. Chlorophyll was measured photometrically (E = 680 nm) every 24 hours until test ended at day three (after 72 hours).

Inhibition is evaluated by the reduction in specific growth rate relative to the cultures of the control. In case growth inhibition is observed, the results are expressed as the lowest ineffective dilution (LID) that is the highest sample concentration at which growth inhibition is < 5%.

### Ceriodaphnia dubia reproduction test

This reproduction test measures the chronic toxicity of water samples (e.g. surface water, secondary effluent of WWTP) to the daphnia *Ceriodaphnia dubia* (*C. dubia*) using less than 24 h old neonates during a three-brood, static renewal test. *Ceriodaphnia dubia* are exposed to different concentrations according native samples, until 60% or more of surviving control females have three broods of offspring. Determination of the chronic toxicity is based on reproduction inhibition of the test organisms at different sample concentrations compared to control.

*Ceriodaphnia dubia* reproduction test was conducted according to ISO 20665:2008. Reproduction test was carried out at five different concentrations of the according samples (6.15%, 12.34%, 24.67%, 49.34% and 98.87%) in addition to the control (no sample added). Ten *C. dubia* were separately exposed to each test concentrations using glass trays. The standardized test medium was changed, and organisms fed with *Raphidocelis subcapitata* and YCT (yeast, cerophyll and trout chow) on a daily basis. During this process the reproduction and mortality of *C. dubia* was recorded along with other parameters such as dissolved oxygen concentration, temperature and pH of the old and new solutions. Results were expressed by the concentration of the sample that induces reproduction inhibition of 50% ($EC_{50}$) of the population compared to the control at the end of the test. Lower $EC_{50}$ values indicate higher toxic effects.

The duration of chronic reproduction test was 7 ± 1 days, when control group has produced third brood of offspring. For each tested sample concentration, reproduction inhibition was evaluated based on arithmetic mean of the counted living young *C. dubia*.

Test was considered valid if the following criteria were met in the control:

- 60% of the control organisms have produced their third brood after 7±1 days
- Average of born offspring per alive adult female at the end of the test is ≥ 15
- Average mortality rate of adult females at the end of the test does not exceed 20%
- The proportion of adult males does not exceed 10%

During ecotoxicological tests with wastewater samples, sensitivity tests of *C. dubia* were performed with $CuSO_4$ * $5H_20$. $EC_{50}$ concentrations for performed sensitivity tests were from 114.36 µg/l to 171.51 µg/L. While the assessed concentration of $CuSO_4$ * $5H_20$ that causes 50% inhibition of reproduction ($EC_{50}$) in 7±1 day in interlaboratory tests for ISO standard is in rage from 135 µg/l to 311 µg/l. Test results are reproducible and the culture is slightly more sensitive compared to interlaboratory test results, but similar to other reported values for the same chemical.

## Results of *In-Vitro* tox-tests

### Neurotoxicity

Conducted Acetylcholinesterase (AChE) inhibition tests did not show neurotoxic effects within the tested concentrations (fEF ≤ 10) in any samples from the WWTPs in Berlin, Kalundborg and Linköping (see also SI-Table 1, SI-Table 2 and SI-Table 3 in the appendix).

Studies carried out by Macova et al. (2010) focused on the determination of neurotoxicity using AChE assay. At a two-stage ozonation process at a water reclamation plant, they determined a slight increase compared to the Parathion equivalent concentrations (PTEQ) at the influent and then a significant decrease after the main ozonation stage. PTEQ was decreased below the detection limit by the subsequent GAC-filter. Thus, PTEQ at the effluent of the water reclamation plant was only slightly above the detection limit of the AChE test. Macova et al. (2010) also stated that sand filtration did not change the PTEQ concentration, whereas PTEQ was also efficiently reduced below the detection limit by a biological activated carbon-filter.

In the samples collected from WWTPs Berlin, Kalundborg and Linköping no neurotoxicity effects were detected, in comparison to the cited study of Macova et al. (2010), which can be related to the components of the wastewater samples. AChE is focused on target chemicals as organophosphates and carbamate insecticides. Thus, low concentrations of these substances in the evaluated samples can explain the absence of neurotoxic effects. Thus, it is recommended to use higher concentrated samples (higher final enrichment factor) for the AChE test.

## Mutagenicity

### Ames (TA1535 + TA1537, +/-S9)

For the Berlin site and at a final enrichment factor of 10, occasional mutagenic effects were detected by the *Salmonella typhimurium* TA1535 strain in samples from the 2nd, 3rd and 5th sampling campaign, whereas no mutagenic effects were detected by the *Salmonella typhimurium* TA1537 strain (Table 5; an example raw data can be found in the appendix in SI-Table 4). Secondary effluent in 2nd campaign showed mutagenic potential which was maintained after ozonation treatment. This effect was detected by *S. typhimurium* TA1535 strain without S9 fraction which means that in bacteria DNA base pair substitution mutations took place after contact with mutagens present in the treated wastewater. The mutagenic effect was removed by all post-treatments. In the 3rd and 5th sampling campaign, no mutagenic effects were determined at the secondary effluent, but after ozonation its character changed. As these mutagenic effects were detected by *S. typhimurium* TA1535 strain with metabolic activation (+ S9), it means that promutagens were present in the ozonation effluent. In both cases, mutagenic effects were removed by all post-treatments except for the sand/anthracite filter (S/A) during the 3rd sampling campaign.

It is worth emphasizing that in above mentioned sampling campaigns the constructed wetland was efficient in reducing the mutagenic character, which was occasionally detected at the ozonation effluent. Furthermore, biological activated carbon filter (S/BAC) as well as sand/anthracite filter together with granular activated carbon filter (S/A+GAC) were also effective in removing the mutagenic potential of all three case with positive effects after ozonation. Sand/anthracite filter as post treatment step reduced the ozone induced mutagenicity in two out of three cases.

*Table 5: Results of AMES test using the strains TA1535 (+/- S9) and TA1537 (+/- S9) for samples from WWTP Berlin.*

| Berlin GERMANY | *S. typh.* strain +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|---|
| BLN I | TA1535 -S9 | not mutagenic | not mutagenic | - | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | - | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | - | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | - | not mutagenic | not mutagenic | not mutagenic |
| BLN II | TA1535 -S9 | MUTAGENIC | MUTAGENIC | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| BLN III | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | MUTAGENIC | not mutagenic | not mutagenic | MUTAGENIC | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| BLN IV | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| BLN V | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | MUTAGENIC | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic | not mutagenic |

For WWTP Kalundborg and at a final enrichment factor of 10, occasional mutagenic effects were detected by the *S. typhimurium* TA1535 strain and the TA1537 strain (Table 6). At the 1st campaign ozonation increased the mutagenicity of the samples, which was detected using *Salmonella* TA1535 strain without S9 fraction. This effect was removed by the moving bed biofilm reactor (MBBR). In the 2nd campaign only secondary effluent had mutagenic character (identified by TA1537 strain without S9 fraction) and subsequent stages of wastewater treatment removed the mutagenic potential. In the 3rd campaign the wastewater flowing out of the secondary clarifier had mutagenic potential detected by TA1535 with additional metabolic activation (+S9). After ozonation the character of mutagenicity of samples changed. Mutagenic effect was detected by TA1537 *Salmonella* strain without S9 fraction, but not for TA1535 with additional metabolic activation anymore. It indicates that promutagens in the secondary effluent were affecting base substitution in DNA, whereas mutagens in the ozonation effluent were affecting reading frame. Nevertheless, this negative effect was removed by the MBBR post-treatment.

*Table 6: Results of AMES test using the strains TA1535 (+/- S9) and TA1537 (+/- S9) for samples from WWTP Kalundborg.*

| Kalundborg DENMARK | *S. typhimurium* strain +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | $O_3$ + MBBR (S3) |
|---|---|---|---|---|
| KAL I | TA1535 -S9 | not mutagenic | MUTAGENIC | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic |
| KAL II | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | MUTAGENIC | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic |
| KAL III | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | MUTAGENIC | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | MUTAGENIC | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic |

For WWTP Linköping, no mutagenic effects were detected by the *S. typhimurium* TA1535 and TA1537 strain in any sample using the final enrichment factor of 10, neither with nor without metabolic activation (SI-Table 5).

### Ames (YG7108, +/-S9)

Samples from Linköping and Kalundborg caused no increase of mutant induction factor (MIF) in the Ames test with *Salmonella typhimurium* strain YG7108, neither with nor without metabolic activation (± S9 mix) of the samples at a final enrichment factor of 10. Accordingly, secondary effluents of both WWTPs as well as their ozonation and MBBR effluent, respectively, showed no mutagenic effects regarding alkylating agents. Moreover, the tested field blank showed no mutagenic effects (-S9 as well as +S9, data not shown).

Furthermore, all samples from Berlin campaigns were inconspicuous regarding mutagenicity when the 10-fold enriched samples have been treated with metabolic activation (+S9) as mean MIF did not increase significantly above the MIF of the negative control (SI-Figure 1). Without metabolic activation (-S9), 10-fold enriched samples of ozonation effluent caused a slight increase of MIF above the negative control in only one of the Berlin sampling campaigns (Figure 4), indicating no or only occasional mutagenic effects with fEF 10 as found for the TA1535 strain.

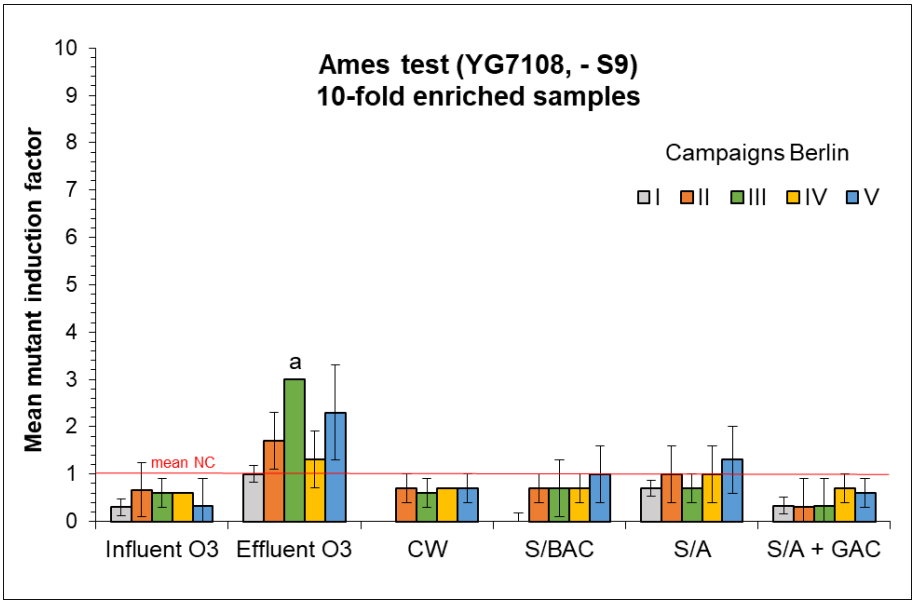


*Figure 4: Mean mutant induction factor (MIF, n = 3) of Salmonella typhimurium strain YG7108 treated with 10-fold enriched samples of five Berlin campaigns at six different sampling points. Samples have not been metabolically activated. Red line is the mean MIF of the negative control. Letter a indicates a p value of 0.074 of an one-way ANOVA followed by a post hoc-test (including correction according Bonferroni) for a comparison to the negative control. Error bars indicate standard deviation.*

Therefore, results with strain YG 7108 and a fEF of 10 did not allow a clear picture of mutagenic effects at different sampling points. To get more insights on potential difference between

treatment stages, samples with the highest possible enrichment factor of EF = 1000 in the -S9 Ames test with strain YG 7108 have additionally been tested.

Samples with EF = 1000 had still no mutagenic effects in the secondary effluent, i.e. the influent of the ozonation, of Berlin WWTP. After ozonation samples of all five Berlin sampling campaigns revealed a significantly increased mean MIF compared to the negative control: The latter indicates a high mutagenic potential of these highly enriched samples regarding alkylating agents. However, all investigated post-treatments were effective in reducing the respective MIF. The constructed wetland as well as the sand/anthracite (S/A) + GAC filter combination reduced the effects even to the level of the negative control (Figure 5). MIF of effluent samples from the sand/biological activated carbon filter (S/BAC) and the sand/anthracite filter (S/A) were still above the negative control, but lower than from the effluent of the ozonation (mean 11.7 ± 4.6 vs. 3.4 ± 1.0 and 5.1 ± 1.5, respectively).

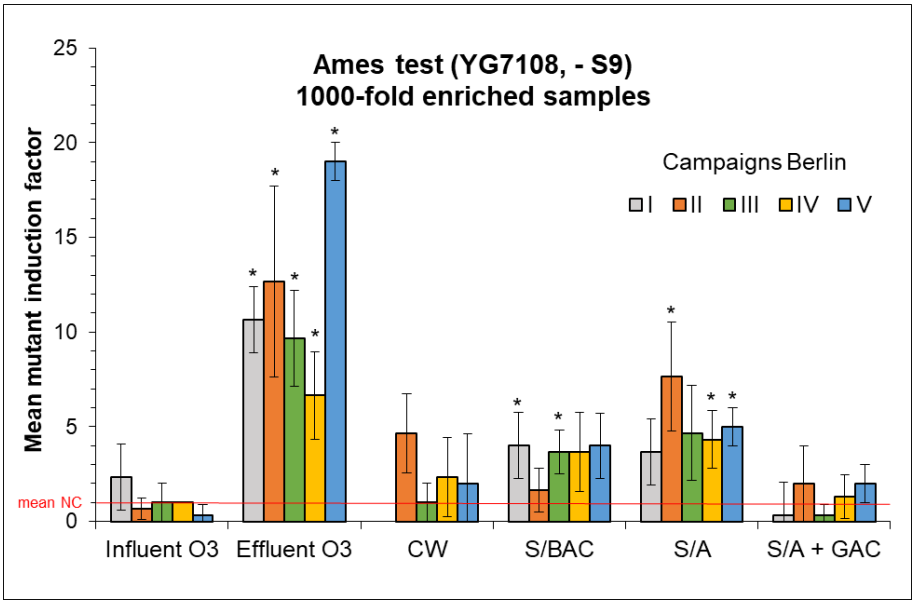


*Figure 5: Mean mutant induction factor (MIF, n = 3) of Salmonella typhimurium strain YG7108 treated with **1000-fold** enriched samples of five Berlin campaigns at six different sampling points. Samples have not been metabolically activated. Red line is the mean MIF of the negative control. Asterisks indicate significant differences to the negative control (p = 0.05; one-way ANOVA followed by a post hoc-test, including correction according Bonferroni). Error bars indicate standard deviation*

### Summary and discussion of mutagenic effects

Ozonation is based on the oxidative transformation of organic matter and is an effective tool for the removal of pathogens as well as a wide range of pharmaceuticals and endocrine disrupting chemicals (EDCs) (Huber et al., 2005; Luddeke et al., 2015). A disadvantage of this technology is the potential formation of toxic transformation products due to partial oxidation of the compounds and reaction with matrix components. Some of these oxidation by-products, e.g. bromate or NDMA, may interact with DNA and show genotoxic and carcinogenic properties. In this study, it was confirmed that ozonation is a factor generating mutagenesis in tested samples. Other authors (Giebner et al., 2018; Magdeburg et al., 2014; Petala et al., 2008) also observed increased mutagenic effects in wastewater samples after ozonation using TA98 and YG7108 *Salmonella* strains. That the mutagenic response can be influenced by the applied ozone dose was also demonstrated by Petala et al. (2008) by ozonation of secondary effluent at a lab-scale semi-batch ozone reactor. They observed for AMES TA98 that the use of lower ozone doses (2.5 and 5.0 $mgO_3/L$) and corresponding low contact times (2 and 5 min) induced an increase of mutagenic response, especially when no metabolic enzymes were added, whereas the application of higher ozone doses (7.3 and 8.0 $mgO_3/L$) resulted in a significant reduction of mutagenicity. Metabolic activation of the bacteria (+ S9) resulted in a further reduction of mutagenic effects.

The present study used three different strains of *Salmonella typhimurium* (TA1535/TA1537/YG7108), each with and without metabolic activation. While none of the three

tests showed mutagenic effects for WWTP Linköping, the results for the two other WWTPs differ. For WWTP Kalundborg, strain YG7108 showed no mutagenic potential of the samples, whereas strains TA1535 and TA1537 delivered occasional effects. Conclusion on the removal of mutagenicity by MBBR cannot be made as structural patterns are missing. Mutagenic effects were observed in some of the WWTP Berlin samples when tested with strain TA1535, but not with strain TA1537. Also strain YG7108 (±S9) revealed no mutagenic samples (only one without S9 in the ozonation effluent) of the WWTP Berlin. Only with highly enriched samples (with fEF = 1000) strain YG7108 (-S9) revealed a systematic mutagenic potential for the samples from Berlin. This mutagenic potential of the ozonation effluents was removed by post-treatment, i.e. constructed wetland as well as S/BAC-, S/A, and S/A+GAC-filters. Furthermore, also occasionally determined mutagenic effects after ozonation (for BLN and KAL with TA-strains) were decreased below the detection limit in a post-treatment. It needs to be highlighted that probably also at least the TA1535 strain would have delivered mutagenic effects more systematically when higher EF would have been used.

Liang et al. (2009) found that constructed wetland systems can be used as a potential treatment technique to remove dibutyl phthalate (reproductive toxic chemical) pollution with a high, nearly 100%, efficiency. Furthermore, other experiences imply that constructed wetlands can effectively remove several different phenolic compounds from wastewaters even at high concentrations (Stefanakis and Thullner, 2016). These compounds are considered priority pollutants and are characterized as toxic, mutagenic, carcinogenic, and teratogenic compounds. Activated carbon is based on sorption processes to the fine pored carbon surface and removes a broad spectrum of organic contaminants and organic carbon in general (Snyder et al., 2007). Ternes et al. (2002) showed that granular activated carbon (GAC) was very effective especially in removing pharmaceuticals. El-taliawy et al. (2018) tested the treatment performance of a MBBR system at lab-scale with respect to ozonation products of pharmaceuticals and showed that MBBRs have the potential to be efficient as ozonation post-treatment. In summary, the obtained results demonstrate that ozonation can result in formation of mutagenic effects, which are effectively removed by most of the post-treatments used.

Mutagenic activity of wastewater samples is usually assessed by different strains of *Salmonella typhimurium*, i.e. TA98 and TA100 (Völker et al., 2019). Among many studies, only a small number of publications detected an increase in mutagenic potential after ozonation using these strains. In the present study, also none of the Ames test with different *S. typhimurium* strains was effective in detecting the mutagenic potential of wastewater samples in a clear and systematic way when using low enrichment factors (here 10). Therefore, a fEF of 10 seems to be too low to evaluate mutagenic potentials properly. Anyhow, for strain YG7108 it was shown that higher EF would allow the detection of mutagenicity and also its lack in samples subjected to most post-treatments. Therefore, an Ames test (e.g. with strain YG7108 or TA1535) is recommended as a suitable test system for monitoring an advanced wastewater treatment, but only with higher enrichment factors (e.g. 1000) to achieve a clear dose-response relationship.

## Genotoxicity

### SOS Chromotest

All samples taken at the WWTPs in Berlin, Kalundborg and Linköping did not show genotoxic properties, which means that Induction Factor (IF) did not exceed 1.5 (see SI-SOS Chromotest). Genotoxicity was not obtained for samples tested with or without liver homogenate activation. Only in one sample (Influent O3, BLN I) the IF slightly exceeded 1.5 and was 1.53. No genotoxic effect was detected, this value meant “unmarked material”.

In the current study, genotoxic effects were not detected, but the study performed by Bourbigot et al. (1986) has shown that an ozone dose of about 3 mg/L causes a decrease in the toxicity of water samples tested using the SOS Chromotest assay compared to samples with a lower level of ozonation (0.75 and 1.5 mg/L). Ozonation can even eliminate the initial genotoxicity of the water. However, an increase in genotoxicity can be detected when low doses of ozone are used. Also the study showed that the combined treatment of ozone followed by activated carbon is a

better process for removing mutagenic compounds than ozone treatment alone (Bourbigot et al., 1986).

### UmuC

Genotoxic effects were not detected in any samples by the umuC test. This holds for all types of samples and all sampling campaigns in Berlin, Linköping and Kalundborg as well as for the field blank. Raw data are available in the appendix (SI-UmuC).

### Summary of genotoxic effects

SOS Chromotest and umuC test are tests for determining the genotoxicity of specific environmental samples. Genotoxic compounds are hazardous due to their ability to react negatively with cellular DNA and induce mutations or other physical damages that alter proper protein production and cellular function. The use of two different organisms (*Escherichia coli* and *Salmonella typhimurium* (TA1535)) and two variants of enzymatic activation (with and without S9) enhance the sensitivity and accuracy of the test. However, none of the tested samples with fEF = 10 revealed genotoxic effects. Therefore, both tests cannot be recommended for the evaluation of wastewater ozonation or the assessment of the according post-treatment.

## Estro-/Androgenicity

### YES/YAS-Test

Estrogens and androgens are some of the most potent endocrine disrupting compounds (EDCs) found in wastewater. 10-fold enriched samples from WWTP Berlin showed an estrogenic potential before ozonation in the range of 0.2 ng EEQ/L to 2.5 ng EEQ/L (Figure 6). The average estradiol equivalent (EEQ) of the five sampling campaigns from Berlin was 1.5 ng EEQ/L. At WWTP Linköping, estrogenic potential was only detected in one out of three ozonation influent samples at a level of 9.4 ng EEQ/L (SI-Table 14), whereas no estrogenic effects were detected in any sample from WWTP Kalundborg (SI-Table 13). However, in all cases ozonation process reduced the estrogenic activity of the secondary effluent below the limit of detection.

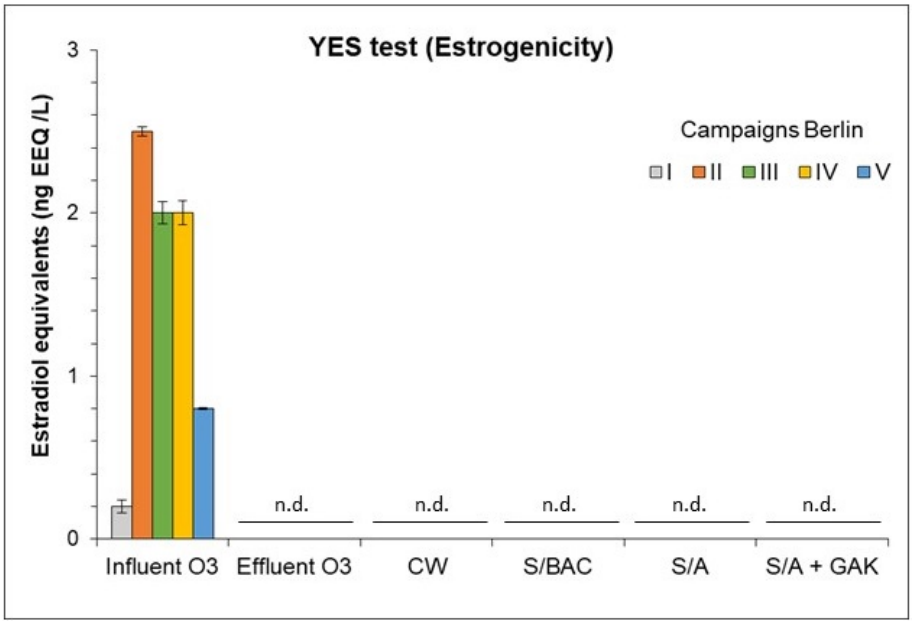


*Figure 6: Estrogenic potential expressed in estradiol equivalents (EEQ, n = 2) at individual stages of the wastewater treatment plant in Berlin. The LOD was 1.14 ng EEQ / L, and the results of 0.2 and 0.8 ng EEQ / L were calculated by extrapolation. Error bars indicate standard deviations.*

Androgenic activity was not detected in any sample of the three WWTPs. A systematic trend was not observed in the YES/YAS antagonistic properties (anti-estrogenic and anti-androgenic) in any of the studied cases, which prevented further evaluation and conclusions from the results obtained. Details of the test results are provided in the appendix (SI-YES/YAS-Test).

### YES/Anti-YES

Estrogenic potential of 10-fold enriched samples from WWTPs Berlin and Linköping has only been detected in the influent of the ozonation, i.e. the secondary effluent of the WWTPs (Figure 7). Average estradiol equivalents (EEQ) of the five sampling campaigns at the ozonation influent from Berlin was determined to be 1.4 ng EEQ/L (0.5 - 2.0 ng EEQ/L). Differences between the five sampling campaigns in Berlin probably resulted from diurnal variations at different sampling dates. The three sampling campaigns at WWTP Linköping delivered different results with high values for the first campaign (1.9 ng EEQ/L), lower for the second (0.2 ng EEQ/L) and values below the limit of quantification (LOQ[6]) for the last campaign. In the effluent of the ozonation and in all post-treatments of WWTP Berlin as well as the MBBR of WWTP Linköping EEQ has been below LOQ. Hence, ozonation reduces the remaining estrogenic potential of the wastewater at the effluent of the conventional treatment below the limit of quantification without any further detectable reduction by the according post-treatments. In contrast, no estrogenic potential was detected in all samples (three sampling points, three sampling campaigns) at WWTP Kalundborg.

Moreover, the field blank revealed no estrogenic effects above the LOQ, indicating that processes such as sampling, transportation and SPE processing did not lead to a background estrogenic potential. Accordingly, detected EEQ values display only estrogenic effects from samples and not from surrounding processes.

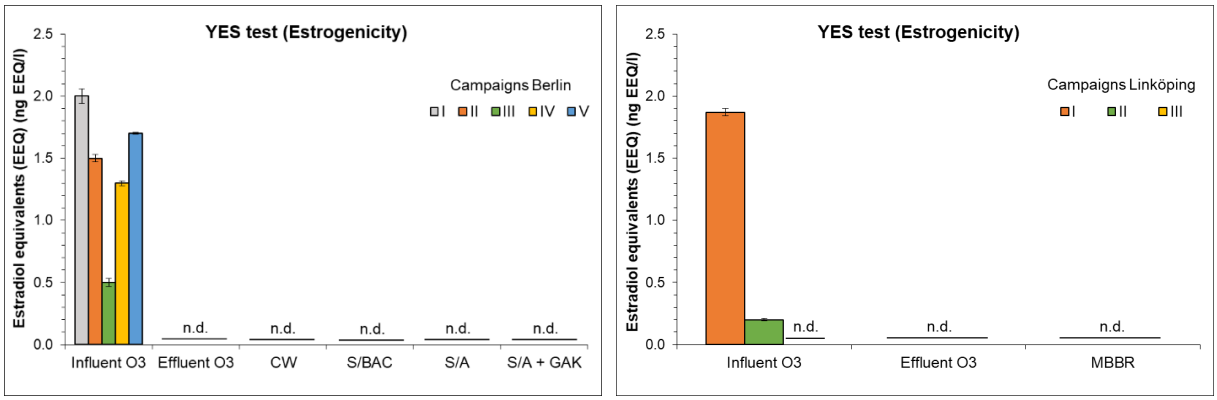


*Figure 7: Estradiol equivalents (EEQ, n = 3) in ng EEQ/L detected by YES test of samples from five sampling campaigns at WWTP Berlin (left) and three sampling campaigns at WWTP Linköping (right). Limit of quantification (LOQ) = 8-15 ng EEQ/l (ISO 19040, 2018). Results have been recalculated for the fEF of 10. Error bars indicate standard deviation.*

YES- and anti-YES-test can only display the strongest of both effects, i.e. either estrogenic or anti-estrogenic effects. Accordingly, samples with estrogenic potential determined with YES test could not detect (lower) anti-estrogenic effects. Therefore, samples from WWTP Berlin show no measurable anti-estrogenic effects at the influent of the ozonation, but at least partly afterwards (Figure 8). While anti-estrogenic potential was determined in all samples from the first campaign (constructed wetland (CW) was not tested), the following campaigns revealed lower values or no effects, without any structural causality. Note that a value of 1 ng EEQ/L in the anti-YES-test would mean that the sample contains compounds which inhibit estrogenic potential equivalent to the effect of 1 ng estradiol per liter.

[6] Each YES-test delivers its own LOQ as each microtiter plate contains a separate reference curve. Lowest LOQ value is 1 ng EEQ/L. Note that samples have been tested with fEF = 10 and recalculated afterwards. Therefore, EEQ values below the LOQ are possible.

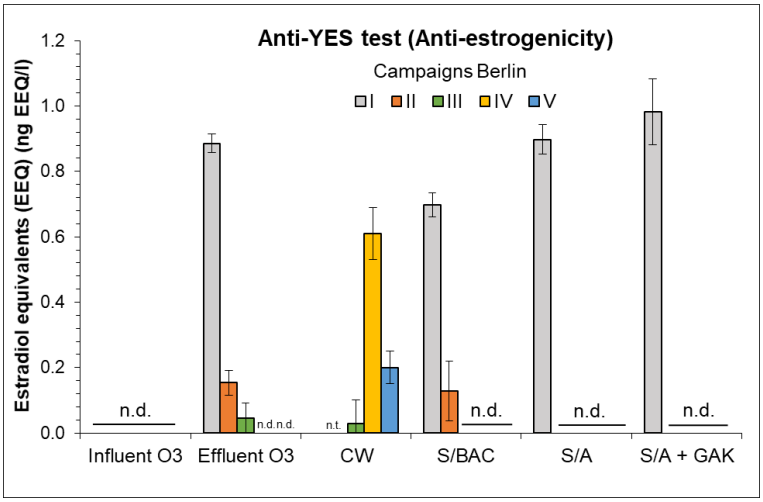


*Figure 8: Anti-estrogenic potential in ng EEQ/L (n = 3) detected by anti-YES test of samples from five campaigns from WWTP in Berlin at six different sampling points. Results have been recalculated for the fEF of 10. Error bars indicate standard deviation.*

Anti-estrogenic effects have also been determined for samples from the WWTPs in Linköping and Kalundborg. For the second campaign in Linköping an anti-estrogenic potential of 2.2 ng EEQ/L in the effluent of the ozonation has been determined, which decreased after the MBBR to 0.4 ng EEQ/L (Figure 9, left). Anyhow, a pattern which was not found in the other two campaigns. Here results indicate an increase of the anti-estrogenic potential by the MBBR post-treatment.

Samples from WWTP Kalundborg showed anti-estrogenic potential at all three sampling points, as there were no estrogenic effects found (Figure 9, right). While in the first campaign values did not differ between all three sampling points, results for second and third sampling campaign indicate lower values after ozonation and further decreased values after the MBBR.

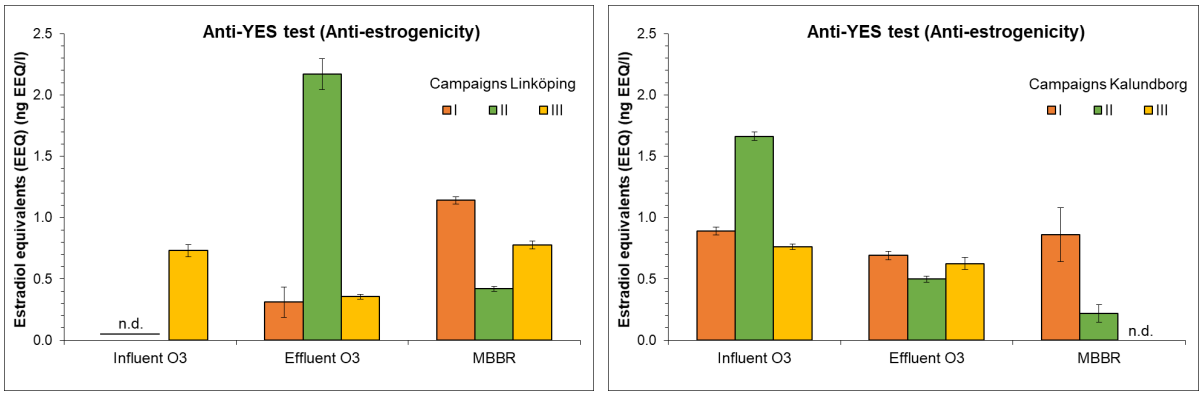


*Figure 9: Anti-estrogenic potential in ng EEQ/L (n = 3) detected by anti-YES test from three sampling campaigns and three sampling points at WWTP Linköping (left) and WWTP Kalundborg (right), respectively. Results have been recalculated for the fEF of 10. Error bars indicate standard deviation.*

### ER-Calux

Estrogenic potential of samples detected via ER-Calux test from the WWTPs in Berlin and Linköping has only been detected in the influent of the ozonation (Figure 10). Average estradiol equivalents (EEQ) of the five sampling campaigns at the ozonation influent from Berlin was determined to be 1.50 ng EEQ/L (0.6 - 2.3 ng EEQ/L). Differences between the five sampling campaigns in Berlin probably resulted from diurnal variations at different sampling dates. The average of the three sampling campaigns at WWTP Linköping was very stable at 1.06 ng EEQ/L (1.0 - 1.1 ng EEQ/L). In the effluent of the ozonation and in all post-treatments of WWTP Berlin as well as WWTP Linköping EEQ have been below the LOD of 0.21 ng EEQ/L. Hence, ozonation reduces the remaining estrogenic potential of wastewater at the effluent of the conventional treatment below the detection limit without any further detectable reduction by post treatments.

Samples from Kalundborg showed no estrogenic potential at none of the three sampling points from all three sampling campaigns. Also, field blank revealed no estrogenic effects above the LOD, indicating that processes such as sampling, transportation and SPE processing did not lead to a background estrogenic potential.

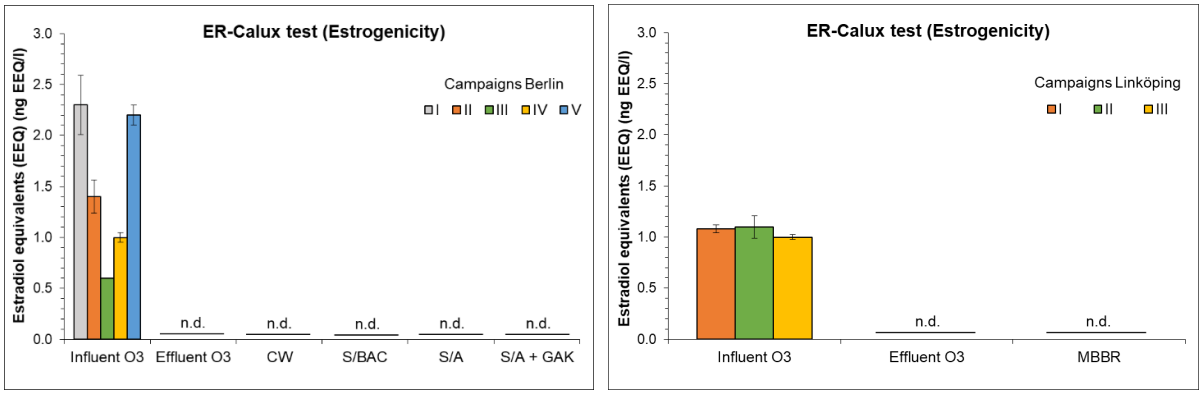


*Figure 10: Estradiol equivalents (EEQ, n = 3) in ng EEQ/l detected by ER-Calux test from five sampling campaigns at WWTP Berlin (left) and three sampling campaigns at WWTP Linköping (right). Long-term limit of detection (LOD) = 0.21 ng EEQ/l. Error bars indicate standard deviation.*

### Summary and discussion of estrogenic effects

Estrogenic potential of 10-fold enriched samples taken from the WWTPs in Berlin and Linköping has been detected by two independent laboratories (IOS and UBA). Three different assays for estrogenicity were applied in this study. The obtained results of estrogen content in the tested wastewater samples, regardless of the test method used, were in most cases comparable. For example, the average estradiol equivalent in the ozonation influent at the WWTP Berlin was determined to be 1.5 ng EEQ/L in the YES-test (IOS), 1.4 ng EEQ/L in the YES-test (UBA) and 1.5 ng EEQ/L with the ER-Calux (UBA), respectively. In contrast, the three sampling campaigns at WWTP Linköping delivered different results for estrogenicity of samples from the secondary influent when analyzed with the YES-test (UBA, Figure 7 right), but almost the same values with the ER-Calux-test (Figure 10, right). Furthermore, the estrogenic potential was only detected in one out of three samples from the secondary effluent with the YES-test by IOS. This indicates that results in estrogenicity tests depend on the used test and probably the given samples. However, all tests confirmed that ozonation is an effective process for removing estrogens from wastewater as samples from the effluent of the ozonation (and further sampling points) from all three WWTPs showed no estrogenic potential. The latter is also confirmed by literature. For example, Koh et al. (2008) showed that by applying ozone to WWTP effluent, a removal of estrone and estradiol of 62% – 98% and 57% – 100%, respectively, was achieved. Another study by Racz and Goel (2010) showed that estrogens have been removed by 90% or more than 99% (20 ng/L to 350 ng/L initial concentration) with an applied ozone doses between 1 mg/L to 1.25 mg/L. These findings are in line with the overview study of Völker et al. (2019), who summarized that conventional wastewater treatment can effectively eliminate estrogenicity at an average of more than 90% but the remaining estrogenicity at the WWTP effluent with an average of 1.8 ng/L would still be high enough to cause adverse effects on organisms in the receiving water. Regarding the effectiveness of advanced wastewater treatment with activated carbon or ozone, data show a clear picture that both technologies are able to lower estrogenic concentrations (Völker et al., 2019).

In sum, all three tests show the removal of estrogenic effects by ozonation and deliver comparable results (e.g. for WWTP Berlin, Figure 11, left). However, particularly for the influent of ozonation for WWTP Linköping (Figure 11, right) all three tests deliver different results compared to each other and only the ER-Calux test showed similar EEQ values for all three sampling campaigns.

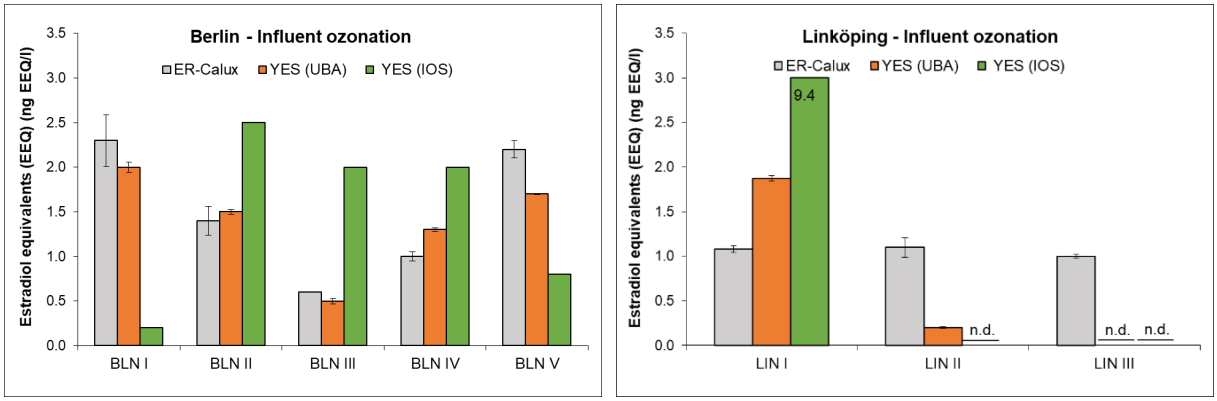


*Figure 11: Estradiol equivalents (EEQ, n = 3 for UBA tests) in ng EEQ/l from five sampling campaigns at WWTP Berlin (left) and from three sampling campaigns at WWTP Linköping (right) detected by ER-Calux test and YES-test at German EPA (UBA) and by YES-test at IOS. Error bars indicate standard deviation.*

Nevertheless, evaluation of estrogenic effects by using one of the three tests is strongly recommended for monitoring WWTPs with an advanced treatment. Regarding the evaluation of antagonist properties there are concerns about the lack of repeatability of the results (no systematic trend). Accordingly, neither the YAS nor the Anti-YES test is recommended as suitable test for WWTP monitoring regarding advanced wastewater treatment.

## Results of *In-Vivo* tests

### Bacteria tests

#### Pseudomonas putida

Based on the results from the three WWTPs Berlin, Kalundborg and Linköping, it can be stated that regardless of the different sampling points and treatment technology used, growth inhibition reached negative values in almost all tested samples. This means that a growth stimulation of *Pseudomonas Putida* bacteria was observed. The test results can be found in the appendix (SI-Pseudomonas putida). At the second sampling campaign in Berlin, some sampling points showed values close to zero (i.e. CW = 2.24%, S/BAC = 0.93% and S/A = 1.6%), which can be interpreted as "no effect" (Figure 12). Negative bacterial growth inhibition values were obtained for the remaining samples in Berlin. Unlike to other tests, in the case of WWTP Berlin only three campaigns were evaluated, because of absence of toxic effects to Pseudomonas putida.

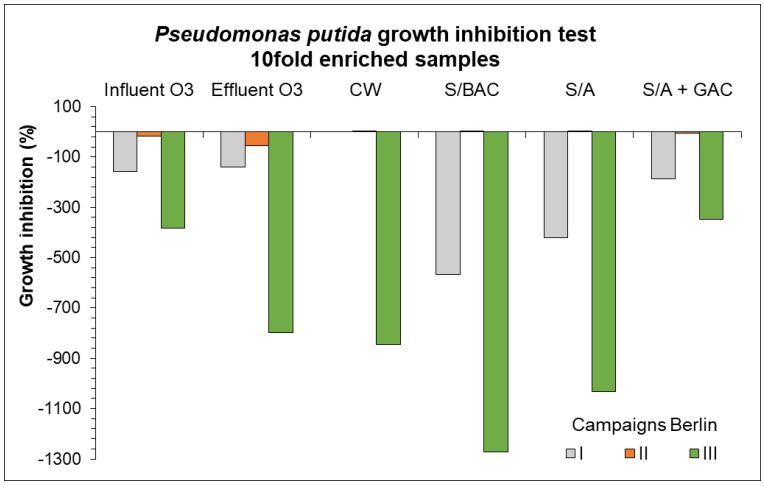


*Figure 12: Results of Pseudomonas putida growth inhibition test of samples taken from WWTP Berlin.*

For the conducted sampling campaigns at WWTP Linköping, *Pseudomonas putida* growth stimulation was observed for all samples (Figure 13, left). For WWTP Kalundborg, the test showed a slight growth inhibition (21.5%) of *Pseudomonas putida* only for one sample (ozonation effluent at first sampling campaign, Figure 13, right). However, this effect was not confirmed at the other sampling campaigns in which a significant bacterial growth stimulation was observed.

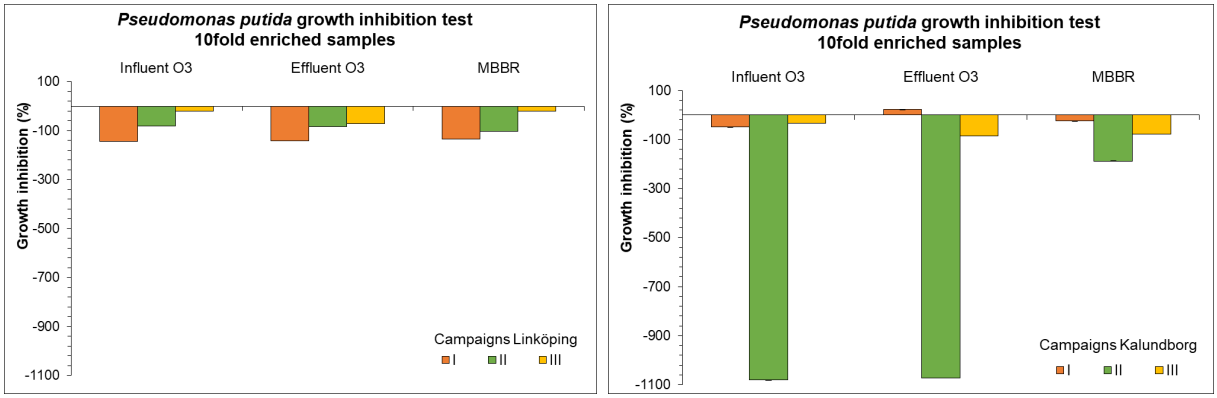


*Figure 13: Results of Pseudomonas putida growth inhibition test of samples taken from WWTP Linköping (left) and WWTP Kalundborg (right).*

Field blank sample revealed stimulation of Pseudomonas putida growth (-250%). If tap water contains sources of carbon and phosphate required for bacterial growth, the biofilm of *Pseudomonas putida* appears (van Nevel et al., 2013).

Due to the lack of a systematic trend in the results, it can be concluded that the *Pseudomonas growth* inhibition test, performed in accordance with the PN-EN ISO 10712 methodology, is not suitable for assessing the toxicity of treated wastewater. *Pseudomonas putida* is a bacteria naturally living in the wastewater and adding it to the wastewater causes its growth. From the literature it is known that for testing the potential toxicity of pharmaceuticals at WWTPs, the *Pseudomonas putida* test was assessed to be not suitable because *Pseudomonas putida* is no model organism for sludge when samples are rich of concentrated organic substances which served as a source of carbon and energy (Kümmerer and Alexy, 2006). Treated wastewater is a good medium for bacterial growth because of the carbon and phosphorous compounds content. Despite using extracted samples and separation of inorganic nutrients from the organic matter in the samples, growth promotion occurred. This might be linked to a bacterial growth stimulated by organic matter itself, which is indicated by the field blank (drinking water).

### Aliivibrio fischeri

On the basis of the percentage inhibition effect (PE), toxicity levels of samples from the WWTPs Berlin and Linköping varied between a PE of 9.00% - 46.47% and a PE of 15.48% - 35.35%, respectively, which are defined as “not toxic” (class I) or “slightly toxic” (Class II, appendix SI-Table 18 and SI-Table 20). Acute toxicity was not observed for samples taken from both WWTPs. In the case of WWTP Kalundborg samples exhibited slight and acute toxicity (PE 20.97% - 52.33%). Acute toxicity (PE > 50%) was determined only in the ozonation influent samples from the first and second sampling campaign (appendix

SI-Table 19). Other parameters for toxicity assessment, such as $EC_{50}$ and TU values, also confirm this classification (Appendix, SI-Table 26 - SI-Table 34). Graphical summaries of the test results are shown in Figure 14 and Figure 15.

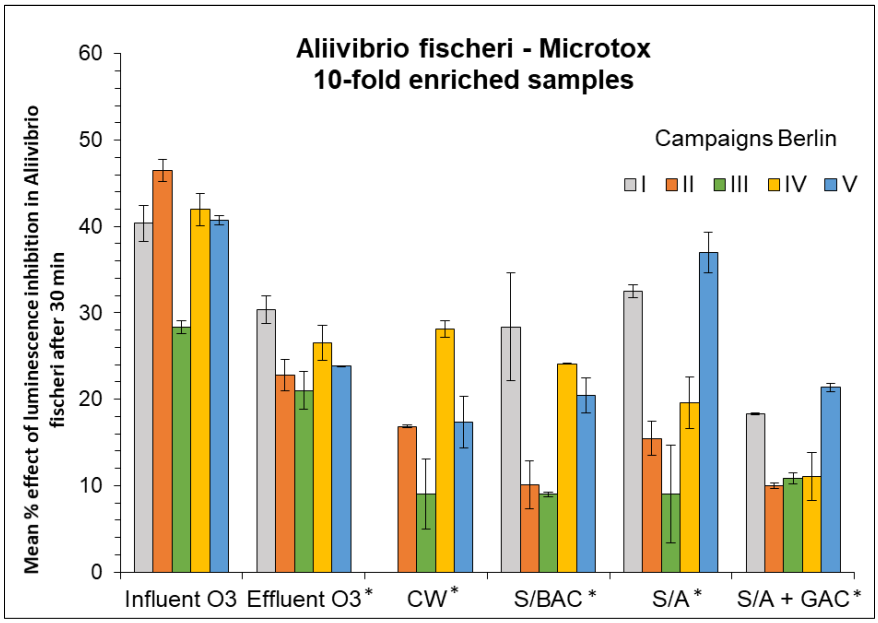


*Figure 14: Mean inhibition effect of Aliivibrio fischeri luminescence at different sampling points at WWTP Berlin. Asterisks indicate "significantly different from ozonation influent" (1-way ANOVA plus post hoc test including Fisher's last significant difference).*

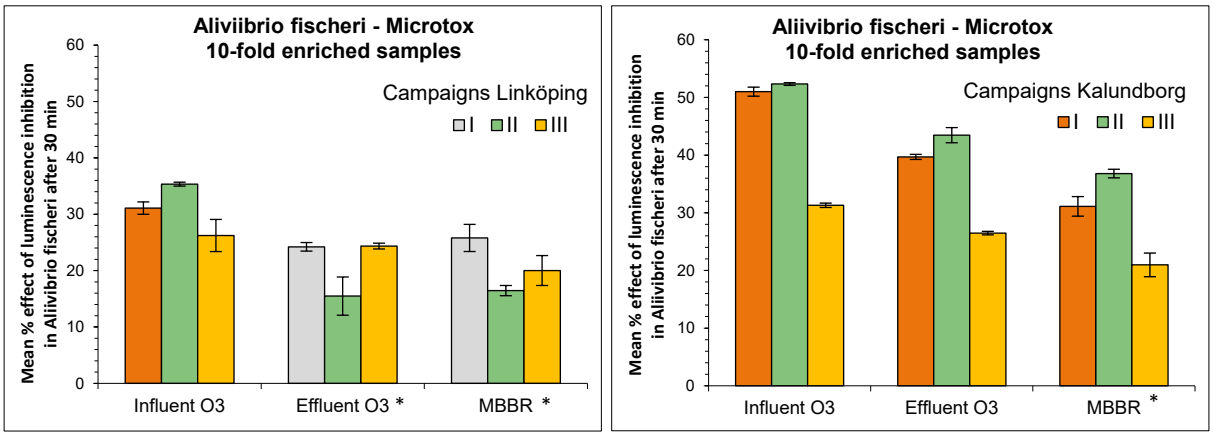


*Figure 15: Mean inhibition effect of Aliivibrio fischeri luminescence at different sampling points of WWTP Linköping (left) and WWTP Kalundborg (right). Asterisks indicate "significantly different from ozonation influent" (1-way ANOVA plus post hoc test including Fisher's last significant difference).*

The highest inhibition effect (PE) to *A. fischeri* was detected in samples at the influent of the ozonation. Based on this study it can be concluded that the ozonation process effectively reduced the level of toxicity towards marine bacteria. Statistical analyses have also confirmed this effect (see statistical analysis section in the appendix) and a statistically significant difference between samples before and after the ozonation process were found. However, no significant difference was found between the influent and the effluent of the ozonation at the WWTP Kalundborg, which means that the ozonation process in this case was not effective in elimination of toxicity (SI-Table 34).

At WWTP Berlin, treatment of the ozonation effluent by the constructed wetland resulted in a reduction of toxicity against *A. fischeri* and most samples were classified as non-toxic. However, no systematic trend was observed in results from all Berlin campaigns at this treatment stage. Li et al. (2014) noted that constructed wetlands hold a great potential as an alternative secondary wastewater treatment system for contaminants removal. However, within this study, the toxicity reduction seems not to be a result of the API reduction, but from the reduction of other compounds (see also section Impact of post-treatment on APIs and transformation products). Similar to the results of the constructed wetland, a toxicity reduction was obtained by the treatment with deep-bed filters using sand/anthracite (S/A) or sand/biological activated carbon (S/BAC) as filter material (see statistical comparison, SI-Table 28). In most cases the samples were non-toxic to bacteria, however, no systematic trend was observed in the results for both

treatment stages. The highest toxicity reduction was observed after the treatment by a combination of a sand/anthracite- and a granular activated carbon-filter (S/A+GAC).

At the WWTPs Linköping and Kalundborg, moving bed biofilm reactors (MBBRs) were used as ozonation post-treatment. The results also show a reduction of the toxicity by the MBBR treatment stage but to a slightly smaller extent than it was achieved when using a sand/anthracite filtration in combination with an active carbon filtration (S/A+GAC) and other post-treatments investigated at WWTP Berlin. The MBBRs used in both WWTPs did not reduce toxicity any further than the ozonation process. The toxicity level towards *Aliivibrio fischeri* between the ozonation effluent and the MBBR effluent at the WWTPs Linköping and Kalundborg was not significantly different. Despite the limited impact regarding the reduction of toxicity towards *Aliivibrio fischeri* and in contrast to the findings in this study, pilot–scale experiments conducted by Ooi (2018) demonstrated the potential usefulness of nitrifying MBBRs as post-treatment stage at WWTPs for API reduction. Most of the analysed pharmaceuticals (17 of 22 compounds studied) were removed with more than 20%, and 8 of these compounds were estimated to be almost completely removed by the MBBR treatment.

In general, it is expected that the probability of the formation of toxic transformation and/or oxidation products that can cause bacterial inhibition increases with the pollutant load at the ozonation influent. Based on research carried out by Petala et al. (Petala et al., 2006a; Petala et al., 2006b) it could be shown that toxicity effect on *Aliivibrio fischeri* was highest (almost 100% inhibition of bioluminescence at the highest ozone dosage) if analysed immediately (during 15 minutes) after taking the sample and decreased with sample storage time until it was almost negligible after 48 h. The researchers confirmed that the toxicity of the stored samples was not due to the presence of ozone, but seems to be caused by residues of transformation and/or oxidation products formed by the ozonation process.

#### Summary of bacteria tests

As it was stated in the description of *Pseudomonas putida* growth inhibition results, this test was not suitable for evaluation of toxicity of treated water.

Bioluminescence inhibition of *Aliivibrio fischeri* was suitable for assessing the toxicity of treated water. The studies confirmed the efficiency of ozonation in purification of treated wastewater, especially when an acute/slight acute toxicity in ozonation influent was determined. There was a statistical difference in inhibition of bioluminescence between ozonation influent samples and the effluent of the ozonation and post-treatments, respectively. The combination of filtration with sand/anthracite and granular activated carbon (S/A + GAC) and constructed wetlands seems to be more effective in toxicity reduction than the other post-treatment technologies. Inhibition of *Aliivibrio fischeri* bioluminescence is a suitable, fast, common and relatively cheap test for assessing wastewater toxicity at every stage of treatment, giving reliable results.

### Chronic tests

#### Algae growth test

Native samples from Berlin campaigns II – V did not indicate algae growth inhibition after ozonation even at high test concentrations (Figure 16). However, at the 1st sampling campaign, the sample at the effluent of the ozonation showed growth inhibition that decreased with both S/BAC and S/A + GAC-filters as post-treatment, whereas treatment with the S/A-filter did not show any impact. The impact of the CW could not be assessed as no sample at the CW effluent was taken at the 1st sampling campaign. Although the growth inhibition was not completely reduced, S/BAC-filter was slightly more efficient than the S/A + GAC-filter combination. It should be noted that water quality at the ozonation influent was unusual at this sampling campaign as water contained such high concentrations of nitrite that even an elevated applied ozone dose of 17 $mgO_3/L$ was not sufficient to achieve the desired specific ozone dose of 0.7 $mgO_3/mgDOC$. Also it is not possible to directly link the results of the ozonation influent and effluent for this sampling campaign due to the issues of the automatic sampler described in the chapter “Sampling and SPE procedure”. In general, WWTP Berlin results show that all the post-

treatments minimize *D. subspicatus* growth overstimulation compared to influent wastewater samples (as seen by the decline of negative inhibition values).

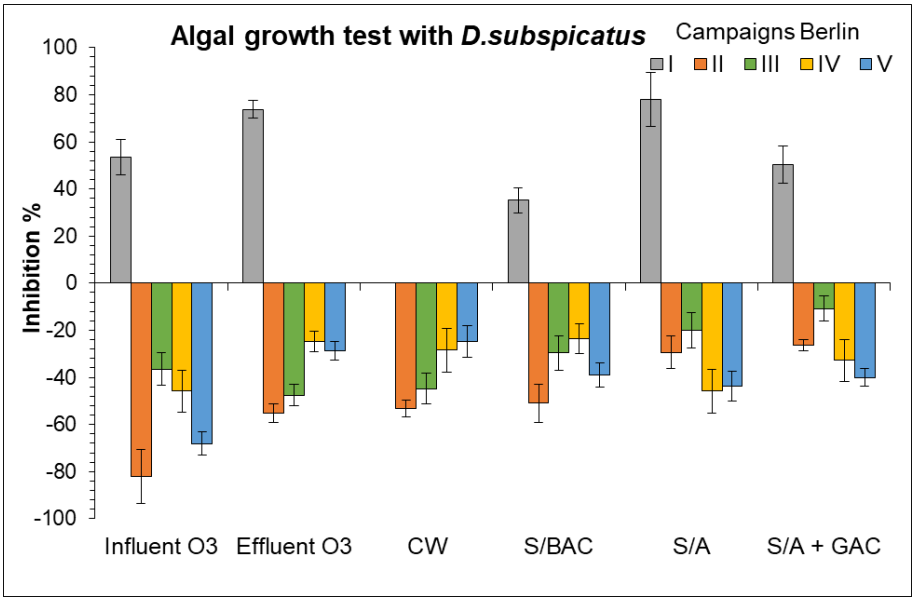


*Figure 16: Overview of growth inhibition mean values with standard deviation (6 replicates) for Berlin native samples (94.3% v/v)*

1st and 3rd of the three sampling campaigns carried out in Kalundborg did not demonstrate an algae growth inhibition before or after ozonation at the highest incubation concentration (Figure 17, left). Growth inhibition effects were seen in the ozonation influent and effluent samples at the 2nd sampling campaign, but were not observed after the MBBR post-treatment. However, the results do not provide clear evidence that MBBR minimizes overstimulation of algae growth, since there was no decline of negative inhibition values in 1st and 3rd sampling campaign, like in the case of post-treatments at WWTP Berlin. For the sampling campaigns at WWTP Linköping, none of the samples indicated growth inhibition. However, decline of negative inhibition value after MBBR treatment in 2nd sampling campaign suggested minimized algae growth promotion (Figure 17, right).

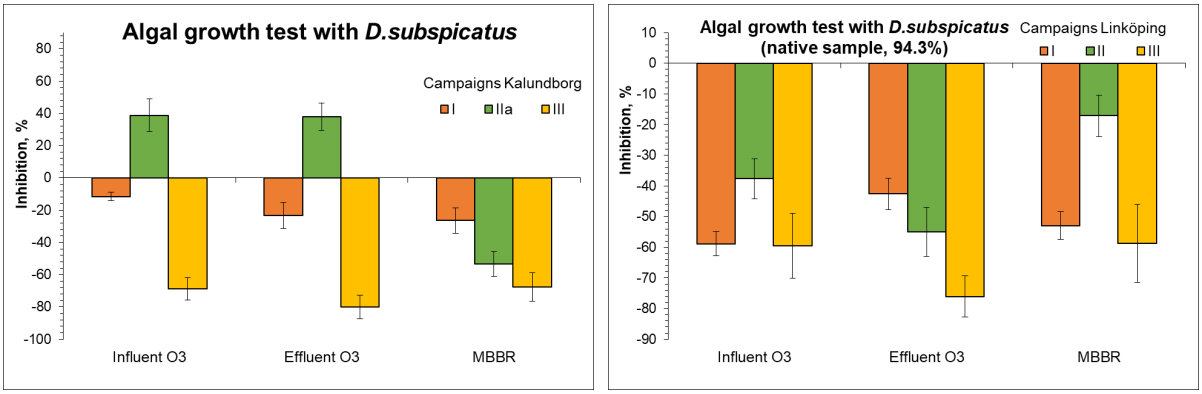


*Figure 17: Overview of growth inhibition mean values with standard deviation (6 replicates) for WWTP Kalundborg (left) and WWTP Linköping (right) using native samples (94.3% v/v)*

To summarize the results of all WWTP sampling campaigns, native samples caused algal growth inhibition only occasionally. Lowest ineffective dilution (LID) was determined for the samples where growth inhibition was detected. Decreased value of LID that can range from <5.9% to 94.3% indicates a higher risk of ecotoxicity. SI-Table 35 in the appendix summarizes the tested sample concentration at which growth inhibition is below 5% inhibition. These results demonstrate that all samples that indicate adverse biological effects (sampling campaigns BLN I and KAL IIa) have a high dilution (≤ 5.9% of wastewater volume per total volume of test solution) and therefore potentially have a profound impact on the environment.

Treated wastewaters are often characterized by high concentrations of nutrients, namely phosphates and nitrates, which are essential for algal growth. These compounds can not only mask toxic effects due to growth stimulation but are also considered as contaminants that are not removed by ozonation (Fatta-Kassinos et al., 2016; Kienle et al., 2011). The results of this study confirm that most of the tested samples enhance algae growth, although it is not possible to determine whether nutrients or other growth factors like trace metals (e.g. Fe, Co, Cu, Mg, Mo, Zn) facilitated this process.

The comparison of post-treatment methods as means of reducing algal growth inhibition is limited based on obtained results since most sampling campaigns (BLN, LIN, KAL) did not show any inhibition. However, 1st campaign of Berlin tests suggests biological activated carbon and granular activated carbon (S/A+GAC) as a promising treatment to reduce ecotoxicological effects. This is supported by an extensive study done by Margot et al. (2013) that evaluated a significant removal of algal growth inhibition by powdered activated carbon.

Wastewater screening for specific environmental contaminants has limitations and does not consider chemical mixture effects, whereas ecotoxicological assays such as algae growth inhibition facilitate combined contaminant risk evaluation for samples in different treatment stages. The algae growth test method is standardized, sensitive and not restricted by the complexity of sample matrix. It is also recommended to use combined ecotoxicological assays that investigate algal growth and photosynthetic activity inhibition (Kienle et al., 2019). Although the algae growth test is widely used in WWTP performance assessment, project results indicate that it is difficult to evaluate the impact of the ozonation process with this tests as in most cases a growth stimulation was determined instead of a growth inhibition.

#### Ceriodaphnia dubia reproduction test

Reproduction inhibition tests (7 +/-1 days) with freshwater cladocerans *C. dubia* were performed with samples from WWTP Berlin from four sampling campaigns (I, II, III and IV) at six sampling points. Test results from the influent and effluent of the ozonation as well as from the effluent of the S/BAC filter at the first sampling campaign were not included because of a decreased amount of neonates in test control. Thus, the test did not meet the requirements of the standard, but as adult mothers for the test (control and concentration) came from the same culture, tests results are indicative.

As shown in Figure 18, for most of the tests performed with samples from WWTP Berlin, reproduction inhibition of *C. dubia* did not reach 50%. However, $EC_{50}$ was reached in 1st campaign for the S/A (15.92%) and S/A + GAC-filter samples (63.68%), in 2nd campaign for the effluents of the ozonation (6.28%) and the S/A + GAC-filter (16.00%) and in the 3rd campaign at the ozonation influent (42.85%) and the S/A-filter samples (77.62%) as well as in the 4th campaign at the ozonation effluent (61.80%) and the S/A-filter samples (75.68%). Raw data can be found in the appendix in SI-Table 36. It is confirmed in literature that tertiary treatment with constructed wetlands (CW) and GAC filters show a decrease in toxicity towards *C. dubia* (Gustavson et al., 2000; Huddleston et al., 2000). Similar results were obtained with samples from WWTP Berlin. Results of the analysed samples from all campaigns could indicate a reduction of toxicity by the constructed wetland. The performance of the three deep-bed filters showed some distinct differences. Compared to the $EC_{50}$ value at the effluent of the ozonation, S/BAC filter was able to consistently remove all adverse effects on C. dubia, whereas S/A filter only had a positive impact in two out of three sampling campaigns. The further treatment with a GAC filter (S/A + GAC) had a positive impact in most cases (three out of four sampling campaigns), but surprisingly showed an adverse effect at the 2nd sampling campaign that couldn't be explained. Also, no conclusion about the treatment with ozone can be made as the overall impact of the ozonation is unclear. Therefore, in order to access the impact of ozonation on reproduction inhibition of *C. dubia* more sampling campaigns and samples should be tested.

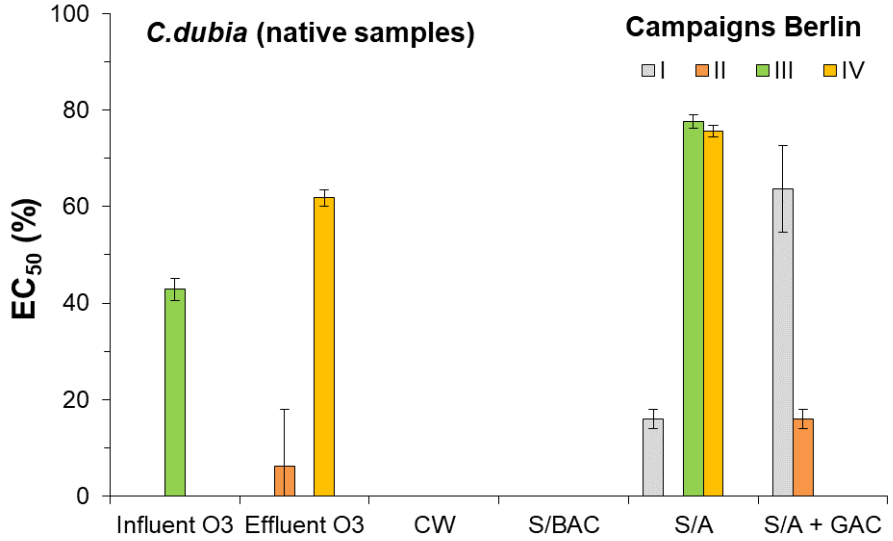


*Figure 18: Results of C. dubia reproduction inhibition test of samples taken from WWTP Berlin. Note that lower $EC_{50}$ values indicate higher toxic effects, whereas no bars indicate $EC_{50}$ was not reached. For the influent and effluent of the ozonation as well as for the S/BAC of the first sampling campaign no results are displayed due to decreased amount of neonates in test control. Also, at the first sampling no sample has been taken for the CW.*

Inhibition tests with *C. dubia* were performed with samples from WWTP Linköping from two sampling campaigns (I and II) at three sampling points. The evaluation of the samples from the 3rd sampling campaign could not be conducted as sample labels went off the bottles during transportation and, thus, a proper identification was not possible.

Reproduction inhibition of *C. dubia* did not reach 50% in any of the tested samples, except for the sample at the ozonation effluent of sampling campaign I for which the EC50 value was 13.77% (Figure 19 left, SI-Table 38). For all other samples, reproduction inhibition was observed at sample concentrations of 28.26% - 44.67% compared to control at a sample concentration of 98.87%. At one of the two sampling campaigns results showed a tendency of negative impact of the ozonation process *on C. dubia*, which could be removed by the MBBR post-treatment. However, more samples should be tested in order to make a clear statement.

Inhibition tests with *C. dubia* were performed with samples from WWTP Kalundborg from three sampling campaigns (I, IIa and IV) at three sampling points. At tests with samples from the ozonation influent, an $EC_{50}$ was observed at sample concentrations between 15.78% and 20.09%. After the ozonation stage $EC_{50}$ values were similar (16.44 – 25.35%; Figure 19 right, SI-Table 37). Therefore, it can be stated that the ozonation process did not influence the reproduction of *C. dubia*. However, in all sampling campaigns the effluent of the MBBR post-treatment showed an increased reproduction inhibition compared to previous treatment stages ($EC_{50}$ at 0.52 and 9.5 % wastewater content in the test) indicating a negative impact of MBBR post-treatment.

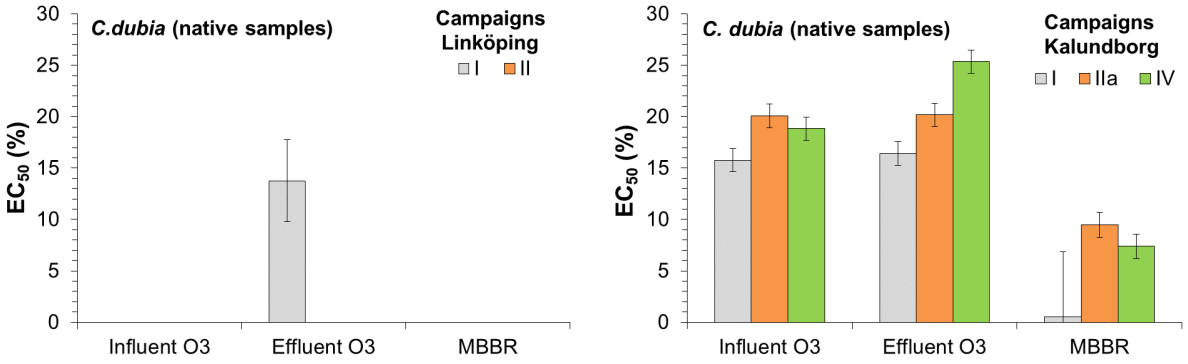


*Figure 19: Results of C. dubia reproduction inhibition test of samples taken from WWTP Linköping (left) and WWTP Kalundborg (right). Note that lower $EC_{50}$ values indicate higher toxic effects, whereas no bars indicate $EC_{50}$ was not reached.*

Moreover, during tests with samples from WWTP Kalundborg, an increased mortality of adult *C. dubia* female organisms was observed depending on wastewater

concentration. The mortality of adult *C. dubia* at the highest used sample concentration (98.87%) always reached 100% (all adults died) between the first and the sixth day of the test. The increased mortality of the mothers affects the $EC_{50}$ value as it is highly dependent of the amount of grown adult *C. dubia* female organisms in the tests. Thus, reproduction inhibition was also 100% as no neonates were produced (see Figure 20). Therefore, calculated $EC_{50}$ values have to be handled with care and cannot directly be compared to the results of the other WWTPs as they might not reflect reproduction toxicity effects in the same way. During the conduction of the tests, an increasing pH drift within the range of 6 to 9 was observed, which might be attributed to the carbonate system (see also GoA 3.2 report). As metal speciation and bioavailability and, thus, metal toxicity changes within this pH range (Schubauer-Berigan et al., 1993), this could have impacted the potential toxicity of the samples.

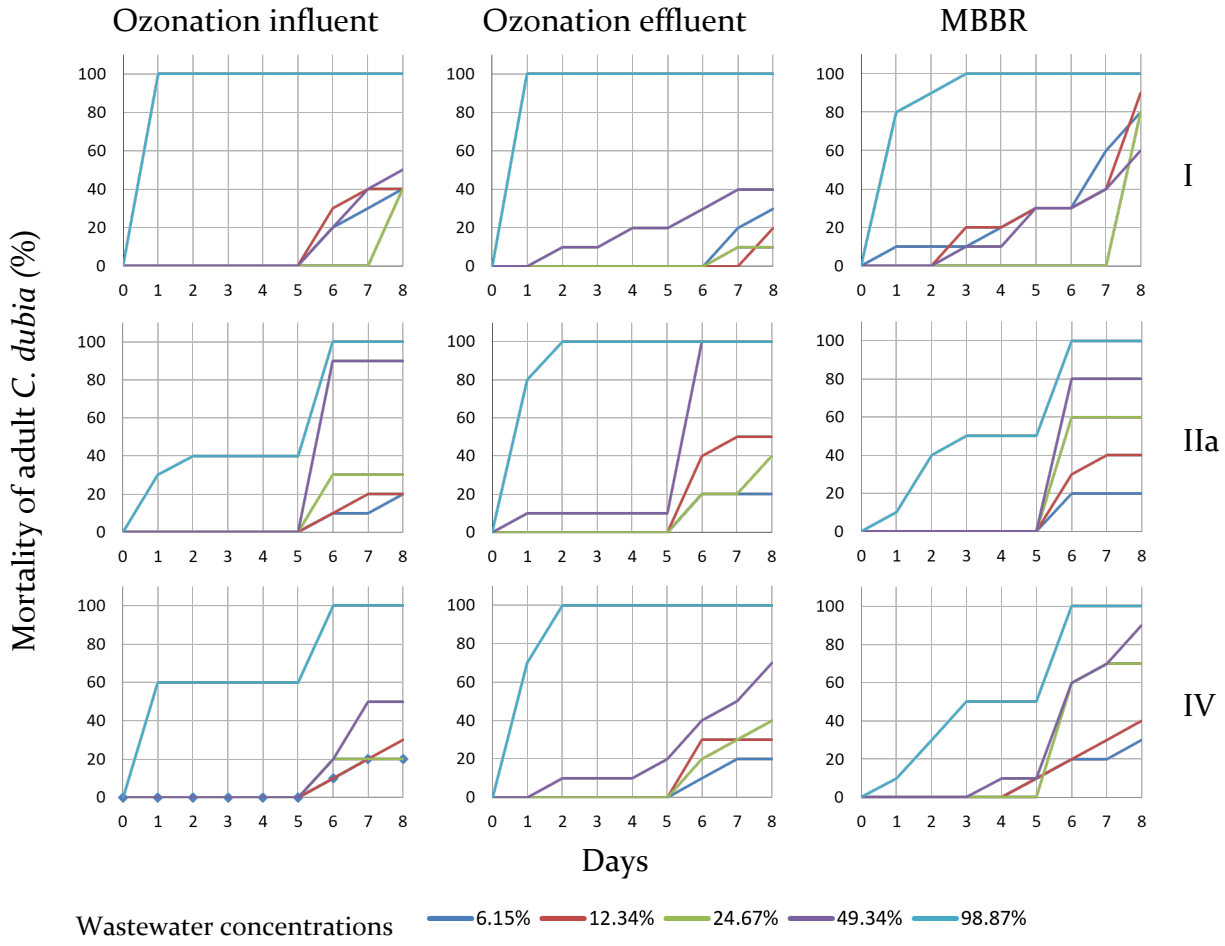


*Figure 20: Results of C. dubia mortality of the three sampling campaigns at WWTP Kalundborg (I, II and IV) at different sampling points.*

Nandini et al. (2004) have investigated the growth of several Cladocera species in different stages of treated wastewater. The results of their study showed that *C. dubia* as well as *Daphnia pulex* showed no population growth in untreated wastewater and partially treated wastewater and only a slight increase in growth in treated wastewater (not chlorinated). However, other studies indicate that acute and chronic toxicity towards *C. dubia* at WWTP effluent can be reduced by a further treatment in a wetland (Hemming et al., 2002). Peitz and Xavier (2016) reported that MBBRs are effective in removing a number of APIs in wastewater and reducing toxicity to crustaceans *Daphnia magna*. Compared to *Daphnia magna* and *D. pulex, C. dubia* is better suited to evaluate toxicity of wastewater, because of the shorter required period for reproduction tests than *Daphnia magna* and *D. pulex* (Tamura et al., 2017). But it is suggested that benthic invertebrate community could be a more sensitive test organism and indicator of effluent toxicity and that the WWTP effluent effect on organisms is the highest in summer (Kosmala et

al., 1999). Based on the evaluation of freshwater samples in the study of Tamura et al. (2017) a contribution for toxic effects on *C. dubia* were found for pharmaceuticals acetaminophen and levofloxacin, whereas for toxicity towards unicellular green algae *Raphidocelis subcapitata* sulfamethoxazole, azithromycin and clarithromycin were identified as relevant APIs. This means that algae are more sensitive to antibiotic presence in water than *C. dubia* (Tamura et al., 2017). An evaluation of eco-genotoxicity caused by anti-cancer drugs, it was revealed that *D. magna* and *C. dubia* were both sensitive to the DNA damage, however, in most of the cases *C. dubia* was slightly more sensitive than *D. magna* (Parrella et al., 2015). It is also been found that the major toxicants in wastewater for *C. dubia* are metals such as zinc and nickel (Viganò et al., 1996). Moreover metal toxicity can change depending of pH values, especially for zinc and nickel where toxicity to *C. dubia* increases with increase to pH value (Schubauer-Berigan et al., 1993).

Blatchley et al. (1997) studied *C. dubia* survival in effluent samples after disinfection processes. Ozonated effluent samples collected from WWTP in same region showed an improved *C. dubia* survival, whereas a repeated test showed an extremely toxic effluent to *C. dubia*. Therefore, the authors suggest to have a large data base for identifying toxicological response trends. Schindler Wildhaber et al. (2015) proposed a modular laboratory decision tool to test the feasibility of an ozonation treatment stage as an option for an advanced wastewater treatment, which also includes a module for bioassays to measure specific and unspecific toxicity and its change by ozonation and biodegradation, respectively. With respect to the impact on *C. dubia*, they reported different results: Effluents of WWTPs, which treat mainly domestic wastewater, showed a reduction in toxic effects due to the ozonation process, whereas ozonation of industrial wastewaters resulted in an increase of toxicity. Biological post-treatment, simulated by spiking of non-ozonated WWTP effluent to the ozonated sample and incubation for 7 days at 25°C, produced mixed results as it could either result in a removal or increase of toxicity, which was also seen by the three sampling campaigns at WWTP Kalundborg which receives a high share of industrial wastewater. However, depending of the type of industry wastewater composition can vary a lot and, thus, a high share of industrial wastewater alone does not directly indicate a potential risk. As possible explanation for the increase of toxicity by the post-treatment sensitivity of daphnids to metals such as copper or zinc was mentioned by Schindler Wildhaber et al. (2015) as a change in dissolved organic matter can alter the metal speciation.

In conclusion of the literature analysis, *C. dubia* is considered suitable for the evaluation of wastewater toxicity. However, numerous authors claim contradicting results indicating that treated wastewater can increase or decrease *C. dubia* survival and reproduction as this species is sensitive to untreated and treated wastewater, especially ozonated wastewater. *C. dubia* is also very sensitive to the environment and certain toxicants in wastewaters such as pharmaceuticals and heavy metals increase the toxicity of the wastewaters. Because of this sensitivity a large data set is needed to draw any conclusions about the effects of wastewaters on *C. dubia*. It is also necessary to evaluate the right choice of test organisms used for ecotoxicological evaluation of wastewaters as different test species could be more sensitive to specific compounds.

### Summary of Chronic tests

Bioassays with algae *D. subspicatus* indicate growth stimulation for an overwhelming number of samples (40 out of 47 tested samples). Overall, ozonation had no measurable effect, however, results for post-treatment with S/BAC-filter as well as the combination of a S/A- and GAC-filter suggest decrease of growth promotion. Similar to *C. dubia*, *Desmodesmus subspicatus* growth inhibition tests are widely used, sensitive and do not require SPE procedure, even if an adaption to SPE extracts is possible. Although response reactions for tested samples could not be interpreted with $EC_{50}$, algae bioassay should be considered in wastewater testing. However, usability regarding the evaluation of the change by ozonation and/or according post-treatment seems to be limited.

*Ceriodaphnia dubia (C. dubia)* reproduction test (ISO 20665:2008) measures the chronic toxicity of water samples (e.g. surface water, secondary effluent of WWTP). Determination of the chronic toxicity is based on reproduction inhibition of the test organisms at different sample concentrations compared to control. Results were expressed by the share of wastewater in the

sample batch that induces reproduction inhibition of 50% ($EC_{50}$) of the population compared to the control at the end of the test. Lower $EC_{50}$ values indicate higher toxic effects. Test results show variable $EC_{50}$ values for ozonated effluents, indicating that ozonation can increase or decrease toxicity for *C. dubia* depending on the water quality of the effluent. However, as $EC_{50}$ values were not always obtained in samples from the three WWTPs results are inconclusive and further sampling campaigns would be necessary to get a clearer picture of the impact of the ozonation and post-treatment, respectively.

## Summary and recommendations for ecotoxicological assessment

The impact of the ozonation and the according post-treatments on the ecotoxicological endpoints neurotoxicity, mutagenicity, genotoxicity, endocrine effects, growth and reproduction are summarized in Table 7. All three tests for estrogenicity (2 x YES and 1 x ER-Calux) show that ozonation reduces the remaining estrogenic potential of the wastewater at the effluent of the conventional treatment below the limit of quantification and without any further detectable reduction by the according post-treatments. For other endocrine endpoints neither effects (androgenicity) nor systematic trends (anti-estrogenicity) could be determined. Beneficial effects of the ozonation were also determined with the *Aliivibrio fischeri* bioluminescence inhibition test. The results also indicate that a further toxicity reduction can be archived by all investigated post-treatments, especially with a constructed wetland and the combination of the sand/anthracite- and granular activated carbon filter (S/A + GAC), respectively.

A systematic increase of the mutagenic potential (Ames test with different strains) by an ozonation stage was not observed with an enrichment factor of 10. A higher EF of 1000 tested by the Ames test with *S. typhimurium* strain YG7108 allowed a more detailed view on mutagenic effects. At WWTP Berlin strain YG7108 without metabolic activation (-S9) revealed such a systematic increase of mutagenicity by ozonation at an enrichment factor of 1000, but not yet at an enrichment factor of 10. However, all post-treatments were able to reduce these mutagenic effects after ozonation, thus, highlighting the need for a post-treatment to minimize potential toxic compounds formed during the ozonation process. No conclusion on the removal of mutagenicity by a MBBR can be made as no systematic patterns of mutagenic effects have been determined at the WWTPs Kalundborg and Linköping. Results obtained by using Ames strains TA1535 and TA1537 with a fEF of 10 indicated occasionally non-systematic effects at the ozonation influent and effluent, respectively, which were not detectable after the according post-treatment stages. The latter highlights the need for higher enrichment factors for evaluating mutagenic effects with Ames strains properly.

Tests for neurotoxicity and genotoxicity showed no ecotoxicological effects at a final enrichment factor of 10. In contrast, tests with *Pseudomonas putida* resulted in most cases in a growth stimulation instead of a growth inhibition. Likewise, bioassays with algae *D. subspicatus* indicated growth stimulation with most samples and no measureable effect of the ozonation. Results of the reproduction tests with *Ceriodaphnia dubia*, which are widely used in determining acute and chronic toxicities of WWTP effluents, showed inconclusive results regarding the impact of the ozonation and post-treatment. Findings at WWTP Kalundborg showed a consistent increased mortality of adult *C. dubia* female organisms regardless of the sampling point, which prevents to draw clear conclusions without a further, more detailed investigation.

*Table 7: Overview of test ecotoxicological test results. Time for the conduction of the test does not include SPE enrichment. Qualitative cost estimation are based on project internal cost comparison. Some test kits allow the assessment of multiple endpoints (e.g. evaluation of YES/YAS at IOS) at the same time (*).*

| Bio-assay | SPE | Lab | Time | Costs | Ozonation | | | MBBR | | Deep-bed filter | | | CW |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | KAL | LIN | BLN | KAL | LIN | S/BAC | S/A | S/A + GAC | BLN |
| **Neurotoxicity** (*In-vitro* ) | | | | | | | | | | | | | |
| Acetylcholinesterase inhibition | Yes | IOS | < 1 day | low | *no toxicological effects found* | | | | | | | | |
| **Mutagenicity** (*In-vitro* ) | | | | | | | | | | | | | |
| Ames (TA1535, -S9) | Yes | IOS | 4 days | medium | →/↑ | → | → | →/↓ | → | →/↓ | →/↓ | →/↓ | →/↓ |
| Ames (TA1535, +S9) | | | | | →/↓ | → | →/↑ | → | → | →/↓ | →/↓ | →/↓ | →/↓ |
| Ames (TA1537, -S9) | Yes | IOS | 4 days | medium | →/↓/↑ | → | → | →/↓ | → | → | → | → | → |
| Ames (TA1537, +S9) | | | | | *no toxicological effects found* | | | | | | | | |
| Ames (YG7108, -S9) | Yes | UBA | 3 days | medium | → | → | ↑ | → | → | ↓ | ↓ | ↓ | ↓ |
| Ames (YG7108, +S9) | | | | | *no toxicological effects found* | | | | | | | | |
| **Genotoxicity** (*In-vitro* ) | | | | | | | | | | | | | |
| SOS Chromotest (+/-S9) | Yes | IOS | < 1 day | medium | *no toxicological effects found* | | | | | | | | |
| UmuC | Yes | UBA | 2 days | medium | *no toxicological effects found* | | | | | | | | |
| **Estrogenicity and androgenicity** (*In-vitro* ) | | | | | | | | | | | | | |
| YES (estrogenicity) | Yes | IOS | 6 days | medium* | → | →/↓ | ↓ | → | → | → | → | → | → |
| YES (anti-estrogenicity) | | | | | →/↓/↑ | →/↑ | →/↓/↑ | →/↓/↑ | →/↓ | →/↓/↑ | →/↓ | →/↑ | →/↓ |
| YAS (androgenicity) | | | | | *no toxicological effects found* | | | | | | | | |
| YAS (anti-androgenicity) | | | | | →/↓ | →/↓ | →/↓ | → | →/↑ | → | → | → | → |
| YES (estrogenicity) | Yes | UBA | 3 days | medium | → | ↓ | ↓ | → | → | → | → | → | → |
| Anti-YES (anti-estrogenicity) | Yes | UBA | 3 days | medium | ↓ | ↓/↑ | →/↑ | ↓/↑ | ↓/↑ | →/↓ | →/↓ | →/↑ | →/↓/↑ |
| ER-Calux (estrogenicity) | Yes | UBA | 3 days | medium | → | ↓ | ↓ | → | → | → | → | → | → |
| **Bacteria tests** (*In-vivo* ) | | | | | | | | | | | | | |
| Growth inhibition (*Pseudomonas putida*) | Yes | IOS | 1 day | low | *results show growth stimulation instead of inhibition* | | | | | | | | |
| Bioluminescence inhibition (*Aliivibrio fischeri*) | Yes | IOS | 2 h | low | ↓ | ↓ | ↓ | ↓ | →/↓ | →/↓ | →/↓ | ↓ | →/↓ |
| **Chronic tests** (*In-vivo* ) | | | | | | | | | | | | | |
| Algae growth inhibition (*Desmodesmus subspicatus*) | No | LIAE | 3 days | low | → | → | → | →/↓ | → | →/↓ | → | →/↓ | → |
| Chronic reproduction (*Ceriodaphnia dubia* ) | No | LIAE | 7 days | high | →/↓ | →/↑ | →/↓/↑ | ↑ | →/↓ | →/↓ | →/↓/↑ | →/↓/↑ | →/↓ |

negative effect / potential postive effect
positive effect / potential positive effect
unclear effect
no effects measured

↑ increase of effect compared to preceeding sampling point
→ no change of effect compared to preceeding sampling point
↓ decrease of effect compared to preceeding sampling point

A set of ecotoxicological tests that can provide reliable systematic results should be used for the evaluation of the ozonation and the according post-treatments. Based on the approach used within this project, it is therefore recommended to use a set of tests, which covers the evaluation of mutagenic effects (AMES), estrogenicity (YES or ER-Calux) and bioluminescence inhibition (Microtox).

All these tests require the use of enriched samples. Due to the screening character of this study, practical limitations required to use a maximal enrichment factor (EF) of 10 in the test. Nevertheless, with this reduced set of bioassays also higher EFs of 100 or 1000 can be feasible and would probably lead to clearer results ('the dose makes the poison'). However, interpretation of ecotoxicological findings would in turn be more abstract as the effluent of full-scale WWTPs is diluted by the recipient and not enriched. Thus, tests with such high enrichment factors should primarily be used to assess the impact of single treatment stages (ozonation, post-treatment) on the different ecotoxicological endpoints and not to determine potential risks of the produced water quality on e.g. aquatic life.

In addition to the qualitative indication of the test costs provided in Table 7 a price check at several laboratories in Germany was conducted to evaluate the costs for the recommended test set. Including the sample enrichment (SPE) assessment of mutagenicity, estrogenicity and bioluminescence inhibition would cost between 810 and 1470 € for one sample. Thus, a full evaluation with samples from the influent and effluent of the ozonation as well as the post-treatment effluent would cost between 2400 and 4400 €. Naturally, provided price range is only valid for Germany and may be different in other countries.

# Water quality parameters

The interaction between water quality parameters and the ozonation process as well as the post-treatment options are summarized within this chapter. Findings are based on the sampling campaigns conduced for the ecotoxicological evaluation as well as on the experiences of the long-term operation, which are described in more detail in the reports of GoA3.1 and GoA3.2, respectively.

## Impact of ozonation on water quality parameters

As can be seen in more detail in the next chapter, performance of the ozonation process in respect to API elimination strongly depends on the specific applied ozone dose, which is the applied ozone dose normalized for the dissolved organic carbon (DOC). In addition, nitrite can have a relevant impact on the ozonation process as it is rapidly transformed into nitrate by consuming about 3.4 $mgO_3$/mg-N. Thus, nitrite concentration of 0.5 mg-N/L can consume about 1.7 $mgO_3$/L, which corresponds to about 20% of the applied ozone at the WWTPs Berlin and Linköping. As a consequence, less ozone is left for the API oxidation and a correction of the applied ozone dose ($mgO_3$/L) by the amount used for nitrite oxidation is necessary to assess the "effective" specific ozone dose. However, nitrite is usually not measured at the WWTP effluent on a regular basis as it is not considered to be a relevant parameter for the general WWTP performance. In case it is planned to evaluate a possible upgrade of an existing WWTP for an advanced wastewater treatment with ozone it is therefore strongly recommended to conduct regular measurements for DOC as well as for nitrite at the secondary effluent in order to get a solid data basis for the evaluation of the required ozone production capacity. In contrast, ammonium is not impacted by the ozonation process directly as its reactivity with ozone is far too low (reaction rate constant of 20 - 40 $M^{-1}s^{-1}$ (von Sonntag and von Gunten, 2012)).

Even though the organic background of the water (DOC) is considered one of the most relevant water quality parameter for the ozonation process, commonly applied specific ozone doses of less than 1 $mgO_3$/mgDOC do not result in a significant reduction (mineralization) of the DOC by the ozonation process. In contrast, COD reduction by the ozonation stage increases with higher specific ozone doses but usually does not exceed 17% when ozonation is used for API elimination. If ozonation is located subsequent of a well functioning clarifier, the remaining low levels of suspended solids do usually not affect the API elimination efficiency of the ozonation process. However, at WWTP Linköping a reduction of the suspended solids by around 10% within the ozone reactor was observed as well as foaming, which can harm the ozone offgas treatment system. Nevertheless, it remains unclear what caused the reduction of the suspended solids as sedimentation within the ozone reactor is not likely due to the good plug flow. But as this effect hasn't occurred at the other two sites, it seems to be a site specific issue. Bromide can be transformed by the ozonation process into cancerogenic bromate depending on the present bromide level as well as the applied specific ozone dose. Operation of the ozonation at the usual setpoint resulted in a bromate formation of up to 6 µg/L at the pilot plant in Berlin (Figure 21) and about 100 µg/L in Kalundborg, respectively, which is higher than the acute as well as chronic quality standard for bromate of 50 µg/L in surface waters proposed by the Swiss Oekotoxzentrum[7]. The reason for this comparable high bromate formation is due to the elevated bromide levels at WWTP Kalundborg (≈ 2 mg/L), which has its origin in industrial wastewater as well as potential sea water intrusion into the municipal sewer system. At WWTP Linköping, no relevant bromate levels (< 5 µg/L) have been detected at the ozonation effluent. In addition to the API elimination, ozonation can also result in a certain disinfection of the wastewater, which depends on the applied specific ozone dose. At WWTP Linköping about 1-log reduction for intestinal enterococci and *E. coli* was observed at a specific ozone dose of 0.5 $mgO_3$/mgDOC. Also results of another research project (AquaNES), which was also conducted at the same pilot

[7] https://www.ecotoxcentre.ch/expert-service/quality-standards/proposals-for-acute-and-chronic-quality-standards, accessed at 29.05.2020

plant at WWTP Berlin, showed a significant reduction of *E. coli* and Enterococci of more than 2 log-units at an applied ozone dose of about 0.7 $mgO_3$/mgDOC (Brunsch et al., 2019).

### Impact of ozonation post-treatment on water quality parameters

In general, the ozonation of the wastewater results in an improved biodegradability of the bulk organic background matrix. In addition, dissolved oxygen concentration are strongly increased at the ozonation effluent (up to 20 mg/L), which affected the MBBRs in Linköping and Kalundborg. In Linköping, high dissolved oxygen peaks reached the denitrification stage during high flow events with a high ozone production, which required an increase of the carbon source dosage. Similarly and due to already very low ammonium levels, the whole MBBR system at WWTP Kalundborg was aerobic. In case of elevated ammonium concentrations at the ozonation effluent, the oxygen oversaturation can support the nitrification at the post-treatment stage. At the Berlin pilot plant, deep-bed filter systems with an empty bed contact time of about 16 minutes were able to reduce ammonium in case present (see Figure 21). As the rapid filters were always completely aerobic, no denitrification occurred. In contrast and due to the long retention time of about 1 day, the wetland was able to perform nitrification as well as denitrification. In addition, the apparently anoxic conditions within the wetland resulted in a reduction of bromate, which can be used as electron acceptor by microorganisms. All post-treatment options at WWTP Berlin were able to achieve a further COD reduction. Based on the measurements shown in Figure 21, a COD reduction of about 50% was achieved by the wetland as well as by the S/BAC- and S/A-filters. The GAC filter, which was operated subsequent of the S/A filter, increased the overall COD removal to an average of 61%. However, it should be noted that due to the elevated COD levels at the ozonation influent (average 41.7 mg/L), overall COD reduction during the conducted sampling campaigns was higher than the one reported one for the long term operation by Sauter et al. (submitted): Starting at an average COD level of 32.4 ± 5.2 mg/L at the ozonation influent, a similar COD (DOC) reduction of 38% (23%) and 35% (19%) was achieved by the combination of the ozonation and the S/BAC- and S/A-filter, respectively. Compared to its influent, wetland reduced the COD and DOC by 32% and 22%, respectively. Thus, in combination with the impact of the ozonation, the wetland showed a slightly better performance than the deep-bed filters, even though no coagulant was used (Brunsch et al., 2019). At WWTP Linköping an average DOC reduction of about 16% was determined for the combination of ozonation and MBBR with aluminium chloride dosing, whereas an evaluation of impact of the MBBR on the COD was not possible due to analytical limitations. Available data at WWTP Kalundborg showed no additional COD reduction by the MBBR post-treatment, which was explained by the low amount of active biomass on the MBBR carriers.

Besides improvement of some water quality parameters, ozonation post-treatment can also have beneficial effects regarding pathogen indicators such as *E. coli* and Enterococci. At the Berlin site, all post-treatment options (wetland, S/BAC-, S/A- and S/A + GAC-filter) showed a consistent reduction of both indicator parameters, in many cases even below LOQ (Brunsch et al., 2019). In contrast, measurements at WWTP Linköping also indicate a further reduction of Enterococci, coliform bacteria and *E. Coli* by up to 1 log, however, with an strong variation.

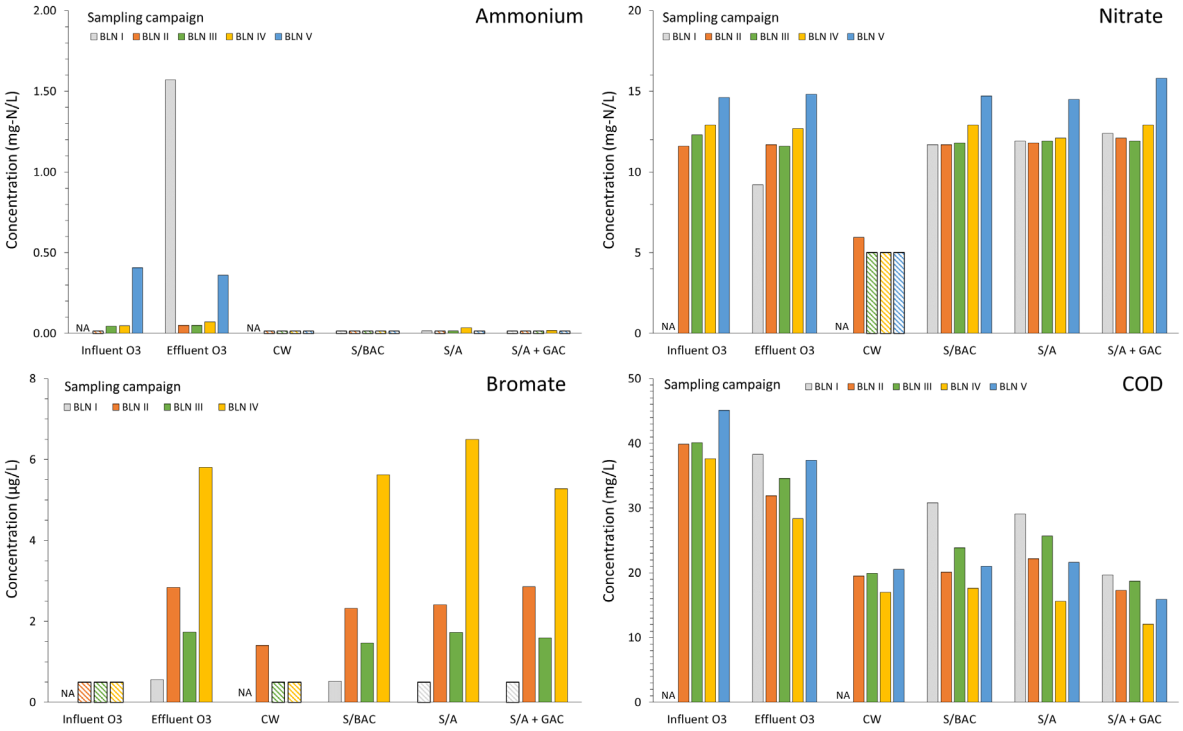


*Figure 21: Concentrations of ammonium, nitrate, COD and bromate measured at the six measurement points at the pilot plant at WWTP Berlin. Shaded bars indicate that results are < LOQ. Note that parameters at the ozonation influent are not included as they did not directly correspond to the ozonation effluent.*

# APIs and transformation products

This chapter summarizes the API elimination and formation of transformation products (TPs) by the ozonation process at the three sites based on the sampling campaigns conduced for the ecotoxicological evaluation as well as on the experiences of the long-term operation, which are described in more detail in the other reports of GoA3.1 and GoA3.2, respectively. In addition, potential benefits of the different post-treatments with respect to APIs and TPs will be evaluated. Details regarding the analytical procedure as well as an overview of the measured APIs and TPs can be found in the appendix section (APIs and transformation products).

## Impact of ozonation on APIs

A demonstration of the correlation between the specific applied ozone dose and the substance specific API reduction at WWTP Berlin was derived by conducting an ozone dose step-response test. For that, the applied ozone dose was varied between 1.4 and 13.2 $mgO_3/L$, which corresponds to a specific ozone dose between 0.1 and 1.3 $mgO_3/mgDOC$, and corresponding grab samples were taken at the influent and effluent of the ozonation plant. Results shown in Figure 22 reveal that APIs that react very fast with ozone such as diclofenac, trimethoprim, carbamazepine, propranolol, phenazone and sulfamethoxazole can already be reduced by more than 90% at a specific ozone dose of 0.3 $mgO_3/mgDOC$. For APIs with a medium reactivity such as citalopram, venlafaxine, tramadol, metoprolol as well as the corrosion inhibitor benzotriazole specific ozone doses between 0.4 and 0.7 $mgO_3/mgDOC$ are required to achieve an 80% reduction by the ozonation process. Some compounds such as the x-ray contrast media diatrizoic acid, iomeprol, iopromide and iopamidol have a very low reactivity with ozone. As a consequence, they can only be attacked by OH-radicals that are always produced during the ozonation of wastewater. However, as the amount of produced OH-radicals is several magnitudes lower than the amount of applied ozone, the reduction of these compounds by the ozonation process is limited and even at elevated specific ozone doses of 1 $mgO_3/mgDOC$ only a reduction by 60% or less can be achieved.

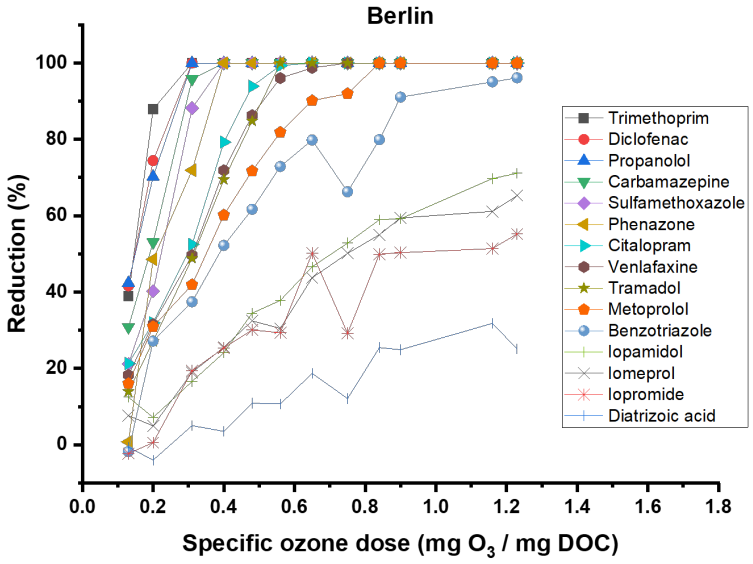


*Figure 22. Reduction of pharmaceuticals in dependence of the specific ozone dose at WWTP Berlin. In case concentration at the ozonation effluent was below LOQ, API reduction is indicated as 100%.*

Similar variations of the ozone dose were also conducted at the other sites and results can be found in the appendix (SI-Figure 2 and SI-Figure 3). In order to take into account the site-specific differences of the ozone dose range (Berlin: 1.4 and 13.2 $mgO_3/L$, Kalundborg: 8.8 – 28.6 $mgO_3/L$, Linköping: 4 – 10 $mgO_3/L$) and water quality, applied ozone doses were corrected for present nitrite and normalized for DOC. In doing so, correlation of the API reduction with the specific ozone dose show a good agreement at all sites and follows the pattern that already has been described for WWTP Berlin (Figure 23).

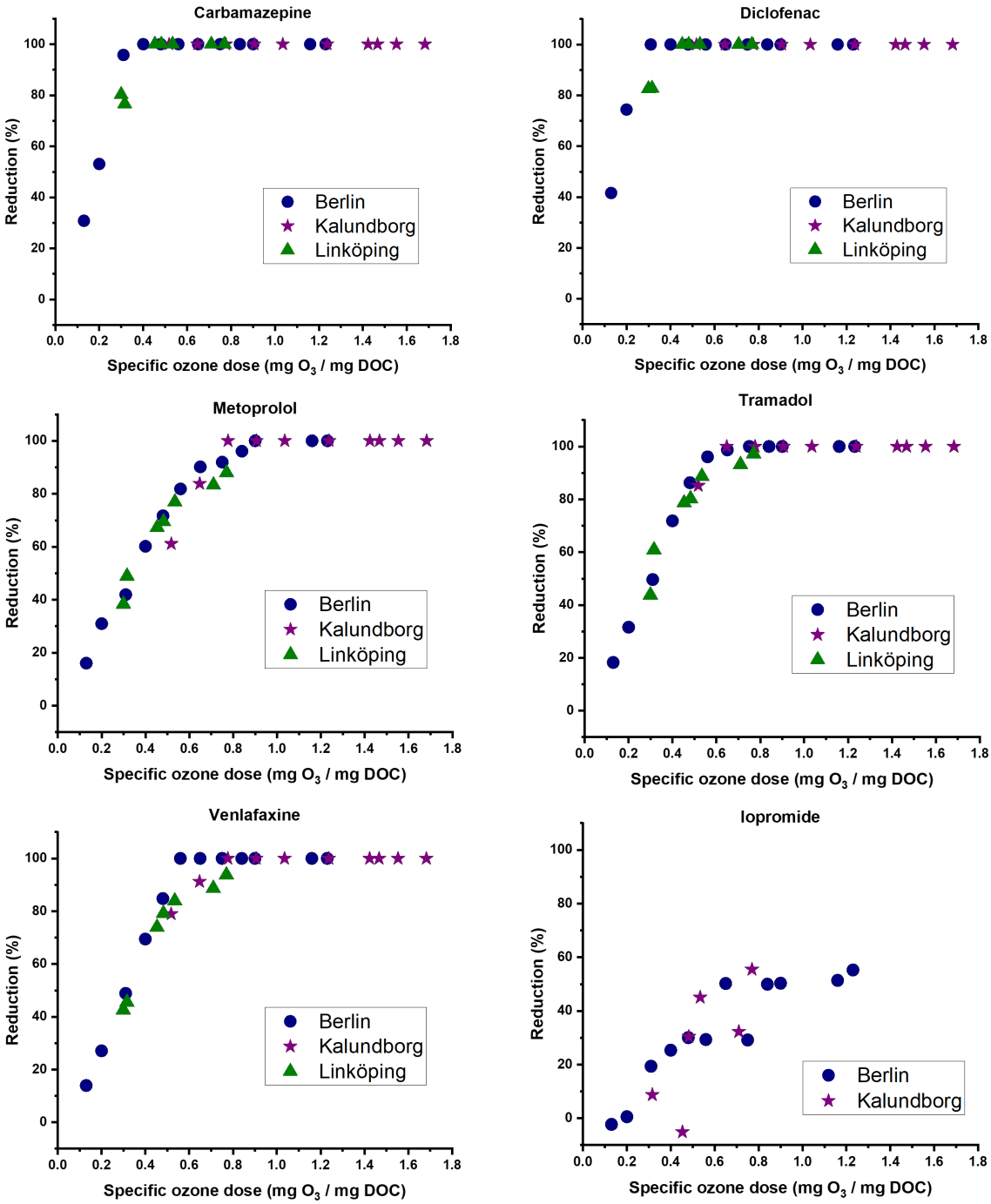


*Figure 23: Comparison of the reduction of carbamazepine and diclofenac (fast reacting compounds), metoprolol, tramadol, and venlafaxine (medium reacting compounds) as well as iopromide (slow reacting compound) in respect to the specific ozone dose at the three evaluated WWTPs in Berlin, Kalundborg and Linköping. In case concentration at the ozonation effluent was below LOQ, API reduction is indicated as 100%.*

## Impact of ozonation on transformation products

In general, ozone transforms APIs into transformation products (TPs) rather than to mineralize them completely. Also intermediate TPs can be formed which can then be further transformed into other TPs. Thus, often it is not possible to derive a closed mass balance and identify all possible TPs formed. What kind of TP is formed from the original API (parent) depends on the reaction site ozone attacks the molecule structure, which are primarily olefins, amines, aromatics or sulfur containing compounds (Hübner et al., 2015). N-oxides, for example, are a major group of TPs that are formed by the reaction of ozone and tertiary amines that are a part of the chemical structure of tramadol or venlafaxine. Within CWPharma, a total of 15 different TPs have been measured. Focus was on TPs of diclofenac (six TPs), carbamazepine (three TPs) as well as on N-oxides of azithromycin, clarithromycin, erythromycin, tramadol, and

venlafaxine. In addition, N-desmethyl tramadol was measured. An overview of the TPs including their according LOQs (limit of quantification) is provided in the appendix (SI-Table 39).

The concentrations of tramadol *N*-oxide and venlafaxine *N*-oxide in the effluent of the three WWPTs with respect to the specific ozone dose are shown in Figure 24. Both N-oxides were below LOQ at the influent of the ozonation, but could be detected in the effluent and, thus, clearly showing their formation by the ozonation process. The total amount of formed N-oxides depends on the applied specific ozone dose. N-oxide concentrations increase with the specific ozone dose and reaches a maximum at around 0.5 to 0.6 $mgO_3$/mg DOC, which corresponds to the ozone dose required for a (almost) complete reduction of the corresponding parent compound (see Figure 23). In case higher specific ozone doses are used, N-oxide concentrations start to gradually decrease, indicating that N-oxides are further oxidized. The same behaviour of the formation and reduction was found for the transformation products BaQD (parent = carbamazepine) and DCF 2,5-Quinone imine (DCF-QIM, parent = diclofenac). However, due to the higher reactivity of ozone with the corresponding parent compounds (see Figure 23), highest concentrations were measured at a specific ozone dose of about 0.3 $mgO_3$/mgDOC (Figure 24). In contrast to the N-oxides, BaQD and DCF-QIM could already be detected in the ozonation influent and, as an example for WWTP Berlin, a specific ozone dose of 0.6 and 0.8 $mgO_3$/mgDOC would be necessary to reduce the TP concentrations below this level again. However, in order to reduce the formed N-oxides completely, very high specific ozone doses of more than 1.2 $mgO_3$/mgDOC would be required that would cause a distinct increase of operational costs and an elevated bromate formation. The formation and removal of TP from APIs was described in more detail in the study of Kharel et al. (2020).

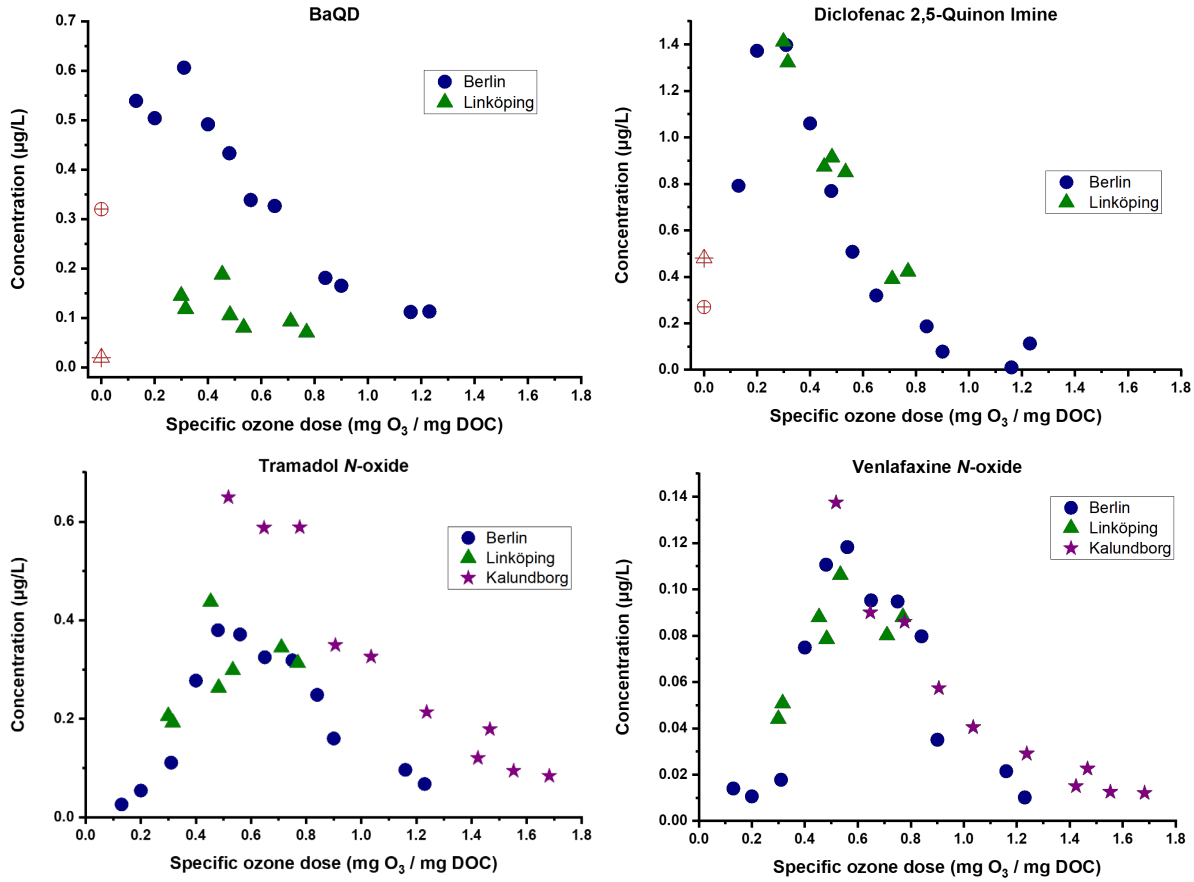


*Figure 24: Concentration of transformation products in the effluent of the ozonation plants. Concentrations at the influent of the ozonation is marked with a '+' sign inside the according symbols. N-oxide concentrations at ozonation influent were below LOQ and, thus, are not shown. At WWTP Kalundborg, BaQD and DCF-QIM was always below LOQ.*

## Impact of post-treatment on APIs and transformation products

Even though applied specific ozone doses between 0.5 and 0.8 $mgO_3$/mgDOC are sufficient to completely remove fast reactive substances such as diclofenac and carbamazepine, residuals of

medium (e.g. gabapentin, benzotriazole, metoprolol) and slow reactive substances (e.g. x-ray contrast media iopromide, iohexol, iomeprol) are still present at the ozonation effluent along with formed transformation products. Thus, their fate at the different post-treatment types at the three sites along with the results of the lab-scale MBBR experiments (see GoA3.2 report) are summarized as residual percentage ($C/C_0$) in Table 8. In total, biological post-treatment types such as the S/A-filter, constructed wetland and MBBR do not show a relevant API reduction, except for an apparent reduction of some x-ray contrast media (iohexol, iomeprol and iopromide) by the wetland. Also, results of the lab-scale MBBR tests calculated for a comparable long retention time of 4 hours indicate only reduction for gabapentin and metoprolol by 50% and 34%, respectively. In contrast, deep-bed filters using granular activated carbon as filter material showed a clear reduction for some compounds. As also known by literature activated carbon is based on sorption processes to the fine pored carbon surface and removes a broad spectrum of organic contaminants and organic carbon in general (Snyder et al., 2007). Ternes et al. (2002) showed that granular activated carbon (GAC) was very effective especially in removing pharmaceuticals. While the S/BAC-filter was only able to reduce benzotriazole and metoprolol by about 40% and 60%, respectively, the GAC filter could also reduce benzotriazole, irbesartan, metoprolol and tramadol by an average of around 80% or more. Considering that the S/BAC filter has already treated more than 4-fold the water volume than the GAC-filter (average 13,500 BV vs. 60,000 BV) it is not surprising that the adsorption capacity of the GAC at the S/BAC-filter is more exhausted.

Likewise, GAC-filtration was also able to reduce transformation products such as N-oxides, which were not affected by the MBBR, S/BAC- and S/A-filter. Treatment by the constructed wetland resulted in a reduction of N-oxides for up to 60%, thus, indicating that these N-oxides are potentially biodegradable. However, it remains unclear if this biodegradation was due to the long retention time of about 1 day or the aerobic/anoxic condition. DCF-QIM was reduced by all post-treatments. While occasionally increasing API concentration (e.g. x-ray contrast media at S/A- and S/BAC-filter, tramadol at S/A-filter and CW) by biotransformation is unlikely and probably an analytical artefact, BaQD formation from BQD[8] (another transformation product formed during ozonation) within biological post-treatment was also described in literature (Hübner et al., 2014). Surprisingly, the BaQD concentration within the GAC-filter was stable, indicating adsorption of either BQD or parts of BaQD (or both in parallel).

In order to highlight the fate of APIs and transformation products within the GAC-filter, a one-time sampling campaign was carried out at which samples were taken at different heights of the GAC-filter bed by using pre-installed sampling points. At the time the sampling campaign was conducted GAC-filter was operated at a low filter velocity of 2.3 m/h, corresponding to an empty bed contact time (EBCT) of about 46 min, and has already treated about 17,130 bed volumes. In Figure 25, relative concentrations ($C/C_0$) of APIs and TPs are shown which were still detectable at the GAC influent. For benzotriazole and metoprolol as well as for the TPs N-desmethyl tramadol and the azithromycin N-oxide, a reduction by 80% or more was achieved within the first 68 cm of the filter bed, indicating that these compounds can easily be adsorbed by GAC. With the exception of the carbamazepine transformation product BaQD, which does not seem to be affected by the GAC-filter, the relative concentrations of all other measured compounds show an almost linear relationship with the filter bed-depth. As deep-bed filters are usually operated at higher filter velocities and the filter bed depth at a fixed flow directly corresponds to the EBCT, a lower API / TP reduction is expected. For example, a filter operation at 8 m/h would correspond to an EBCT of around 13 min and, thus, a filter bed depth of 50 cm in Figure 25. Accordingly, compounds with a very good adsorbability would still be reduced by more than 70%, whereas most of the other compounds would only be reduced by 15% to 40%.

[8] 1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-one

*Table 8. Summary of the residual concentration normalized for concentration at the influent of the post-treatment ($C/C_0$, %) for the different sites along with calculated results for a retention time of 4 hours based on lab-scale MBBR experiments. Colour code is used to highlight decrease ($C/C_0 \leq 75\%$, green) or increase ($C/C_0 > 125\%$, orange) of concentration.*

| APIs | S/BAC (BLN) | S/A (BLN) | GAC (BLN) | CW (BLN) | MBBR (LIN) | MBBR (KAL) | MBBR (LAB) |
|---|---|---|---|---|---|---|---|
| Total samples | n = 14 | n = 14 | n = 7 | n = 5 | n = 2 | n = 4 | calculation |
| Benzotriazole | 41 ± 20 (n = 14) | 106 ± 10 (n = 13) | 11 ± 3 (n = 7) | 83 ± 37 (n = 5) | 92 ± 1 (n = 2) | 83 ± 15 (n = 4) | 96 ± 0.7 |
| Diatrizoic acid | 106 ± 9 (n = 5) | 109 ± 17 (n = 5) | 142 ± 16 (n = 5) | 144 ± 38 (n = 5) | < LOQ | < LOQ | ≈ 100 |
| Gabapentin | 104 ± 18 (n = 14) | 107 ± 15 (n = 14) | 80 ± 12 (n = 7) | 133 ± 26 (n = 5) | 103 ± 7 (n = 2) | 91 ± 15 (n = 4) | 50 ± 9.9 |
| Iohexol | 128 ± 42 (n = 12) | 150 ± 34 (n = 11) | 24 ± 10 (n = 4) | 50 ± 30 (n = 3) | 96 ± 2 (n = 2) | < LOQ | 93 ± 4.3 |
| Iomeprol | 122 ± 33 (n = 14) | 134 ± 28 (n = 13) | 37 ± 19 (n = 7) | 40 ± 28 (n = 5) | < LOQ | 90 ± 46 (n = 4) | 95 ± 3.2 |
| Iopamidol | 119 ± 37 (n = 13) | 134 ± 24 (n = 12) | 23 ± 3 (n = 3) | 182 ± 123 (n = 4) | < LOQ | < LOQ | 97 ± 1.5 |
| Iopromide | 145 ± 44 (n = 11) | 148 ± 27 (n = 10) | 62 ± 17 (n = 4) | 53 ± 37 (n = 2) | < LOQ | < LOQ | 76.9 ± 11 |
| Irbesartan | 96 ± 14 (n = 14) | 101 ± 10 (n = 13) | 6 ± 5 (n = 4) | 114 ± 28 (n = 5) | 90 ± 0 (n = 2) | < LOQ | 96 ± 0.5 |
| Metoprolol | 60 ± 23 (n = 14) | 108 ± 16 (n = 13) | 9 ± 6 (n = 4) | 99 ± 51 (n = 4) | 89 ± 4 (n = 2) | 84 ± 17 (n = 4) | 66 ± 7.8 |
| Tramadol | 109 ± 62 (n = 13) | 150 ± 70 (n = 12) | 22 ± 12 (n = 3) | 206 ± 148 (n = 4) | 111 ± 8 (n = 2) | 98 ± 23 (n = 4) | 98 ± 1.9 |
| TPs | | | | | | | |
| AZI-NOX | 85 ± 28 (n = 9) | 108 ± 35 (n = 9) | < 22 ± 4 (n = 3) | NA | NA | NA | 98 ± 0.3 |
| BaQD | 147 ± 69 (n = 14) | 136 ± 42 (n = 14) | 96 ± 12 (n = 7) | 366 ± 135 (n = 5) | 195 (n = 1) | 87 ± 19 (n = 3) | 94 ± 0.4 |
| CLM-NOX | 101 ± 22 (n = 9) | 87 ± 28 (n = 10) | < 85 ± 3 (n = 7) | 75 ± 20 (n = 4) | 69 (n = 1) | < LOQ | 95 ± 0.4 |
| DCF-QIM | 58 ± 14 (n = 5) | 59 ± 31 (n = 4) | < 7 ± 3 (n = 4) | 77 ± 26 (n = 5) | 70 ± 6 (n = 2) | 55 (n = 1) | NA |
| TRA-NOX | 94 ± 11 (n = 14) | 94 ± 21 (n = 14) | 26 ± 7 (n = 7) | 47 ± 29 (n = 5) | 93 ± 3 (n = 2) | 104 ± 9 (n = 2) | 99 ± 0.1 |
| VLX-NOX | 88 ± 11 (n = 14) | 96 ± 19 (n = 14) | 21 ± 8 (n = 7) | 40 ± 21 (n = 4) | 86 ± 1 (n = 2) | 109 ± 7 (n = 2) | 99 ± 0.1 |

< LOQ means that concentration was below LOQ in at the influent of the according post-treatment, whereas NA represent no data.
< prior to $C/C_0$ value indicate that concentration after the post-treatment was below LOQ; $C/C_0$ was calculated by using C = LOQ
n below C/Co value indicate the amount of samples with concentrations above LOQ

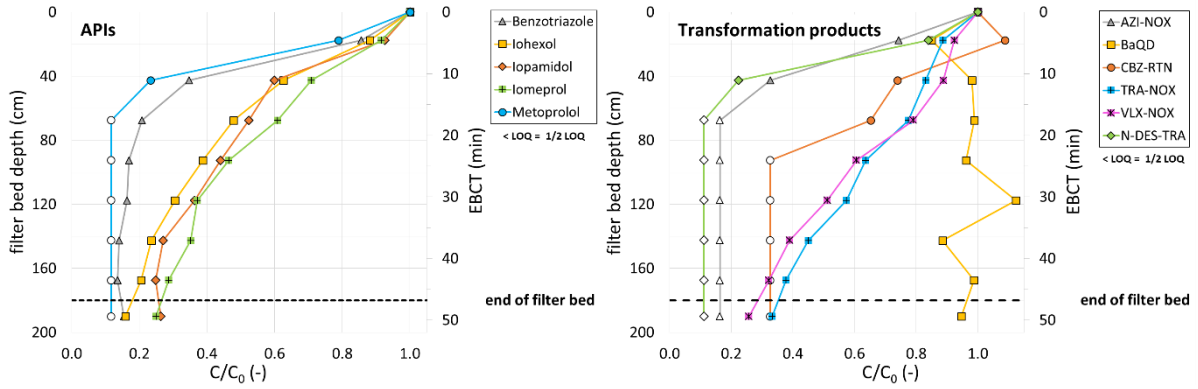


*Figure 25: Relative concentration change ($C/C_0$) in dependency of the GAC filter bed depth for APIs (left) and the following transformation products (TPs, right) at 17,130 treated bed volumes: N-Oxides of Azithromycin (AZI-NOX), Tramadol (TRA-NOX) and Venlafaxine (VLX-NOX), TPs of Carbamazepine (BaQD and CBZ-RTN) as well as N-Desmethyl tramadol (N-DES-TRA). If concentration C was below limit of quantification (LOQ) $C/C_0$ was calculated by using C = ½ LOQ (empty symbols).*

# Summary and recommendations

**Water quality parameters:** At commonly applied ozone doses used for API elimination, no relevant impact on DOC (mineralization) and only a slight reduction of the COD can be expected. A further COD and DOC reduction can be achieved in combination with biological post-treatment options (wetland, deep-bed filter). However, impact of MBBR post-treatment on DOC and COD was limited. Nitrite present at the ozonation influent increases the ozone demand or, in case a constant ozone dosage is used, reduces the performance of the ozonation. WWTPs with high shares of wastewater and/or located close to the sea can have elevated levels of bromide, which can result in a relevant formation of cancerogenic bromate.

The ozonation process results in an oversaturation of the water with oxygen (up to 20 mg/L), which might be beneficial for nitrification processes, but can also have a negative impact on denitrifying post-treatment stages when the oxygen is not completely consumed at a subsequent treatment stage. The constructed wetland at the Berlin site with a retention time of about 1 day was able to perform nitrification as well as denitrification. Measurements at the wetland also indicate that the apparent anoxic conditions result in a bromate reduction. Thus, a post-treatment with anoxic conditions (e.g. wetland, MBBR) might be beneficial in case a relevant bromate formation occurs at the ozonation stage. However, this aspect was not investigated in detail and therefore more research is needed to support this statement.

In addition, a certain wastewater disinfection by the ozonation process can be achieved. At the WWTPs Linköping and Berlin, a reduction *E. coli* and Enterococci between 1 and 2 log units could be achieved. Post-treatment might also be beneficial for the reduction of pathogen indicators. Even though available data do not allow a cross-comparison of the post-treatment options, wetland and the deep-bed filters at WWTP Berlin with a good particle separation showed a more consistent reduction of microbial indicators than the MBBR system at WWTP Linköping with high variations.

**APIs and TPs:** API elimination achieved by an ozonation stage depends primarily on the applied specific ozone dose (nitrite corrected) and is compound specific. Fast reacting APIs such as carbamazepine and diclofenac can be reduced by 90% at a specific ozone dose of 0.3 $mgO_3$/mgDOC, whereas medium reactive compounds such as benzotriazole, metoprolol, tramadol and venlafaxine require specific ozone doses between 0.4 and 0.8 $mgO_3$/mgDOC to achieve an 80% reduction. Compounds with a very low reactivity towards ozone and OH-radicals (e.g. x-ray contrast media) can hardly be reduced by the ozonation process. Ozonation results in the formation of transformation products (TPs) that can also be further oxidized depending on the applied ozone dose. TPs from parent compounds with a high reactivity towards ozone (e.g. carbamazepine and diclofenac) can be reduced below the concentration at the ozonation influent at specific ozone doses of less than 0.8 $mgO_3$/mgDOC. In contrast, ozone doses of more than 1.2 $mgO_3$/mgDOC would be required for the reduction of the N-oxides of Tramadol and Venlafaxine, which would drastically increase the operational costs and pose a risk for an enhanced formation of other ozonation by-products such as bromate.

Fate of APIs and TPs in the ozonation post-treatment was evaluated for a constructed wetland, deep-bed filters using sand/anthracite or granular activated carbon as filter material as well as for two full-scale MBBRs. In total, only the GAC filter was able to significantly remove a broad spectrum of the analyzed APIs and TPs due to adsorption. Nevertheless, adsorption of APIs onto GAC strongly depends on treated bed volumes, which correlate to the overall filter runtime. Thus, long-term filter operation for adsorption requires a regular exchange of the GAC material. MBBRs, which were designed for nitrification and denitrification of WWTP effluent, as well as the S/A-filter had only a very limited impact on APIs and TPs if used as ozonation post-treatment. For the constructed wetland at least a distinct reduction of N-oxides was observed.

**Bio-assays:** A total of 17 ecotoxicological tests were used to investigate the impact of ozonation and post-treatment on a broad range of toxicological endpoints such as neurotoxicity, mutagenicity, genotoxicity, endocrine effects, growth and reproduction. Most of the

ecotoxicological tests were conducted with a final enrichment factor of 10-fold based on extracts from a solid phase extraction (1000-fold enrichment). As dissolved or suspended compounds have been separated from other compounds in the samples (according to their physical and chemical properties), the ecotoxicity results of enriched samples cannot be compared one-to-one to those which used native samples. Samples were taken at the ozonation influent and effluent as well as the according post-treatments at the WWTPs Berlin, Linköping and Kalundborg. As post-treatment three deep-bed filters with different filter material (sand/anthracite, granular activated carbon), a constructed wetland as well as MBBRs have been used.

In summary, a clear beneficial impact of the ozonation process was found for estrogenic effects (YES, ER-Calux) and inhibition of *Aliivibrio fischeri* bioluminescence. In contrast, a systematic increase of the mutagenic potential due to the ozonation was detected at WWTP Berlin using the Ames test with *S. typhimurium* strain YG7108 without metabolic activation (-S9), when using an enrichment factor of 1000 (instead of 10 for the other strains). However, all post-treatments were able to reduce these mutagenic effects after ozonation. The evaluation of mutagenic effects with Ames strains TA1535 and TA1537 showed occasionally non-systematic effects at the ozonation influent and effluent, respectively, but not in the effluent of the according post-treatment. This is probably caused by the low enrichment factor of 10 used in the tests with these two strains and might be different with higher enrichment factor. Thus, detection of mutagenic potential (Ames) is strongly depended on the used enrichment factor, the chosen test organism and whether the samples have been metabolically activated or not. For other endpoints such as neurotoxicity, androgenicity and genotoxicity, no ecotoxicological effects were determined in all samples. Growth inhibition tests with the algae *D. subspicatus* or the bacterium *Pseudomonas putida* resulted in most cases in a growth stimulation instead of a growth inhibition. Also, no systematic trend was observed for the anti-estrogenic and anti-androgenic effects as well as for the chronic reproduction tests with *Ceriodaphnia dubia*, which prevents to draw clear conclusions without a further, more detailed investigation.

For the evaluation of the ozonation and the according post-treatments a set of ecotoxicological tests should be used that is able to provide reliable systematic results. Based on the approach used within this project, it is therefore recommended to use the following set of tests, which covers the evaluation of mutagenic effects (AMES YG7108 or TA1535), estrogenicity (YES or ER-Calux) and bioluminescence inhibition (Microtox). With this reduced set of bioassays also higher final enrichment factors (e.g. 100 or 1000) in the tests are practical feasible that would probably lead to clearer results e.g. when comparing different post-treatment types. However, using such high enrichment factors should primarily be used to assess the impact of single treatment stages (ozonation, post-treatment) on the different ecotoxicological endpoints and not to determine potential risks of the produced water quality on e.g. aquatic life.

In general, ozonation post-treatment can reduce adverse effects that might result from the ozonation process. However, based on the available data no clear conclusion can be derived what kind of post-treatment is the most optimal one to remove potentially formed ecotoxicity.

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# Appendix

Please note that if not stated otherwise, all presented results in the appendix are from tests conducted with a final enrichment factor (fEF) of 10.

## Neurotoxicity

*SI-Table 1: Results of Neurotoxicity for samples from WWTP Berlin. Green fields indicate no effect.*

| Berlin GERMANY | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|
| BLN I | no effect | no effect | N/A | no effect | no effect | no effect |
| BLN II | no effect | no effect | no effect | no effect | no effect | no effect |
| BLN III | no effect | no effect | no effect | no effect | no effect | no effect |
| BLN IV | no effect | no effect | no effect | no effect | no effect | no effect |
| BLN V | no effect | no effect | no effect | no effect | no effect | no effect |

*SI-Table 2: Results of Neurotoxicity for samples from WWTP Kalundborg. Green fields indicate no effect.*

| Kalundborg DENMARK | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|
| KAL I | no effect | no effect | no effect |
| KAL II | no effect | no effect | no effect |
| KAL III | no effect | no effect | no effect |

*SI-Table 3: Results of Neurotoxicity for samples from WWTP Linköping. Green fields indicate no effect.*

| Linköping SWEDEN | Influent O3 (S1) | Effluent O3 (S2) | O3 + MBBR (S3) |
|---|---|---|---|
| LIN I | no effect | no effect | no effect |
| LIN II | no effect | no effect | no effect |
| LIN III | no effect | no effect | no effect |

## Mutagenicity

### SI-AMES TA1535 (+/- S9)

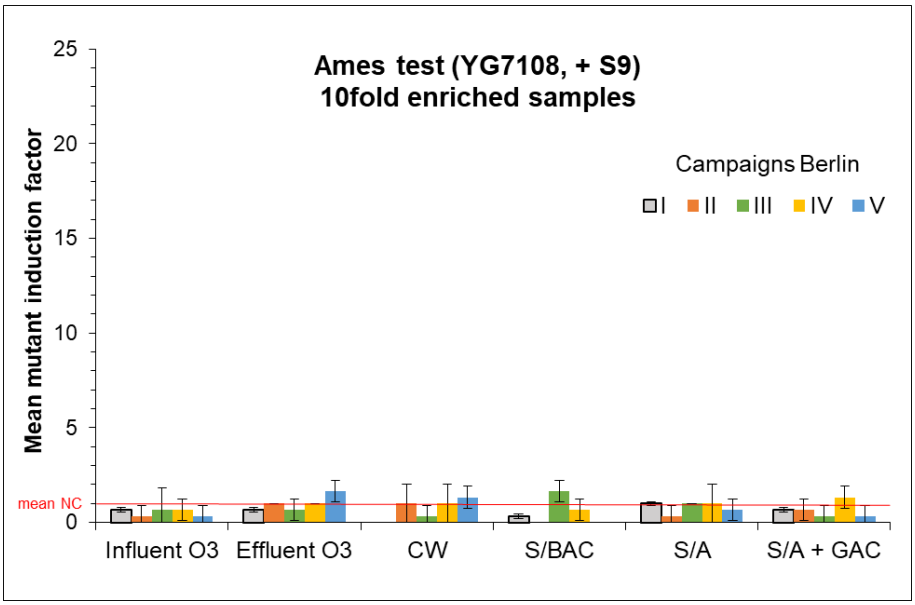
*SI-Table 4: Raw data example of the S/A-filter sample at the 3rd sampling campaign at WWTP Berlin (BLN III).*

| TA1535 +S9 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| BLN-C3-S7 | | | | | | | | | |
| Conc. () | Precipitation | n | Mean # positive wells | SD | Base-line | Fold increase over baseline | Binomial B-value | Mutagenic Conc. / Cytotoxic Effect | Numerical limit based on historical values* |
| 0 | | 3 | 0,33 | 0,58 | 1,00 | | | | none set |
| 0,31 | None | 3 | 0,67 | 0,58 | | 0,67 | 0,9203 | | |
| 0,63 | None | 3 | 1,33 | 0,58 | | 1,33 | **0,9965** | | |
| 1,25 | None | 3 | 0,67 | 1,15 | | 0,67 | 0,9203 | | |
| 2,5 | None | 3 | 2,00 | 1,73 | | **2,00** | **0,9999** | **Mutagenic Conc.** | |
| 5 | None | 3 | 2,67 | 0,58 | | **2,67** | **1,0000** | **Mutagenic Conc.** | |
| 10 | None | 3 | 3,33 | 1,15 | | **3,33** | **1,0000** | **Mutagenic Conc.** | |
| 2-AA | | 3 | 43,00 | 1,00 | | **43,00** | **1,0000** | **Mutagenic Conc.** | |
| *Limit for "Pass":* | | | | | | *3,00* | *0,99* | | *20* |

*SI-Table 5: Results of AMES test using the strains TA1535 (+/- S9) and TA 1537 (+/- S9) for samples from WWTP Linköping.*

| Linköping SWEDEN | *S. typhimurium* strain +/- S9 fraction | Influent $O_3$ ($S_1$) | Effluent $O_3$ ($S_2$) | $O_3$ + MBBR ($S_3$) |
|---|---|---|---|---|
| LIN I | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic |
| LIN II | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic |
| LIN III | TA1535 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1535 +S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 -S9 | not mutagenic | not mutagenic | not mutagenic |
| | TA1537 +S9 | not mutagenic | not mutagenic | not mutagenic |

## SI-AMES YG7108 (+ S9)




*SI-Figure 1: Mean mutant induction factor (MIF, n = 3) of Salmonella typhimurium strain YG7108 treated with 10fold enriched samples of five Berlin sampling campaigns at six different sampling points. Samples have been metabolically activated with S9 enzyme mix. Red line is the mean MIF of the negative control. Error bars indicate standard deviation.*

## Genotoxicity

### SI-SOS Chromotest

*SI-Table 6: Results of SOS Chromotest (genotoxicity) provided as unitless induction rates for samples from WWTP Berlin. Green fields indicate no effect, whereas yellow indicates "unmarked material". No samples have been analysed for CW at sampling campaign BLN I.*

| Berlin GERMANY | *Escherichia coli* +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|---|
| BLN I | + S9 | 1.15 | 1.04 | N/A | 1.39 | 1.22 | 0.96 |
| | - S9 | 1.53 | 1.06 | N/A | 1.18 | 1.23 | 1.03 |
| BLN II | + S9 | 1.36 | 1.17 | 1.15 | 1,15 | 1.04 | 1.20 |
| | - S9 | 0.53 | 0.46 | 0.71 | 0.40 | 0.42 | 0.46 |
| BLN III | + S9 | 0.95 | 0.95 | 1.10 | 1.02 | 0.86 | 0.90 |
| | - S9 | 1.32 | 1.19 | 1.18 | 1.18 | 1.08 | 1.12 |
| BLN IV | + S9 | 0.60 | 0.99 | 0.90 | 1.15 | 0.94 | 0.98 |
| | - S9 | 0.89 | 0.74 | 0.80 | 0.71 | 0.78 | 0.75 |
| BLN V | + S9 | 0.87 | 1.18 | 0.90 | 1.02 | 0.99 | 0.94 |
| | - S9 | 0.87 | 0.98 | 0.91 | 0.95 | 0.97 | 0.89 |

*SI-Table 7: Results of SOS Chromotest (genotoxicity) provided as unitless induction rates for samples from WWTP Kalundborg. Green fields indicate no effect.*

| Kalundborg DENMARK | *Escherichia coli* +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|---|
| KAL I | + S9 | 1.10 | 0.72 | 1.10 |
| | - S9 | 1.13 | 0.97 | 0.89 |
| KAL II | + S9 | 0.61 | 1.27 | 1.07 |
| | - S9 | 0.96 | 0.88 | 0.95 |
| KAL III | + S9 | 1.09 | 1.06 | 1.06 |
| | - S9 | 0.87 | 0.93 | 0.87 |

*SI-Table 8: Results of SOS Chromotest (genotoxicity) provided as unitless induction rates for samples from WWTP Linköping. Green fields indicate no effect.*

| Linköping SWEDEN | *Escherichia coli* +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|---|
| LIN I | + S9 | 1.08 | 1.41 | 1.23 |
| | - S9 | 0.55 | 0.50 | 0.55 |
| LIN II | + S9 | 1.11 | 0.97 | 1.05 |
| | - S9 | 1.23 | 1.19 | 1.23 |
| LIN III | + S9 | 1.18 | 0.98 | 0.86 |
| | - S9 | 0.89 | 0.90 | 0.75 |

## SI-UmuC

*SI-Table 9: Results of UmuC test (genotoxicity) for samples from WWTP Berlin. Given numbers are the induction rates (without units). Green fields indicate no effect. No samples have been analysed for CW at sampling campaign BLN I and for campaign BLN V.*

| Berlin GERMANY | *Salmonella typhimurium (TA1535)* +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|---|
| BLN I | + S9 | 1.25 | 1.10 | N/A | 1.17 | 1.05 | 1.10 |
| | - S9 | 1.40 | 1.31 | N/A | 1.15 | 1.13 | 1.16 |
| BLN II | + S9 | 1.49 | 1.02 | 1.05 | 0.91 | 0.93 | 1.00 |
| | - S9 | 1.35 | 1.06 | 1.04 | 1.00 | 0.97 | 1.07 |
| BLN III | + S9 | 1.41 | 1.01 | 1.00 | 1.05 | 0.99 | 1.00 |
| | - S9 | 1.43 | 1.18 | 1.15 | 1.13 | 0.96 | 1.20 |
| BLN IV | + S9 | 1.43 | 1.32 | 1.45 | 1.29 | 1.48 | 1.37 |
| | - S9 | 1.37 | 1.18 | 1.20 | 1.18 | 1.17 | 1.12 |
| BLN V | + S9 | N/A | N/A | N/A | N/A | N/A | N/A |
| | - S9 | N/A | N/A | N/A | N/A | N/A | N/A |

*SI-Table 10: Results of UmuC test (genotoxicity) for samples from WWTP Kalundborg. Given numbers are the induction rates (without units). Green fields indicate no effect.*

| Kalundborg DENMARK | *Salmonella typhimurium (TA1535)* +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|---|
| KAL I | + S9 | 1.08 | 1.06 | 1.04 |
| | - S9 | 1.22 | 1.19 | 1.10 |
| KAL II | + S9 | 1.33 | 1.03 | 1.14 |
| | - S9 | 1.39 | 1.15 | 1.13 |
| KAL III | + S9 | 1.37 | 1.29 | 1.36 |
| | - S9 | 1.42 | 1.42 | 1.38 |

*SI-Table 11: Results of UmuC test (genotoxicity) for samples from WWTP Linköping. Given numbers are the induction rates (without units). Green fields indicate no effect.*

| Linköping SWEDEN | *Salmonella typhimurium (TA1535)* +/- S9 fraction | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|---|
| LIN I | + S9 | 1.02 | 1.04 | 1.02 |
| | - S9 | 1.14 | 1.10 | 1.06 |
| LIN II | + S9 | 1.06 | 1.02 | 0.94 |
| | - S9 | 1.11 | 1.03 | 0.94 |
| LIN III | + S9 | 1.24 | 1.25 | 1.15 |
| | - S9 | 1.40 | 1.27 | 1.44 |

## Estro-/Androgenicity

### SI-YES/YAS-Test

*SI-Table 12: Endocrine potential of samples taken from WWTP Berlin. No samples have been analysed for CW at sampling campaign BLN I. Red highlighted fields indicate effects, whereas yellow fields indicate that effects were still present after post-treatment but lower compared to the ozonation influent. Green fields represent no effects.*

| Berlin GERMANY | Endocrine test | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|---|
| BLN I | Estrogenic (YES) | 0.2 ng EEQ/L | | N/A | | | |
| | Anti-Estrogenic (YES) | | 7.6 mg EEQ/L | N/A | | 7.6 mg EEQ/L | 67.3 mg EEQ/L |
| | Androgenic (YAS) | | | N/A | | | |
| | Anti-Androgenic (YAS) | 0.1 mg AEQ/L | | N/A | | | |
| BLN II | Estrogenic (YES) | 2.5 ng EEQ/L | | | | | |
| | Anti-Estrogenic (YES) | | | | | | |
| | Androgenic (YAS) | | | | | | |
| | Anti-Androgenic (YAS) | | | | | | |
| BLN III | Estrogenic (YES) | 2.0 ng EEQ/L | | | | | |
| | Anti-Estrogenic (YES) | 3.0 mg EEQ/L | | | | | |
| | Androgenic (YAS) | | | | | | |
| | Anti-Androgenic (YAS) | | | | | | |
| BLN IV | Estrogenic (YES) | 2.0 ng EEQ/L | | | | | |
| | Anti-Estrogenic (YES) | 36.7 mg EEQ/L | | | 21.9 mg EEQ/L | | 1.3 mg EEQ/L |
| | Androgenic (YAS) | | | | | | |
| | Anti-Androgenic (YAS) | | | | | | |
| BLN V | Estrogenic (YES) | 0.8 ng EEQ/L | | | | | |
| | Anti-Estrogenic (YES) | | 17.9 mg EEQ/L | | 23.3 mg EEQ/L | | 2.1 mg EEQ/L |
| | Androgenic (YAS) | | | | | | |
| | Anti-Androgenic (YAS) | 130 mg AEQ/L | | | | | |

*SI-Table 13: Endocrine potential of samples taken from WWTP Kalundborg. Red highlighted fields indicate effects, whereas yellow fields indicate that effects were still present after post-treatment but lower compared to the ozonation influent. Green fields represent no effects.*

| **Kalundborg DENMARK** | **Endocrine test** | **Influent $O_3$ (S1)** | **Effluent $O_3$ (S2)** | **MBBR (S3)** |
|---|---|---|---|---|
| **KAL I** | Estrogenic (YES) | | | |
| | Anti-Estrogenic (YES) | 8.4 mg EEQ/L | 8.1 mg EEQ/L | 8.7 mg EEQ/L |
| | Androgenic (YAS) | | | |
| | Anti-Androgenic (YAS) | 1.7 mg AEQ/L | 1.5 mg AEQ/L | |
| **KAL II** | Estrogenic (YES) | | | |
| | Anti-Estrogenic (YES) | 1.4 mg EEQ/L | 30.8 mg EEQ/L | |
| | Androgenic (YAS) | | | |
| | Anti-Androgenic (YAS) | 3.5 mg AEQ/L | | |
| **KAL III** | Estrogenic (YES) | | | |
| | Anti-Estrogenic (YES) | | | |
| | Androgenic (YAS) | | | |
| | Anti-Androgenic (YAS) | | | |

*SI-Table 14: Endocrine potential of samples taken from WWTP Linköping. Red highlighted fields indicate effects, whereas yellow fields indicate that effects were still present after post-treatment but lower compared to the ozonation influent. Green fields represent no effects.*

| **Linköping SWEDEN** | **Endocrine test** | **Influent $O_3$ (S1)** | **Effluent $O_3$ (S2)** | **MBBR (S3)** |
|---|---|---|---|---|
| **LIN I** | Estrogenic (YES) | 9.4 ng EEQ/L | | |
| | Anti-Estrogenic (YES) | | | |
| | Androgenic (YAS) | | | |
| | Anti-Androgenic (YAS) | 39.1 mg AEQ/L | | |
| **LIN II** | Estrogenic (YES) | | | |
| | Anti-Estrogenic (YES) | 9.6 mg EEQ/L | 41.2 mg EEQ/L | 19.5 mg EEQ/L |
| | Androgenic (YAS) | | | |
| | Anti-Androgenic (YAS) | | | 0.3 mg AEQ/L |
| **LIN III** | Estrogenic (YES) | | | |
| | Anti-Estrogenic (YES) | | | |
| | Androgenic (YAS) | | | |
| | Anti-Androgenic (YAS) | 42.6 mg AEQ/L | 0.11 mg AEQ/L | 5.9 mg AEQ/L |

## Bacteria tests

### SI-Pseudomonas putida

*SI-Table 15: Results of Pseudomonas Putida growth inhibition test of samples taken from WWTP Berlin. Values are provided as growth inhibition (%). Green fields indicate negative growth inhibition (growth promotion), whereas grey fields indicate "no effect". No samples have been analysed for CW at sampling campaign BLN I. Also, evaluation of sampling campaigns BLN IV and BLN V have been skipped due to absence of toxic effects in the first three sampling campaigns.*

| Berlin GERMANY | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|
| BLN I | -158 ± 0.4 | -141.2 ± 0.1 | N/A | -567.9 ± 0.03 | -421 ± 0.03 | -186.3 ± 0.01 |
| BLN II | -17.2 ± 0.1 | -54.4 ± 0.1 | 2.2 ± 0.04 | 0.93 ± 0.04 | 1.6 ± 0.03 | -6.15 ± 0.1 |
| BLN III | -383.1 ± 0.3 | -798.3 ± 0.3 | -845.9 ± 0.1 | -1271 ± 0.1 | -1032.4 ± 0.01 | -347 ± 0.003 |

*SI-Table 16: Results of Pseudomonas Putida growth inhibition test of samples taken from WWTP Kalundborg. Values are provided as growth inhibition (%). Green fields indicate negative growth inhibition (growth promotion), whereas red fields indicate a "slight growth inhibition".*

| Kalundborg DENMARK | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|
| KAL I | -50.0 ± 0.2 | 21.5 ± 0.3 | -25.4 ± 0.4 |
| KAL II | -1081.2 ± 0.2 | -1071.7 ± 0.1 | -188.0 ± 0.1 |
| KAL III | -33.1 ± 0.1 | -86.1 ± 0.1 | -77.3 ± 0.1 |

*SI-Table 17: Results of Pseudomonas Putida growth inhibition test of samples taken from WWTP Linköping. Values are provided as growth inhibition (%). Green fields indicate negative growth inhibition (growth promotion).*

| Linköping SWEDEN | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|
| LIN I | -144.5 ± 0.2 | -141.2 ± 0.1 | -134.3 ± 0.02 |
| LIN II | -81.5 ± 0.2 | -82.5 ± 0.1 | -102.1 ± 0.02 |
| LIN III | -21.4 ± 0.1 | -71.3 ± 0.1 | -20.9 ± 0.1 |

## SI-Aliivibrio fischeri

*SI-Table 18: Percentage inhibition effect (PE) of Aliivibrio fischeri bioluminescence (%) in samples from WWTP Berlin after 30 minutes of application. Green fields indicate "no acute toxicity", whereas yellow fields indicate a "slight acute toxicity". No samples have been analyzed for CW at sampling campaign BLN I. SD = standard deviation.*

| Berlin GERMANY | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|
| BLN I | 42.47 | 31.95 | N/A | 34.61 | 33.2 | 18.43 |
| | 38.31 | 28.76 | N/A | 22.15 | 31.71 | 18.26 |
| mean effect ± SD | **40.39±2.08** | **30.36±1.60** | **N/A** | **28.38±6.23** | **32.46±0.75** | **18.35±0.08** |
| BLN II | 47.71 | 24.60 | 16.99 | 12..90 | 17.42 | 10.35 |
| | 45.23 | 20.97 | 16.72 | 7.37 | 13.50 | 9.63 |
| mean effect | **46.47±1.24** | **22.79±1.82** | **16.86±0.14** | **10.14±2.77** | **15.46±1.96** | **9.99±0.36** |
| BLN III | 29.14 | 23.18 | 13.11 | 9.27 | 14.71 | 11.44 |
| | 27.64 | 18.86 | 4.95 | 8.72 | 3.40 | 10.23 |
| mean effect | **28.39±0.75** | **21.02±2.16** | **9.03±4.08** | **9.00±0.27** | **9.06±5.66** | **10.84±0.61** |
| BLN IV | 43.84 | 28.54 | 29.11 | 24.16 | 22.62 | 13.88 |
| | 40.09 | 24.55 | 27.15 | 24.09 | 16.65 | 8.32 |
| mean effect | **41.97±1.88** | **26.55±2.00** | **28.13±0.98** | **24.13±0.04** | **19.64±2.99** | **11.10±2.78** |
| BLN V | 41.25 | 23.86 | 20.34 | 22.46 | 39.30 | 21.85 |
| | 40.20 | 23.76 | 14.34 | 18.41 | 34.63 | 20.90 |
| mean effect | **40.73±0.52** | **23.81±0.05** | **17.34±3.00** | **20.44±2.03** | **36.97±2.34** | **21.38±0.48** |

*SI-Table 19: Percentage inhibition effect (PE) of Aliivibrio fischeri bioluminescence (%) in samples from WWTP Kalundborg after 30 minutes of application. Yellow fields indicate a "slight acute toxicity" and red fields indicate an "acute toxicity". SD = standard deviation.*

| Kalundborg DENMARK | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|
| KAL I | 51.79 | 40.13 | 32.81 |
| | 50.22 | 39.25 | 29.42 |
| mean effect ± SD | **51.01±0.79** | **39.69±0.44** | **31.12±1.70** |
| KAL II | 52.56 | 44.77 | 37.55 |
| | 52.09 | 42.14 | 36.05 |
| mean effect ± SD | **52.33±0.23** | **43.46±1.32** | **36.80±0.75** |
| KAL III | 31.68 | 26.78 | 23.02 |
| | 30.92 | 26.17 | 18.91 |
| mean effect ± SD | **31.30±0.38** | **26.48±0.31** | **20.97±2.06** |

*SI-Table 20: Percentage inhibition effect (PE) of Aliivibrio fischeri bioluminescence (%) in samples from WWTP Linköping after 30 minutes of application. Green fields indicate "no acute toxicity", whereas yellow fields indicate a "slight acute toxicity".*

| Linköping SWEDEN | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|
| LIN I | 32,19 | 24,98 | 28,20 |
| | 29,99 | 23,46 | 23,39 |
| mean effect ± SD | 31,09±1.10 | 24,22±0,76 | 25,80±2,41 |
| LIN II | 35,69 | 18,87 | 17,36 |
| | 35,00 | 12,09 | 15,55 |
| mean effect ± SD | 35,35±0,34 | 15,48±3,39 | 16,46±0,90 |
| LIN III | 29,08 | 24,87 | 22,67 |
| | 23,38 | 23,84 | 17,36 |
| mean effect ± SD | 26,23±2,85 | 24,36±0,52 | 20,02±2,66 |

*SI-Table 21: Toxic effect of wastewaters samples from WWTP Berlin (fEF=10) at differ sampling points on the luminescent properties of Aliivibrio fischeri after 5 minutes of exposition: percentage inhibition effects [PE, %], $EC_{50}$-t values and toxicity units (TU). SD = Standard deviation.*

| Sampling campaign | Sample | $EC_{50}$ (%) | TU | PE after time (fEF=10) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Repetition 1 | Repetition 2 | Mean | SD |
| **Time 5 min** | | | | | | | |
| **BLN I** | Field Blank (FB) | 449.40 | 0.223 | 13.99 | 12.26 | 13.13 | 0.87 |
| | Influent O3 (S1) | 104.80 | 0.954 | 43.67 | 41.67 | 42.67 | 1.00 |
| | Effluent O3 (S2) | 130.60 | 0.766 | 33.18 | 32.03 | 32.61 | 0.57 |
| | S/BAC (S6) | 142.00 | 0.704 | 35.93 | 23.70 | 29.82 | 6.12 |
| | S/A (S7) | 143.90 | 0.695 | 33.97 | 26.31 | 30.14 | 3.83 |
| | S/A + GAC (S8) | 189.60 | 0.528 | 20.99 | 18.43 | 19.71 | 1.28 |
| **BLN II** | Influent O3 (S1) | 100.00 | 0.999 | 44.29 | 43.52 | 43.91 | 0.38 |
| | Effluent O3 (S2) | 380.00 | 0.263 | 21.32 | 20.33 | 20.83 | 0.50 |
| | O3 + CW (S3) | 348.90 | 0.287 | 19.02 | 15.86 | 17.44 | 1.58 |
| | S/BAC (S6) | 501.60 | 0.199 | 13.10 | 11.76 | 12.43 | 0.67 |
| | S/A (S7) | 211.50 | 0.473 | 22.44 | 19.35 | 20.90 | 1.55 |
| | S/A + GAC (S8) | 314.80 | 0.318 | 18.46 | 17.05 | 17.76 | 0.71 |
| **BLN III** | Influent O3 (S1) | 215.70 | 0.464 | 24.72 | 22.87 | 23.80 | 0.92 |
| | Effluent O3 (S2) | 374.00 | 0.267 | 23.54 | 21.80 | 22.67 | 0.87 |
| | O3 + CW (S3) | n.d. | n.d. | 12.42 | 3.33 | 7.88 | 4.55 |
| | S/BAC (S6) | 398.90 | 0.251 | 11.76 | 10.45 | 11.11 | 0.66 |
| | S/A (S7) | n.d. | n.d. | 14.47 | 9.34 | 11.91 | 2.57 |
| | S/A + GAC (S8) | 408.10 | 0.245 | 16.79 | 16.46 | 16.63 | 0.16 |
| **BLN IV** | Influent O3 (S1) | 99.04 | 1.010 | 45.80 | 43.21 | 44.51 | 1.30 |
| | Effluent O3 (S2) | 172.50 | 0.579 | 30.42 | 27.94 | 29.18 | 1.24 |
| | O3 + CW (S3) | 243.90 | 0.410 | 29.89 | 29.16 | 29.53 | 0.37 |
| | S/BAC (S6) | 175.50 | 0.570 | 27.75 | 26.68 | 27.22 | 0.54 |
| | S/A (S7) | 277.40 | 0.360 | 23.18 | 18.80 | 20.99 | 2.19 |
| | S/A + GAC (S8) | 250.80 | 0.399 | 17.67 | 14.00 | 15.84 | 1.84 |
| **BLN V** | Influent O3 (S1) | 114.00 | 0.877 | 39.85 | 39.03 | 39.44 | 0.41 |
| | Effluent O3 (S2) | 193.60 | 0.516 | 26.33 | 25.52 | 25.93 | 0.40 |
| | O3 + CW (S3) | 299.30 | 0.334 | 23.96 | 20.79 | 22.38 | 1.59 |
| | S/BAC (S6) | 318.20 | 0.314 | 28.03 | 26.71 | 27.37 | 0.66 |
| | S/A (S7) | 110.60 | 0.904 | 43.64 | 38.40 | 41.02 | 2.62 |
| | S/A + GAC (S8) | 129.20 | 0.774 | 23.03 | 22.35 | 22.69 | 0.34 |

*SI-Table 22: Toxic effect of wastewaters samples from WWTP Berlin (fEF=10) at differ sampling points on the luminescent properties of Aliivibrio fischeri after 15 minutes of exposition: percentage inhibition effects [PE, %], $EC_{50}$-t values and toxicity units (TU). SD = Standard deviation.*

| Sampling campaign | Sample | $EC_{50}$ (%) | TU | PE after time (fEF=10) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Repetition 1 | Repetition 2 | Mean | SD |
| **Time 15 min** | | | | | | | |
| **BLN I** | Field Blank | 410.90 | 0.24 | 13.84 | 11.05 | 12.45 | 1.40 |
| | Influent O3 (S1) | 104.80 | 0.95 | 44.22 | 40.63 | 42.43 | 1.80 |
| | Effluent O3 (S2) | 138.60 | 0.72 | 31.82 | 29.67 | 30.75 | 1.08 |
| | S/BAC (S6) | 119.80 | 0.83 | 36.44 | 23.21 | 29.83 | 6.61 |
| | S/A (S7) | 156.70 | 0.64 | 32.69 | 31.71 | 32.20 | 0.49 |
| | S/A + GAC (S8) | 190.30 | 0.53 | 18.52 | 17.59 | 18.06 | 0.47 |
| **BLN II** | Influent O3 (S1) | 93.86 | 0.91 | 47.96 | 46.03 | 47.00 | 0.97 |
| | Effluent O3 (S2) | 419.80 | 0.24 | 22.37 | 18.11 | 20.24 | 2.13 |
| | O3 + CW (S3) | 245.80 | 0.41 | 17.69 | 15.49 | 16.59 | 1.10 |
| | S/BAC (S6) | 4305.00 | 0.02 | 11.62 | 9.95 | 10.79 | 0.84 |
| | S/A (S7) | 189.80 | 0.53 | 18.37 | 16.10 | 17.24 | 1.14 |
| | S/A + GAC (S8) | 876.70 | 0.11 | 14.37 | 14.28 | 14.33 | 0.04 |
| **BLN III** | Influent O3 (S1) | 189.50 | 0.53 | 28.23 | 25.10 | 26.67 | 1.57 |
| | Effluent O3 (S2) | 281.50 | 0.36 | 23.18 | 20.13 | 21.66 | 1.53 |
| | O3 + CW (S3) | n.d. | n.d. | 9.94 | -1.05 | 4.45 | 5.50 |
| | S/BAC (S6) | n.d. | n.d. | 9.39 | 6.53 | 7.96 | 1.43 |
| | S/A (S7) | n.d. | n.d. | 11.54 | 4.36 | 7.95 | 3.59 |
| | S/A + GAC (S8) | n.d. | n.d. | 12.36 | 11.65 | 12.01 | 0.36 |
| **BLN IV** | Influent O3 (S1) | 107.00 | 0.94 | 44.77 | 40.83 | 42.80 | 1.97 |
| | Effluent O3 (S2) | 177.60 | 0.56 | 28.64 | 26.40 | 27.52 | 1.12 |
| | O3 + CW (S3) | 182.60 | 0.55 | 31.54 | 30.52 | 31.03 | 0.51 |
| | S/BAC (S6) | 168.40 | 0.59 | 27.31 | 26.65 | 26.98 | 0.33 |
| | S/A (S7) | 278.30 | 0.36 | 25.78 | 19.78 | 22.78 | 3.00 |
| | S/A + GAC (S8) | 517.10 | 0.19 | 15.24 | 10.62 | 12.93 | 2.31 |
| **BLN V** | Influent O3 (S1) | 124.80 | 0.80 | 40.03 | 38.73 | 39.38 | 0.65 |
| | Effluent O3 (S2) | 224.50 | 0.45 | 25.27 | 24.19 | 24.73 | 0.54 |
| | O3 + CW (S3) | 234.20 | 0.43 | 23.79 | 17.93 | 20.86 | 2.93 |
| | S/BAC (S6) | 305.20 | 0.33 | 25.67 | 23.60 | 24.64 | 1.04 |
| | S/A (S7) | 115.70 | 0.86 | 41.69 | 37.57 | 39.63 | 2.06 |
| | S/A + GAC (S8) | 140.10 | 0.71 | 21.46 | 20.29 | 20.88 | 0.59 |

*SI-Table 23: Toxic effect of wastewaters samples from WWTP Berlin (fEF=10) at differ sampling points on the luminescent properties of Aliivibrio fischeri after 30 minutes of exposition: percentage inhibition effects [PE, %], $EC_{50}$-t values and toxicity units (TU). SD = Standard deviation.*

| Sampling campaign | Sample | $EC_{50}$ (%) | TU | PE after time (fEF=10) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Repetition 1 | Repetition | Mean | SD |
| **Time 30 min** | | | | | | | |
| **BLN I** | Field Blank (FB) | n.d. | n.d. | 14.31 | 10.24 | 12.28 | 2.04 |
| | Influent $O_3$ ($S_1$) | 107.90 | 0.93 | 42.47 | 38.31 | 40.39 | 2.08 |
| | Effluent $O_3$ ($S_2$) | 142.40 | 0.70 | 31.95 | 28.76 | 30.36 | 1.60 |
| | S/BAC ($S_6$) | 119.80 | 0.83 | 34.61 | 22.15 | 28.38 | 6.23 |
| | S/A ($S_7$) | 148.20 | 0.67 | 33.20 | 31.71 | 32.46 | 0.75 |
| | S/A + GAC ($S_8$) | 188.90 | 0.53 | 18.43 | 18.26 | 18.35 | 0.08 |
| **BLN II** | Influent $O_3$ ($S_1$) | 91.94 | 1.09 | 47.71 | 45.23 | 46.47 | 1.24 |
| | Effluent $O_3$ ($S_2$) | 380.40 | 0.26 | 24.60 | 20.97 | 22.79 | 1.82 |
| | $O_3$ + CW ($S_3$) | 361.20 | 0.28 | 16.99 | 16.72 | 16.86 | 0.14 |
| | S/BAC ($S_6$) | 360.30 | 0.28 | 12.90 | 7.37 | 10.14 | 2.77 |
| | S/A ($S_7$) | 291.90 | 0.34 | 17.42 | 13.50 | 15.46 | 1.96 |
| | S/A + GAC ($S_8$) | 770.40 | 0.13 | 10.35 | 9.63 | 9.99 | 0.36 |
| **BLN III** | Influent $O_3$ ($S_1$) | 176.50 | 0.57 | 29.14 | 27.64 | 28.39 | 0.75 |
| | Effluent $O_3$ ($S_2$) | 312.00 | 0.32 | 23.18 | 18.86 | 21.02 | 2.16 |
| | $O_3$ + CW ($S_3$) | n.d. | n.d. | 13.11 | 4.95 | 9.03 | 4.08 |
| | S/BAC ($S_6$) | n.d. | n.d. | 9.27 | 8.72 | 9.00 | 0.27 |
| | S/A ($S_7$) | n.d. | n.d. | 14.71 | 3.40 | 9.06 | 5.66 |
| | S/A + GAC ($S_8$) | n.d. | n.d. | 11.44 | 10.23 | 10.84 | 0.61 |
| **BLN IV** | Influent $O_3$ ($S_1$) | 108.90 | 0.92 | 43.84 | 40.09 | 41.97 | 1.88 |
| | Effluent $O_3$ ($S_2$) | 159.10 | 0.63 | 28.54 | 24.55 | 26.55 | 2.00 |
| | $O_3$ + CW ($S_3$) | 213.20 | 0.47 | 29.11 | 27.15 | 28.13 | 0.98 |
| | S/BAC ($S_6$) | 249.70 | 0.40 | 24.16 | 24.09 | 24.13 | 0.04 |
| | S/A ($S_7$) | 240.70 | 0.42 | 22.62 | 16.65 | 19.64 | 2.99 |
| | S/A + GAC ($S_8$) | 344.50 | 0.29 | 13.88 | 8.32 | 11.10 | 2.78 |
| **BLN V** | Influent $O_3$ ($S_1$) | 110.10 | 0.91 | 41.25 | 40.20 | 40.73 | 0.52 |
| | Effluent $O_3$ ($S_2$) | 212.90 | 0.47 | 23.86 | 23.76 | 23.81 | 0.05 |
| | $O_3$ + CW ($S_3$) | 415.00 | 0.24 | 20.34 | 14.34 | 17.34 | 3.00 |
| | S/BAC ($S_6$) | 475.80 | 0.21 | 22.46 | 18.41 | 20.44 | 2.03 |
| | S/A ($S_7$) | 125.30 | 0.80 | 39.30 | 34.63 | 36.97 | 2.34 |
| | S/A + GAC ($S_8$) | 248.80 | 0.40 | 21.85 | 20.90 | 21.38 | 0.48 |

*SI-Table 24: Toxic effect of wastewaters samples from WWTP Linköping (fEF=10) at differ sampling points on the luminescent properties of Aliivibrio fischeri after 5, 15 and 30 minutes of exposition: percentage inhibition effects [PE, %], $EC_{50}$-t values and toxicity units (TU). SD = Standard deviation.*

| Sampling campaign | Sample | $EC_{50}$ (%) | TU | PE after time (fEF=10) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Repetition 1 | Repetition 2 | Mean | SD |
| **Time 5 min** | | | | | | | |
| **LIN I** | Influent O3 (S1) | 137.50 | 0.727 | 36.95 | 36.06 | 36.51 | 0.45 |
| | Effluent O3 (S2) | 216.70 | 0.462 | 28.45 | 27.21 | 27.83 | 0.62 |
| | MBBR (S3) | 163.90 | 0.610 | 22.41 | 20.47 | 21.44 | 0.97 |
| **LIN II** | Influent O3 (S1) | 141.60 | 0.706 | 34.37 | 33.91 | 34.14 | 0.23 |
| | Effluent O3 (S2) | 310.80 | 0.322 | 15.36 | 12.19 | 13.78 | 1.59 |
| | MBBR (S3) | 975.80 | 0.103 | 12.49 | 9.26 | 10.88 | 1.62 |
| **LIN III** | Influent O3 (S1) | 225.40 | 0.444 | 24.72 | 24.04 | 24.38 | 0.34 |
| | Effluent O3 (S2) | 346.30 | 0.289 | 20.16 | 17.76 | 18.96 | 1.20 |
| | MBBR (S3) | 222.90 | 0.449 | 19.51 | 11.99 | 15.75 | 3.76 |
| **Time 15 min** | | | | | | | |
| **LIN I** | Influent O3 (S1) | 144.40 | 0.693 | 35.91 | 33.43 | 34.67 | 1.24 |
| | Effluent O3 (S2) | 190.70 | 0.524 | 27.68 | 26.01 | 26.85 | 0.83 |
| | MBBR (S3) | 189.50 | 0.528 | 24.82 | 21.83 | 23.33 | 1.50 |
| **LIN II** | Influent O3 (S1) | 136.30 | 0.734 | 34.48 | 33.50 | 33.99 | 0.49 |
| | Effluent O3 (S2) | 163.50 | 0.612 | 14.77 | 7.96 | 11.37 | 3.41 |
| | MBBR (S3) | 416.70 | 0.240 | 14.95 | 13.20 | 14.08 | 0.88 |
| **LIN III** | Influent O3 (S1) | 202.40 | 0.494 | 25.09 | 20.10 | 22.60 | 2.50 |
| | Effluent O3 (S2) | 489.30 | 0.201 | 22.25 | 20.32 | 21.29 | 0.97 |
| | MBBR (S3) | 336.00 | 0.298 | 21.67 | 16.75 | 19.210 | 2.460 |
| **Time 30 min** | | | | | | | |
| **LIN I** | Influent O3 (S1) | 151.80 | 0.659 | 32.19 | 29.99 | 31.09 | 1.10 |
| | Effluent O3 (S2) | 206.30 | 0.485 | 24.98 | 23.46 | 24.22 | 0.76 |
| | MBBR (S3) | 146.90 | 0.681 | 28.20 | 23.39 | 25.80 | 2.41 |
| **LIN II** | Influent O3 (S1) | 126.00 | 0.794 | 35.69 | 35.00 | 35.35 | 0.34 |
| | Effluent O3 (S2) | 171.10 | 0.584 | 18.87 | 12.09 | 15.48 | 3.39 |
| | MBBR (S3) | 204.00 | 0.490 | 17.36 | 15.55 | 16.46 | 0.90 |
| **LIN III** | Influent O3 (S1) | 177.80 | 0.562 | 29.08 | 23.38 | 26.23 | 2.85 |
| | Effluent O3 (S2) | 165.00 | 0.606 | 24.87 | 23.84 | 24.36 | 0.52 |
| | MBBR (S3) | 188.80 | 0.529 | 22.67 | 17.36 | 20.02 | 2.66 |

*SI-Table 25: Toxic effect of wastewaters samples from WWTP Kalundborg (fEF=10) at differ sampling points on the luminescent properties of Aliivibrio fischeri after 5, 15 and 30 minutes of exposition: percentage inhibition effects [PE, %], $EC_{50}$-t values and toxicity units (TU). SD = Standard deviation.*

| Sampling campaign | Sample | $EC_{50}$ (%) | TU | PE after time (fEF=10) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Repetition 1 | Repetition 2 | Mean | SD |
| **Time 5 min** | | | | | | | |
| **KAL I** | Influent O3 (S1) | 91.71 | 1.090 | 45.43 | 42.67 | 44.05 | 1.38 |
| | Effluent O3 (S2) | 140.60 | 0.711 | 37.60 | 37.42 | 37.51 | 0.09 |
| | MBBR (S3) | 227.00 | 0.441 | 30.48 | 27.70 | 29.09 | 1.39 |
| **KAL II** | Influent O3 (S1) | 103.60 | 0.965 | 42.13 | 40.92 | 41.53 | 0.61 |
| | Effluent O3 (S2) | 100.10 | 0.999 | 41.34 | 38.31 | 39.83 | 1.52 |
| | MBBR (S3) | 150.10 | 0.666 | 33.08 | 33.07 | 33.08 | 0.00 |
| **KAL III** | Influent O3 (S1) | 172.40 | 0.579 | 30.33 | 28.08 | 29.21 | 1.13 |
| | Effluent O3 (S2) | 196.90 | 0.508 | 26.79 | 25.76 | 26.28 | 0.51 |
| | MBBR (S3) | 280.50 | 0.356 | 22.00 | 18.83 | 20.42 | 1.59 |
| **Time 15 min** | | | | | | | |
| **KAL I** | Influent O3 (S1) | 86.12 | 1.161 | 48.55 | 46.68 | 47.62 | 0.93 |
| | Effluent O3 (S2) | 128.30 | 0.780 | 38.27 | 37.81 | 38.04 | 0.23 |
| | MBBR (S3) | 220.20 | 0.454 | 29.86 | 27.41 | 28.64 | 1.23 |
| **KAL II** | Influent O3 (S1) | 84.79 | 1.179 | 48.01 | 47.51 | 47.76 | 0.25 |
| | Effluent O3 (S2) | 103.40 | 0.967 | 42.92 | 41.43 | 42.18 | 0.75 |
| | MBBR (S3) | 137.20 | 0.729 | 33.58 | 33.58 | 33.58 | 0.00 |
| **KAL III** | Influent O3 (S1) | 193.30 | 0.517 | 30.13 | 28.86 | 29.50 | 0.64 |
| | Effluent O3 (S2) | 155.60 | 0.643 | 26.75 | 25.17 | 25.96 | 0.79 |
| | MBBR (S3) | 489.20 | 0.204 | 19.48 | 16.49 | 17.99 | 1.50 |
| **Time 30 min** | | | | | | | |
| **KAL I** | Influent O3 (S1) | 79.55 | 1.257 | 51.79 | 50.22 | 51.01 | 0.79 |
| | Effluent O3 (S2) | 118.50 | 0.844 | 40.13 | 39.25 | 39.69 | 0.44 |
| | MBBR (S3) | 184.60 | 0.542 | 32.81 | 29.42 | 31.12 | 1.70 |
| **KAL II** | Influent O3 (S1) | 75.22 | 1.329 | 52.56 | 52.09 | 52.33 | 0.23 |
| | Effluent O3 (S2) | 92.82 | 1.077 | 44.77 | 42.14 | 43.46 | 1.32 |
| | MBBR (S3) | 116.30 | 0.859 | 37.55 | 36.05 | 36.80 | 0.75 |
| **KAL III** | Influent O3 (S1) | 165.10 | 0.606 | 31.68 | 30.92 | 31.30 | 0.38 |
| | Effluent O3 (S2) | 170.10 | 0.588 | 26.78 | 26.17 | 26.48 | 0.31 |
| | MBBR (S3) | 280.00 | 0.357 | 23.02 | 18.91 | 20.97 | 2.06 |

**Statistical analysis**

The ANOVA table composes the variance of max % effect into two components: between group component and within group component (SI-Table 26). The F – ratio, which in this case equals 13.499, is a ratio of the between-group estimate to the within-group estimate. Since the P-value of the F-test is less than 0.05, there is a statistically significant difference between the mean max % effect from one level of sample to another at the 95.0% confidence level. To determine which means are significantly different from which others, select Multiple Range Test was made.

*SI-Table 26: Statistical analysis of Aliivibrio fischeri test results from WWTP Berlin samples: analysis of variance ANOVA*

| *Source of variance* | *SS (sum of square)* | *df* | *MS (Mean square)* | *F - ratio* | *P - value* | *Test F* |
|---|---|---|---|---|---|---|
| Between groups | 3962.777 | 5 | 792.555 | 13.499 | 0.00000002 | 2.39 |
| Within groups | 3053.131 | 52 | 58.714 | | | |
| Total | 7015.908 | 57 | | | | |

SI-Table 27 and SI-Table 28 show results of a multiple comparison procedure to determine which means was significantly different from the others. In the SI-Table 27, three homogenous groups were identified using columns of X's. Within each column, the levels containing X's form a group of means within which there were no statistically significant differences.

SI-Table 28 showed the estimated difference between each pair of means. An asterisk (*) marks the seven pairs, indicating that these pairs show statistically significant difference at a 95.0% confidence level. For comparison, the Fisher's last significant difference (LSD) was calculated. If difference between samples was higher that the LSD value it shows that they differed significantly from each other. With this method there was a 5% risk of calling each pairs of mean significantly different when the actual difference equals 0.

*SI-Table 27: Statistical analysis of Aliivibrio fischeri test results from WWTP Berlin samples: post hoc Multiple Range Test*

| Sample | Count | Mean | Homogenous groups |
|---|---|---|---|
| S8 | 10 | 14.329 | X |
| S3 | 8 | 17.839 | X X |
| S6 | 10 | 18.414 | X X |
| S7 | 10 | 22.714 | X |
| S2 | 10 | 24.903 | X |
| S1 | 10 | 39.588 | X |

*SI-Table 28: Statistical analysis of Aliivibrio fischeri test results from WWTP Berlin samples: Last Significant Differences (LSD) according Fisher's procedure*

| Contrast | Difference | LSD |
|---|---|---|
| S1 - S2 | 14.685* | 6.87635 |
| S1 - S3 | 21.7493* | 7.29347 |
| S1 - S6 | 21.174* | 6.87635 |
| S1 - S7 | 16.874* | 6.87635 |
| S1 - S8 | 25.259* | 6.87635 |
| S2 - S3 | 7.06425 | 7.29347 |
| S2 - S6 | 6.489 | 6.87635 |
| S2 - S7 | 2.189 | 6.87635 |
| S2 - S8 | 10.574* | 6.87635 |
| S3 - S6 | -0.57525 | 7.29347 |
| S3 - S7 | -4.87525 | 7.29347 |
| S3 - S8 | 3.50975 | 7.29347 |
| S6 - S7 | -4.3 | 6.87635 |
| S6 - S8 | 4.085 | 6.87635 |
| S7 - S8 | 8.385* | 6.87635 |

|a – b| < LSD means no significant difference; |a – b| > LSD means significant difference (*)

*SI-Table 29: Statistical analysis of Aliivibrio fischeri test results from WWTP Linköping samples: analysis of variance ANOVA*

| *Source of variance* | *SS (sum of square)* | *df* | *MS (Mean square)* | *F - ratio* | *P - value* | *Test F* |
|---|---|---|---|---|---|---|
| Between groups | 387.977 | 2 | 193.988 | 8.400 | 0.004 | 3.682 |
| Within groups | 346.408 | 15 | 23.094 | | | |
| Total | 734.385 | 17 | | | | |

*SI-Table 30: Statistical analysis of Aliivibrio fischeri test results from WWTP Linköping samples: post hoc Multiple Range Test*

| *Sample* | *Count* | *Mean* | *Homogenous groups* |
|---|---|---|---|
| S2 | 3 | 20.755 | X |
| S3 | 3 | 21.352 | X |
| S1 | 3 | 30.888 | X |

*SI-Table 31: Statistical analysis of Aliivibrio fischeri test results from WWTP Linköping samples: Last Significant Differences (LSD) according Fisher's procedure*

| *Contrast* | *Difference* | *LSD* |
|---|---|---|
| S1 - S2 | 9.537* | 5.914 |
| S1 - S3 | 10.133* | 5.914 |
| S2 – S3 | 0.597 | 5.914 |

|a – b| < LSD means no significant difference; |a – b| > LSD means significant difference (*)

*SI-Table 32: Statistical analysis of Aliivibrio fischeri test results from WWTP Kalundborg samples: analysis of variance ANOVA*

| *Source of variance* | *SS (sum of square)* | *df* | *MS (Mean square)* | *F - ratio* | *P - value* | *Test F* |
|---|---|---|---|---|---|---|
| Between groups | 699.713 | 2 | 349.857 | 4.559 | 0.028 | 3.682 |
| Within groups | 1151.181 | 15 | 76.745 | | | |
| Total | 1850.894 | 8 | | | | |

*SI-Table 33: Statistical analysis of Aliivibrio fischeri test results from WWTP Kalundborg samples: post hoc Multiple Range Test*

| *Sample* | *Count* | *Mean* | *Homogenous groups* |
|---|---|---|---|
| S2 | 3 | 29.627 | X |
| S3 | 3 | 36.540 | X X |
| S1 | 3 | 44.877 | X |

*SI-Table 34: Statistical analysis of Aliivibrio fischeri test results from WWTP Kalundborg samples: Last Significant Differences (LSD) according Fisher's procedure*

| *Contrast* | *Difference* | *LSD* |
|---|---|---|
| S1 - S2 | 8.33667 | 10.7806 |
| S1 - S3 | 15.25* | 10.7806 |
| S2 – S3 | 6.91333 | 10.7806 |

|a – b| < LSD means no significant difference; |a – b| > LSD means significant difference (*)

## Chronic tests

### SI-Algae growth test

*SI-Table 35: Overview of evaluated ecotoxicological test systems and toxicological endpoints. Note that LID of BLN I Influent O3 was not included as for BLN I samples of the influent and effluent of the ozonation might not directly correspond (see section Sampling and SPE procedure).*

| Campaign | Sample | LID |
|---|---|---|
| BLN I | Effluent O3 | < 5.9% |
| | S/BAC | < 5.9% |
| | S/A | < 5.9% |
| | S/A+GAC | < 5.9% |
| KAL IIa | Influent O3 | 5.9% |
| | Effluent O3 | < 5.9% |

### SI-Ceriodaphnia dubia reproduction test

*SI-Table 36: Results of C. dubia reproduction inhibition test of samples taken from WWTP Berlin shown as $EC_{50}$ (%). Note that for the influent and effluent of the ozonation as well as for the S/BAC of the first sampling campaign no results are displayed due to decreased amount of neonates in test control. No sample at the CW was taken at the sampling campaign BLN I.*

| Berlin GERMANY | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | CW (S3) | S/BAC (S6) | S/A (S7) | S/A + GAC (S8) |
|---|---|---|---|---|---|---|
| BLN I | N/A | N/A | N/A | N/A | 15.92 | 63.68 |
| BLN II | Does not reach EC50 | 6.28 | Does not reach EC50 | Does not reach EC50 | Does not reach EC50 | 16.00 |
| BLN III | 42.85 | Does not reach EC50 | Does not reach EC50 | Does not reach EC50 | 77.62 | Does not reach EC50 |
| BLN IV | Does not reach EC50 | 61.80 | Does not reach EC50 | Does not reach EC50 | 75.68 | Does not reach EC50 |

*SI-Table 37: Results of C. dubia reproduction inhibition test of samples taken from WWTP Kalundborg shown as $EC_{50}$ (%).*

| Kalundborg DENMARK | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|
| KAL I | 15.78 | 16.44 | 0.52 |
| KAL II | 20.09 | 20.18 | 9.50 |
| KAL III | 18.84 | 25.35 | 7.39 |

*SI-Table 38: Results of C. dubia reproduction inhibition test of samples taken from WWTP Linköping shown as $EC_{50}$ (%). Samples from the campaign LIN III were not analysed.*

| Linköping SWEDEN | Influent $O_3$ (S1) | Effluent $O_3$ (S2) | MBBR (S3) |
|---|---|---|---|
| LIN I | Does not reach EC50 | 13.77 | Does not reach EC50 |
| LIN II | Does not reach EC50 | Does not reach EC50 | Does not reach EC50 |

**APIs and transformation products**

Water samples from the ozonation of effluent wastewater were centrifuged for 10 minutes at 6000 rpm in order to separate potential particles from the samples. Afterwards, 900 µL aliquots of the samples are placed in a 1 mL vial into which 100 µL internal standard is added. 100 µL samples are directly injected into the HPLC-MS/MS for analysis. The separations were conducted using a Synergi polar-RP column (150*2 mm I.D., particle size 4 um, Phenomenex, Torrance, California, USA). The chromatographic separations for transformation products analysis were achieved using a multi-step gradient with acidic elution (0.2% formic acid) of water (A) and methanol (B) as follows: 0→ 1.5 min: 0%B, 1.5→ 2 min 0→ 60%B, 2→ 8 min 60→ 100%B, 8→ 10 min 100%B, 10→ 12 min 100→ 0%B and 12→ 16 min 0%B at a flow of 250 µL/min. Parent compounds were measured with different gradient: a multi-step gradient with acidic elution (0.2% formic acid) of water (A) and methanol (B) as follows: 0→ 1.5 min: 0%B, 1.5→ 3 min 0→ 40%B, 3→ 9 min 40→ 60%B, 9→ 12 min 60→ 80%B, 12→ 12.5 min 80→ 100%B, 12→ 18 min 100%B, 18→ 19 min 100→ 0%B and 19→ 22.5 min 0%B at a flow of 350 µL/min. 100 µL samples were injected.

An overview on the measures transformation products (TPs) and APIs can be found in the subsequent tables SI-Table 39 and SI-Table 40.

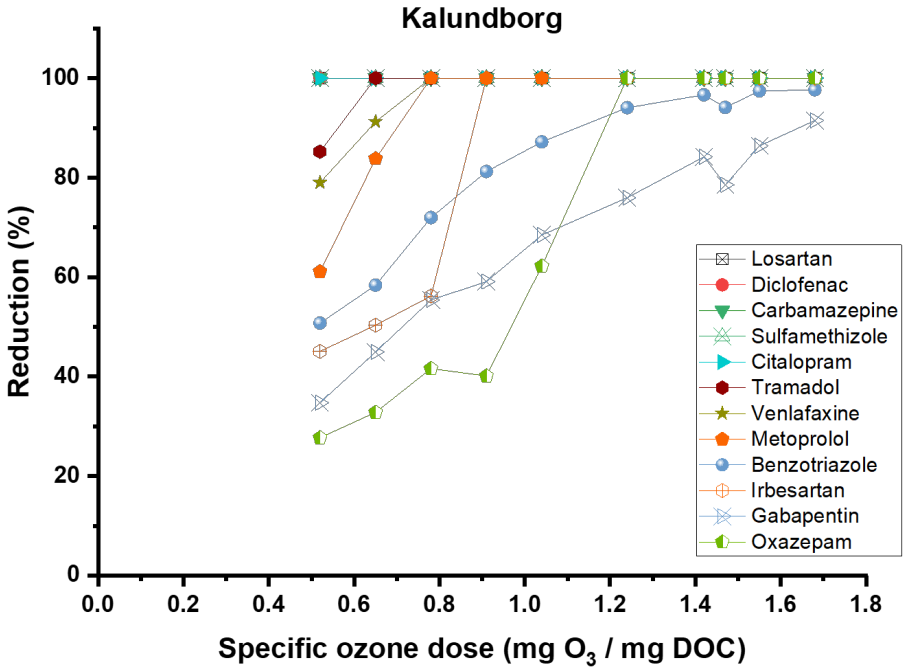
*SI-Table 39: Overview on evaluated transformation products, with the according limit of quantification (LOQ) of the analytical method used at Aarhus University.*

| Parent API | Transformation product | Abbreviation | LOQ (µg/L) |
|---|---|---|---|
| Azithromycin | Azithromycin N-oxides | AZI-NOX | 0.2 |
| Carbamazepine | 1-(2-benzoic acid)-(1H,3H)-quinazoline-2,4-dione | BaQD | 0.05 |
| | CBZ 10,11 epoxides | CBZ-EPX | 0.0125 |
| | rac trans 10,11 (dihyro, dihydroxy) CBZ | CBZ-RTN | 0.0125 |
| Clarithromycin | Clarithromycin N-oxides | CLM-NOX | 0.0125 |
| Diclofenac | Hydroxy diclofenac (mix of 3,4,5 ) | DCF-OH | 0.075 |
| | DCF 2,5 quinone imine | DCF-QIM | 0.1 |
| | DCF amide | DCF-AMD | 0.05 |
| | DCF benzoic acid | DCF-BZA | 0.05 |
| | 1-(2,6-dichlorophenyl)indolin-2,3-dione | DCPID | 0.5 |
| | 2,6-dichlorodiphenylamine | DCPA | 0.2 |
| Erythromycin | Erythromycin N-oxides | ERY-NOX | 0.05 |
| Tramadol | Tramadol N-oxide | TRA-NOX | 0.0125 |
| | N-Desmethyl tramadol | N-DES-TRA | 0.0125 |
| Venlafaxine | Venlafaxin N-oxide | VLX-NOX | 0.00625 |

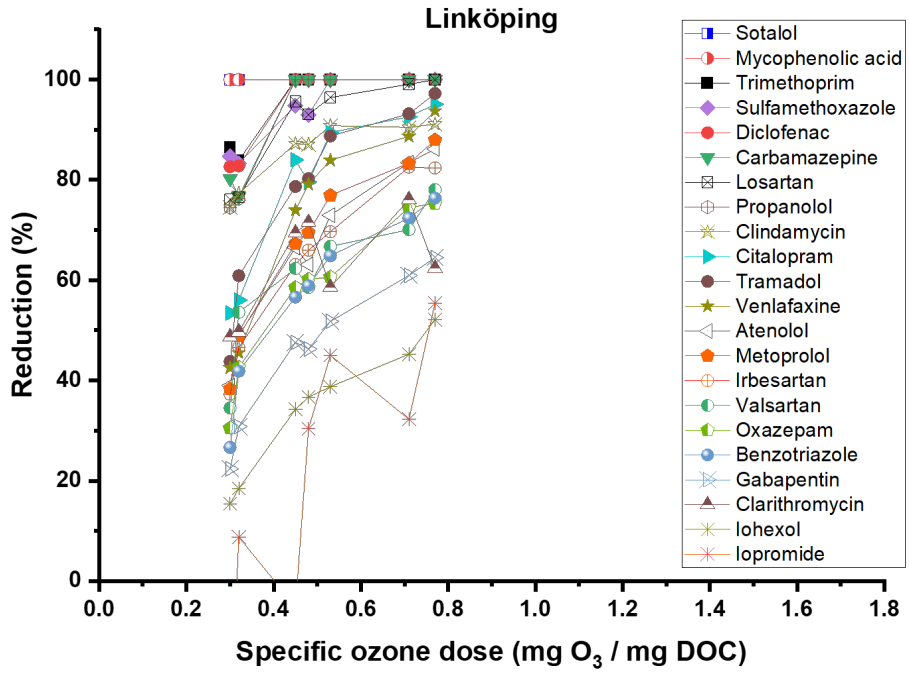
*SI-Table 40: Overview on evaluated APIs as well as other substances such as x-ray contrast agents or corrosion inhibitor, which are highlighted in italic. If not stated otherwise, PNEC and assessment factors are based on CWPharma GoA2.2 report.*

| Active pharmaceutical ingredient (API) | LOQ (µg/L) | PNEC (µg/L) | Assessment factor | CAS Number | Typical API usage |
|---|---|---|---|---|---|
| Atenolol (ATE) | 0.025 | 194 | SSD | 29122-68-7 | antihypertensive |
| Azithromycin (AZI) | 10 | N/A | N/A | 83905-01-5 | antibiotic |
| *Benzotriazole (BTZ)* | *0.025* | *19*[a] | *50*[a] | *95-14-7* | *corrosion inhibitor, antifreezes* |
| Candesartan (CSC) | 0.025 | 0.42 | 1000 | 139481-59-7 | antihypertensive |
| Carbamazepine (CBZ) | 0.05 | 1.28 | SSD | 298-46-4 | antiepileptic |
| Ciprofloxacin (CFX) | 1 | 0.00511 | SSD | 85721-33-1 | antibiotic |
| Citalopram (CIT) | 0.05 | 15.4 | SSD | 59729-33-8 | antidepressant |
| Clarithromycin (CLM) | 0.0125 | 0.00391 | SSD | 81103-11-9 | antibiotic |
| Clindamycin (CDM) | 0.0125 | 0.014[b] | 1000[b] | 18323-44-9 | antibiotic |
| Diatrizoic acid (DZA) | 0.06 | N/A | N/A | 117-96-4 | x-ray contrast agent |
| Diclofenac (DCF) | 0.025 | 0.0852 | SSD | 15307-86-5 | analgesic and anti-inflammatory |
| Eprosartan (ESM) | 0.05 | 100 | 1000 | 133040-01-4 | antihypertensive |
| Erythromycin (ERY) | 0.00625 | 0.0835 | SSD | 114-07-8 | antibiotic |
| Gabapentin (GPN) | 0.05 | 100 | 1000 | 60142-96-3 | antiepileptic |
| Ibuprofen (IBP) | 0.1 | 0.00012 | SSD | 15687-27-1 | analgesic and anti-inflammatory |
| *Iohexol (IHX)* | *0.12* | *N/A* | *N/A* | *66108-95-0* | *x-ray contrast agent* |
| *Iomeprol (IMP)* | *0.12* | *N/A* | *N/A* | *78649-41-9* | *x-ray contrast agent* |
| *Iopamidol (IPD)* | *0.25* | *N/A* | *N/A* | *60166-93-0* | *x-ray contrast agent* |
| *Iopromide (IPR)* | *0.25* | *N/A* | *N/A* | *73334-07-3* | *x-ray contrast agent* |
| Irbesartan (IBS) | 0.00625 | 100 | 1000 | 138402-11-6 | antihypertensive |
| Losartan (LSP) | 0.0125 | 7.8 | 100 | 114798-26-4 | antihypertensive |
| Metoprolol (MET) | 0.05 | 4.38 | SSD | 51384-51-1 | antihypertensive |
| Mycophenolic acid (MPA) | 0.025 | 4.2[b] | 50[b] | 24280-93-1 | immunosuppressant |
| Olmesartan (OLS) | 0.025 | N/A | N/A | 144689-63-4 | antihypertensive |
| Oxazepam (OXA) | 0.025 | 0.81 | 100 | 604-75-1 | treatment of anxiety, insomnia, and alcohol withdrawal syndrome |
| Phenazone (PNZ) | 0.05 | N/A | N/A | 60-80-0 | anti-inflammatory |
| Propranolol (PRO) | 0.025 | 0.01[b] | 50b | 525-66-6 | antihypertensive |
| Roxithromycin (RXM) | 0.3 | N/A | N/A | 80214-83-1 | antibiotic |
| Sotalol (SOT) | 0.025 | 300 | 1000 | 3930-20-9 | antiarrhythmic agent |
| Sulfadiazine (SDZ) | 0.025 | 0.135 | 1000 | 68-35-9 | antibiotic |
| Sulfamethizole (SMZ) | 0.1 | N/A | N/A | 144-82-1 | antibiotic |
| Sulfamethoxazole (SMX) | 0.025 | 0.0438 | SSD | 723-46-6 | antibiotic |
| Tramadol (TRA) | 0.00625 | 170 | 1000 | 27203-92-5 | analgesic and anti-inflammatory |
| Trimethoprim (TRI) | 0.023 | 508 | SSD | 738-70-5 | antibiotic |
| Valsartan (VLS) | 0.05 | 125 | 100 | 137862-53-4 | antihypertensive |
| Venlafaxine (VLX) | 0.0125 | 3.22 | 1000 | 93413-69-5 | antidepressant |

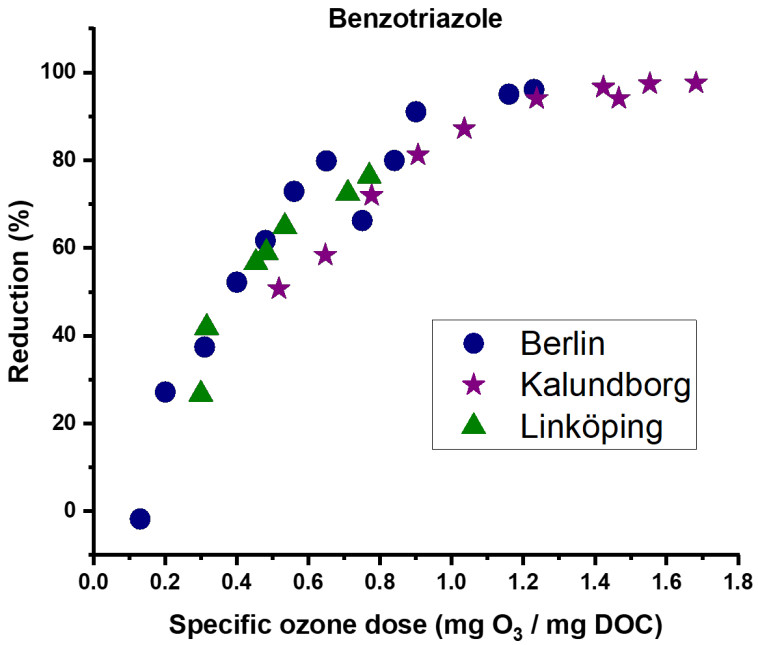
LOQ = limit of quantification of the analytical method used at Aarhus University
PNEC = predicted no effect concentration
SSD = Species Sensitivity Distribution
a) based on European Chemicals Agency (ECHA), date: 14. April 2020. https://echa.europa.eu/registration-dossier/-/registered-dossier/14234/6/1
b) based on Ågerstrand, M. Derivation of PNECs for 39 pharmaceutical substances. ACES report number 36. Stockholm University. Table 4.




*SI-Figure 2: Reduction of pharmaceuticals in dependence of the specific ozone dose at WWTP Kalundborg. In case concentration at the ozonation effluent was below LOQ, API reduction is indicated as 100%. Note that the analytical method was updated with several more compounds compared the step-response experiment at WWTP Berlin.*




*SI-Figure 3: Reduction of pharmaceuticals in dependence of the specific ozone dose at WWTP Kalundborg. In case concentration at the ozonation effluent was below LOQ, API reduction is indicated as 100%. Note that the analytical method was updated with several more compounds compared the step-response experiment at WWTP Berlin.*




*SI-Figure 4: Comparison of the reduction of the corrosion inhibitor benzotriazole (medium reacting compounds) in respect to the specific ozone dose at the three evaluated WWTPs in Berlin, Kalundborg and Linköping. In case concentration at the ozonation effluent was below LOQ, reduction was considered to be 100%.*