CWPharma 2
CLEAR WATERS FROM PHARMACEUTICALS

O2.3 Testing and developing the CWPharma suggestions for the removal of pharmaceuticals - example Hillerød WWTP

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1 Glossary

Parameter	Unit	Explanation
API	µg/L	Active pharmaceutical ingredient
Applied ozone dose	mgO_3/L	Ozone dose corrected by the amount of ozone that left the ozone reactor without any reaction with the water matrix (e.g. via off-gas)
BV		Bed volumes treated
CAS		Conventional Activated Sludge treatment
COD	mg/L	Chemical Oxygen Demand
DOC	mg/L	Dissolved organic carbon
EC_{50}	Vol %	Half maximal effect concentration
K_{O3}	$M^{-1}s^{-1}$	Substance specific reaction rate constant with ozone
K_{OH}	$M^{-1}s^{-1}$	Substance specific reaction rate constant with hydroxyl radicals
LOD		Limit of detection (signal/noise=3/1)
LOQ		Limit of quantification (signal/noise=10/1) (usually 3 times LOD)
NO_2^-	mg-N/L	Nitrite
Ozone dose	mgO_3/L	Amount of ozone added to the ozone reactor normalized for the water flow
PE		Person equivalent
PI	%	Percentage inhibition effect
Q	m^3/day	Flow
Specific ozone dose	mgO_3/mgDOC	Applied ozone dose normalized for the DOC concentration at the ozonation influent
SS	mg/L	Suspended solids
TN	mg/L	Total nitrogen
TP	mg/L	Total phosphorous
TU	-	Toxicity unit

This report is result of the CWPharma 2 project which was funded by the EU's Interreg Baltic Sea Region Programme.

2 General introduction

In CW Pharma it was suggested to remove pharmaceuticals either by ozonation, activated carbon or biofilm reactors. CW Pharma also suggested to go through a process involving i) fitness check, ii) feasibility study, iii)piloting before performing the detailed planning for investments full scale Guideline (2020).

In CW Pharma 2 these recommendations were tested, besides others, at Hillerød WWTP (Hillerød utility), which needs to be ready to conduct full scale removal of pharmaceuticals in the near future due to new legislation in Denmark. Hillerød WWTP will in the future be loaded with significant amounts of wastewater from both a new hospital and even more pharmaceutical industries in the catchment.

Due to space restrictions and due to expected little flexibility to optimise the plant, once established, the pre-decision was to focus on ozonation combined with filtration with granulated activated carbon (GAC) as a post treatment. According to the results of CW Pharma it was assumed that this solution was more ready to be used than other competing technologies.

3 At a glance

- **With ozone and GAC the set targets for HFORS can be achieved**
  With ozonation at a specific ozone dose of 0.5 mg O3/mg DOC and well-functioning granulated activated carbon (GAC) post treatment, all target values for pharmaceuticals for Hillerød wastewater treatment plant can be achieved.
- **The lifetime of GAC can at this moment only be assumed roughly**
  Within the short project it could not be explored whether all compounds needing removal by GAC can be removed over the expected service time (i.e. 20.000 empty bed volumes)
- **In comparison to other wastewater treatment plants used bioassays detected little ecotoxicity of sampled Hillerød wastewater.**
  No effects regarding estrogenicity were detected in this WWTP. Also, opposite to other WWTPs, no issues with ecotoxicity generation during ozonation (especially no mutagenic toxicity) at this plant was detected. In turn, bioluminescence inhibition of enriched samples were generally decreased due to ozonation – as experienced in most other ozonation experiments.
- **PNEC (Predicted No Effect Concentrations) for dimensioning WWTPs**
  PNECs from different data bases can be quite different (up to factor 1000 was experienced in this study). PNECs have shown high value in assessing single chemicals rapidly for regulatory purposes. However, assessing wastewater pollution with organic micropollutants is a complex business as virtually hundreds or thousands of compounds need to be addressed. Using PNECs of multitudes of compounds for dimensioning WWTPs produce difficulties as there is no unified database to refer to and PEC PNEC assessments need to be developed from scientific literature for each WWTP.

4 Hillerød WWTP design and results

4.1 Border conditions at Hillerød WWTP

4.1.1 Design of the classical activated sludge treatment (upfront to possible advanced removal)

The Hillerød Central Wastewater Treatment plant (HCR Syd) is a mechanical – biological – chemical multistage plant taken into service in 2018. A flow diagram is shown in **Figure 1**. Larger materials are removed in the inlet grid followed by sand and grease removal in a trap. Instead of a conventional primary clarifier, 6 Salsnes filters are installed for pre-treatment. In the Salsnes filters the rotating filtermesh removes >50% TSS, and >20% BOD from the primary wastewater and produces drier sludge (4-6% dry matter) for feeding to the digester. Primary sludge from pre-treatment is sent to the anaerobic digester. The biological treatment is handled in 3 process lines each consisting of a selector tank (S), a hydrolysis tank (H) for bio-P enhancement, 3 step denitrification tanks (DN1/2/3), nitrification tank (N) and one swing zone for both nitrification and denitrification (N/DN). Internal recirculation from the N-tank to the DN1 tank is possible up to 5 x process flow. It is possible to dose coagulation and precipitation chemicals in the inlet to the secondary clarifiers where sludge is settled, and phosphorus precipitated. The last step before the treated wastewater is discharged into the recipient is polishing by disc filters to ensure a low content of suspended solids and phosphorus. Secondary sludge from the clarifiers is dewatered in drum filters and sent to the anaerobic digester to produce gas. Heat produced from the gas is used to heat the process buildings and processes where needed and the rest, about half, is sold to the district heating network. Sludge from the anaerobic digester is dewatered in screw presses. The N-rich reject water from the dewatering is treated in an Anammox process to reduce the N-load before circulated back to the process tanks. Currently the final dewatered sludge is incinerated before depositing it.

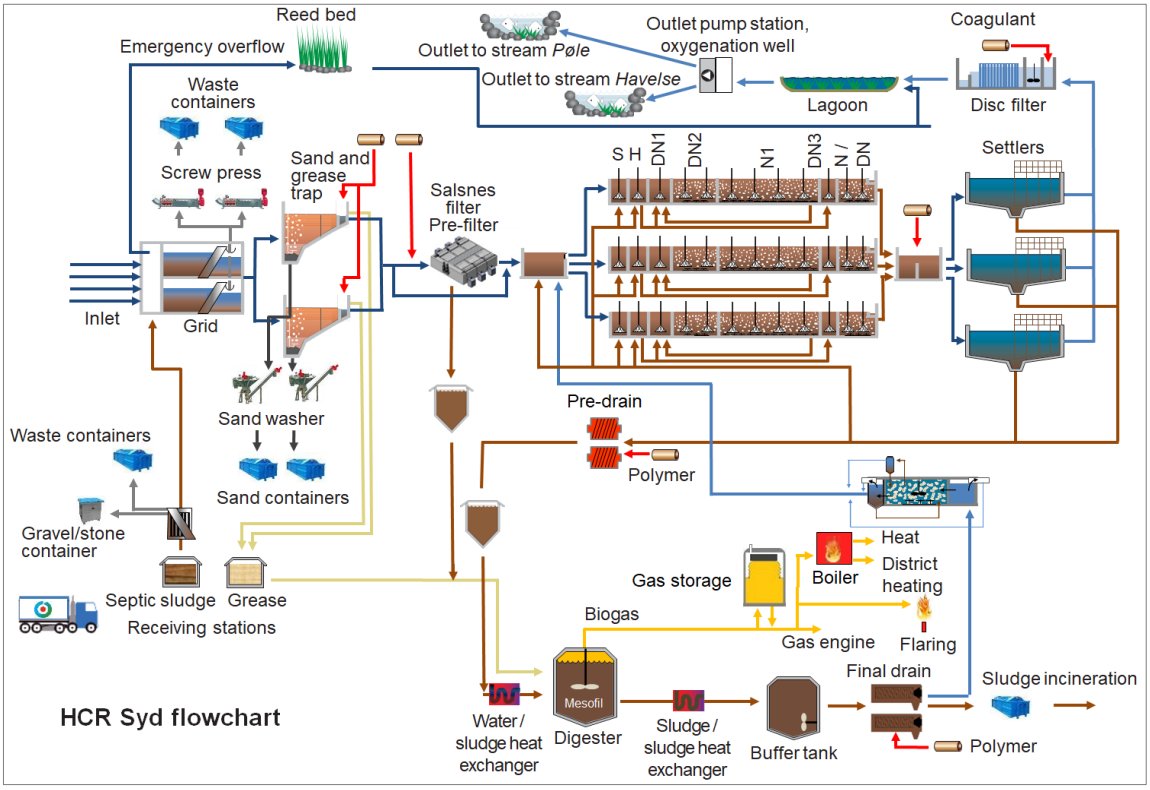

Figure 1. Flow diagram of HCR Syd WWTP in Hillerød, Denmark. Currently the coagulant dosing is only implemented as an option and not operated on a regular basis.

The WWTP is fully covered and build with a sedum green roof for ecological improvement and biodiversity and to impose less nuisance to surroundings and future neighbours. See **Figure 2**.

Figure 2. WWTP in Hillerød. The pretreatment, process tanks and clarifiers with pumps etc. are fully covered inside two buildings built in the landscape with green roofs.

HCR Syd handles wastewater from around 23 000 households and several industries, of which two are large pharmaceutical companies adding substantially to the hydraulic, BOD and pharmaceuticals load of the plant. The current treatment capacity corresponds to 68 000 PE with the possibility to expand to 100 000 PE to make room for a developing city and new industries.

Table 1. Average load and discharge January '21 -October '21 (incl.), and discharge permits, HCR Syd, Hillerød, Denmark.

Parameter, [unit]	2021 average load	2021 average discharge	Discharge permit
Q [m^3/day]	15 919	16 195	18 356
SS [mg/l]	277	3.0	5.0
COD [mg/l]	548	24.6	75
TP [mg/l]	8.09	0.134	0.182
TN [mg/l]	42.6	2.34	3.66

The plant discharges to a local freshwater system which is classified as environmentally vulnerable, thus strict requirements to the discharge quality are necessary. **Table 1** shows the current load, discharge quality and discharge permits at HCR Syd.

4.1.2 New hospital and cleaning for pharmaceuticals

Currently the HCR Syd WWTP is receiving wastewater from a local hospital, which is to be replaced by a large centralized regional hospital in 2025. In Denmark there are no national demands for treatment of pharmaceuticals in wastewater and currently there is no treatment in this regard at HCR Syd. The legal demand on removing the pollution lies on the polluter, in this case the hospital. However, there are negotiations under which conditions HFORS can help to solve the issue for the hospital. The pharmaceutical removal could be achieved by treating the wastewater at the hospital (Scenario 1) (**Figure 3**). However, HCR Syd also discharges to a freshwater system classified as environmentally vulnerable, leading to strict effluent quality requirements. Therefore, it has been also assessed whether the municipal wastewater should be treated to decrease the pharmaceutical loads into the vulnerable stream (Scenario 3). It was decided in 2020 by the local authority that the pharmaceuticals in the wastewater from the new central hospital should be treated at HCR Syd. This would benefit the environment by removing much more pharmaceuticals in total due to the fact the major pharmaceutical load occurs from household wastewater which will be included in a central solution (scenario 2).

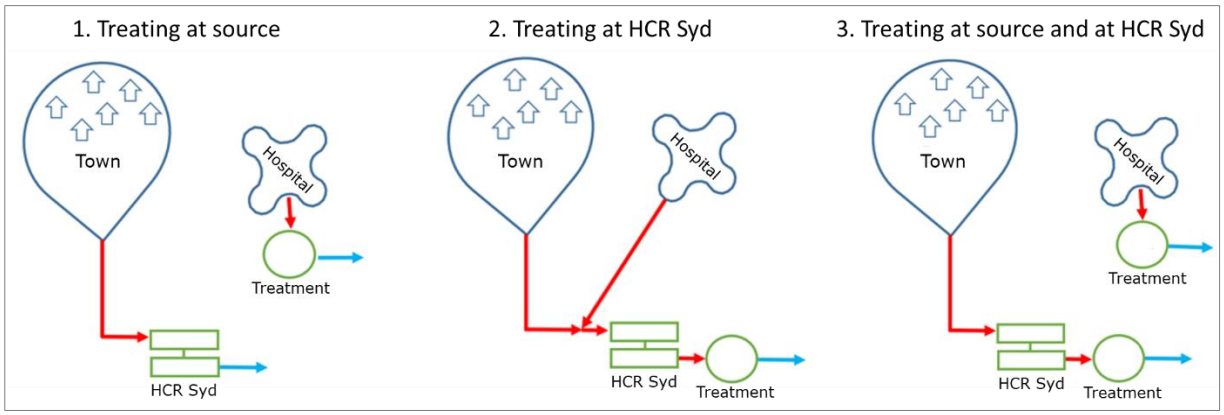

Figure 3. The three scenarios for cleaning pharmaceuticals. The chosen scenario 2 means that the WWTP is going to clean both wastewater streams for Pharmaceuticals.

4.1.3 WWTP HCR Syd, Hillerød pharmaceuticals in effluent

The concentration of active pharmaceuticals ingredients (API) in the effluent of CAS of Hillerød wastewater treatment plant is shown in **Table 2**. Among 53 APIs analyzed, 33 APIs were detected above the limit of quantification. The concentrations of these APIs range from few ng/L to several µg/L. Iodinated x-ray contrast media such as Iohexol was present from 2-20 µg/L, whereas other x-ray contrast media, like Iopamidol and Iomeprol were found to be only around 1 µg/L. Benzotriazole (corrosion inhibitor) was found between 4-15 µg/L. Three pharmaceuticals (Furosemide, Gabapentin and Metoprolol) were found with concentrations exceeding 1 µg/L. The concentrations of the other pharmaceuticals were below the concentration of 1 µg/L.

Besides pharmaceuticals, pharmaceutical metabolites are also present in effluent of CAS. Eleven pharmaceutical metabolites were measured in this experiment above limit of quantification. Among these were *O*-desmethyl -venlafaxine and –tramadol with concentrations above 1 µg/L. The concentrations of the other eight metabolites ranged from a few ng/L to close to 1 µg/L. The measured 10 metabolites are of tramadol, venlafaxine, citalopram, carbamazepine and diclofenac.

Table 2. Pharmaceuticals (API) and pharmaceutical metabolites (*italicized*) measured in the discharge from HCR Syd in April and May 2021.

Pharmaceutical ingredients (including metabolites) and other compounds measured in the HCR Syd discharge April-May 2021	**Discharge HCR Syd mean [µg/l] (n=45) ±SD**				
		Sulfamethizole	0.35 ± 0.08	Erythromycin	0.07 ± 0.01
		Losartan	0.35 ± 0.09	Sertraline	0.07 ± 0.02
		Lidocaine	0.30 ± 0.06	Sulfamethoxazole	0.05 ± 0.01
		Azithromycin	0.28 ± 0.05	Sulfadiazine	0.03 ± 0.01
Benzotriazole (no pharmaceutical: corrosion inhibitor)	8.05 ± 1.81	Bicalutamide	0.25 ± 0.03	Mefenamic acid	0.02 ± 0.00
Iohexol	7.88 ± 3.97	Gemfibrozil	0.25 ± 0.03	*O-Desmethyl Venlafaxine*	2.33 ± 0.26
Furosemide	4.30 ± 0.69	Oxazepam	0.18 ± 0.02	*O-Desmethyl Tramadol*	1.23 ± 0.10
Gabapentin	3.07 ± 0.51	Citalopram	0.16 ± 0.02	*Dihydroxy Carbamazepine*	0.47 ± 0.05
Metoprolol	1.36 ± 0.10	Roxithromycin	0.16 ± 0.02	*Diclofenac Quinone imine*	0.46 ± 0.06
Tramadol	0.90 ± 0.08	Valsartan	0.15 ± 0.04	*N-Desmethyl Tramadol*	0.36 ± 0.05
Iopamidol	0.86 ± 0.51	Clarithromycin	0.15 ± 0.01	*N-Desmethyl Venlafaxine*	0.11 ± 0.01
Iomeprol	0.86 ± 0.52	Atenolol	0.14 ± 0.01	*DES-CIT*	0.11 ± 0.01
Sulphapyridine	0.76 ± 0.08	Trimethoprim	0.13 ± 0.01	*Carbamazepine metabolite BaQD*	0.09 ±0.01
Venlafaxine	0.63 ± 0.07	Carbamazepine	0.10 ± 0.02	*Carbamazepine epoxyde*	0.05 ± 0.01
Diclofenac	0.45 ± 0.05	Rosuvastatin	0.09 ± 0.02	*Bis-Desmethyl Tramadol*	0.02 ± 0.00
Irbesartan	0.37 ± 0.09	Propranolol	0.09 ± 0.01	*Bis-Desmethyl Venlafaxine*	0.02 ± 0.00

As there are no target values for pharmaceuticals (neither for hospital wastewater nor for municipal wastewater or discharge water) the target was set to "no harm", i.e., *a priori* all values in the discharge water should be below Predicted No Effect Concentrations (PNEC). The respective values are shown in **Table 3**. This approach to target values and establish removal of pharmaceuticals is different to those in other regions, e.g., Switzerland and Germany and gave new challenges to CWPharma 2.

Table 3. Proposed demands at the beginning of the project in µg/L

Compound	Best estimate PNEC fresh water [µg/L]	Reference			
Amoxicillin	0.078	BEK nr 1625 from 19/12/2017	Lidocaine	0.002 61	Orias & Perrodin 2013
Ampicillin	0.000 01	Orias & Perrodin 2013	Losartan	331	Fransen Krog *et al*., 2013
Atenolol	128	Fransen Krog *et al*., 2013	Miconazole	0.01	Fransen Krog *et al*., 2013
Azithromycin	0.09	Fransen Krog *et al*., 2013	Mefenamic acid	3.9	Orias & Perrodin 2013
Bicalutamide	0.1	Nielsen *et al*., 2013	Metoprolol	0.1	Orias & Perrodin 2013
Candesartan	0.12	Nielsen *et al*., 2013	Mycophenolic acid	0.1	Nielsen *et al*., 2013
Carbamazepine	0.5	Fransen Krog *et al*., 2013	Olmesartan	60	Asner 2013
Ceftazidime	0.1	Mose Pedersen *et al*., 2007	Propranolol	0.1	Fransen Krog *et al*., 2013
Ciprofloxacin	0.005	Nielsen *et al*., 2013	Phenazone	0.276	Orias & Perrodin 2013
Citalopram	0.000 075	Ekengren *et al*., 2020	Propyphenazone	0.000 086	Orias & Perrodin 2013
Clarithromycin	0.06	Fransen Krog *et al*., 2013	Ranitidine	0.002	Ekengren *et al*., 2020
Clindamycin	0.014	Ekengren *et al*., 2020	Roxithromycin	0.15	Mose Pedersen *et al*., 2007
Codeine	0.06	Orias & Perrodin 2013	Sertraline	0.000 052	Buus Kjær & Ulf Nielsen 2018
Diclofenac	0.1	Fransen Krog *et al*., 2013	Sulfadiazine	4.6	BEK nr 1625 from 19/12/2017
Erythromycin	0.04	Fransen Krog *et al*., 2013	Sulfamethizole	2.54	Orias & Perrodin 2013
Eprosartan	100	Henning *et al*., 2020	Sulfamethoxazole	0.12	Nielsen *et al*., 2013
Estrone	0.000 16	Orias & Perrodin 2013	Sulfapyridine	0.000 122	Orias & Perrodin 2013
Furosemide	31	Nielsen *et al*., 2013	Tramadol	2.25	Fransen Krog *et al*., 2013
Gabapentin	100	Orias & Perrodin 2013	Trimethoprim	10	BEK nr 1625 from 19/12/2017
Ibuprofen	4	Nielsen *et al*., 2013	Venlafaxine	0.1	Biofos's arbejdsgruppe 2013
Irbesartan	700	Schaefer 2016	Oxazepam	0.001 9	Orias & Perrodin 2013
Iohexol	1 000 000	Stuer-Lauridsen *et al*., 2013	Simvastatin	0.000 2	Orias & Perrodin 2013
Iomeprol	1 000 000	Stuer-Lauridsen *et al*., 2013	Sotalol	13	Orias & Perrodin 2013
Iopromide	1 000 000	Stuer-Lauridsen *et al*., 2013	Valsartan	85	Asner 2013

4.2 The Hillerød pilot set ups

4.2.1 Overview on Hillerød pilots

Following the recommendations from "Clear Waters from Pharmaceuticals" (CWPharma) "Guideline for advanced API removal. GoA3.4: Optimization and control of advanced treatment" (December 2020) it was decided that the pilot process design should be treatment of the effluent from HCR Syd with ozonation followed by GAC filtration.

The pilot plant set up was designed to test overall three possible treatment variations: ozonation, GAC, ozonation in combination with GAC See **Figure 4**.

S1. Effluent from conventional activated sludge WWTP/ ozonation influent

S2. Effluent after ozone treatment

S3. Effluent of combined ozonation & GAC

S4. Effluent after GAC filtration without ozonation

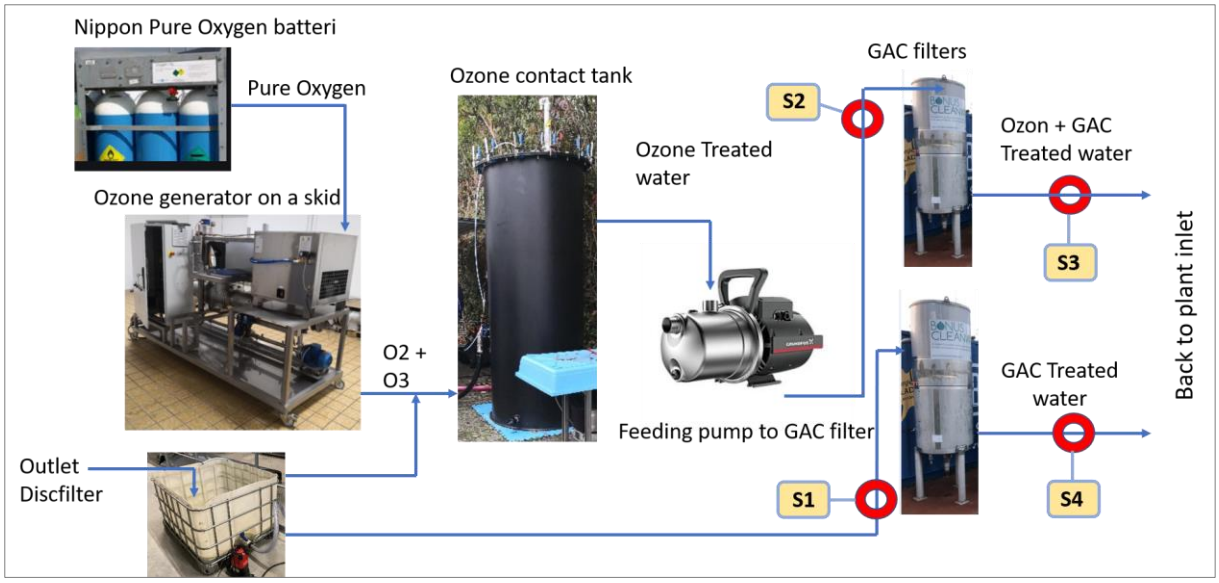

Figure 4. Ozone and GAC pilot plant setup with sampling points at Hillerød WWTP (S1-4).

4.3 Ozone pilot

4.3.1 Set up of ozone pilot

The used ozone reactor had a capacity of 60 g/h ozone rented from Enviroprocess, Odder, Denmark. The ozone treatment process takes place at ambient temperature and pressure with a 1800 L volume of which 420 L are used in the experiment and a 7 minutes hydraulic retention time. Thus, 60 L are treated per minute, corresponding to 3.6 m^3/h. Ozone was transferred into water by using a Roturi® gas mass transfer device also from Enviroprocess. It is based on the generation of a large reaction surface for achieving an instant gas-mass-transfer. The surface

area is created between the water, the equipment's surface and the gas matrix. The ozone dosage in this pilot was determined by controlling gas flow and electrical power settings of the ozone generator. The ozone reactor treated effluent water from the outlet of the disc filters that were operated poststream of the sludge/settler system (**Figure 4**).

4.3.2 Results of ozonation pilot for pharmaceuticals

The future goal for Hillerød WWTP is that the concentrations of the pharmaceuticals that are present in the water that is discharged into the environment should be below the PNEC. **Table 4** shows the PNEC values and concentrations of several APIs in effluent of the current (CAS) WWTP for those compounds that are already below the PNEC and thus do not require further treatment.

Table 4. Pharmaceuticals with CAS effluent concentrations lower than PNEC value

Compounds	PNEC (µg/L)	Inlet concentration (µg/L) (n = 45) ±SD	Benzotriazole	19	8.05 ± 1.81
			Trimethoprim	10	0.13 ± 0.01
Iohexol	1 000	7.88 ± 3.97	Citalopram	0.51	0.16 ± 0.02
Iomeprol	1 000	0.86 ± 0.52	Tramadol	5	0.90 ± 0.08
Iopamidol	1 000	0.86 ± 0.51	Sulfadiazine	4.6	0.03 ± 0.01
Irbesartan	700	0.37 ± 0.09	Sulfamethizole	2.54	0.35 ± 0.08
Valsartan	560	0.15 ± 0.04	Carbamazepine	0.50	0.10 ± 0.02
Losartan	331	0.35 ± 0.09	Erythromycin	0.20	0.07 ± 0.01
Atenolol	128	0.14 ± 0.01	Sulfamethoxazole	0.12	0.05 ± 0.01

The pharmaceutical concentrations presented in **Table 5** were below their limit of quantification (LOQ) and the LOQ for each compound was below PNEC value. Thus, the real concentrations are below PNEC value and no further treatment is required for these compounds.

Table 5. Pharmaceutical compounds with concentrations below PNEC and LOQ below PNEC.

Compounds	PNEC value (µg/L)	LOQ (µg/L)
Iopromide	1 000	0.05
Sotalol	13	0.05
Ibuprofen	4	0.1
Phenazone	0.276	0.1
Candesartan	0.12	0.05
Mycophenolic acid	0.1	0.05
Codeine	0.060	0.05

There were twelve pharmaceuticals detected, for which concentrations were above the PNEC value in the effluent of the conventional WWTP (influent to ozonation) (**Figure 5**). These compounds require further treatment to reach concentrations below PNEC. The resulting concentrations of these compounds after ozonation at five different ozone doses are compared to their PNEC values. With increasing ozone dose, the number of pharmaceuticals exceeding the PNEC decreased, as expected.

In this dataset, most compounds reach concentrations below PNEC when increasing the specific ozone dose to 0.5 (**Figure 5**) which is similar as suggested by Bourgin *et al*., 2018 and on the low side of the range suggested by the CWPharma guideline (2020), while further increase of the ozone dose has no significant effect on the number of compounds reaching concentrations below PNEC. Three pharmaceuticals (Oxazepam, Bicalutamide and Gabapentin) were not removed to the extent required by the PNEC discussed at that time, even at a very high specific ozone dose (1 mg O_3/mg DOC).

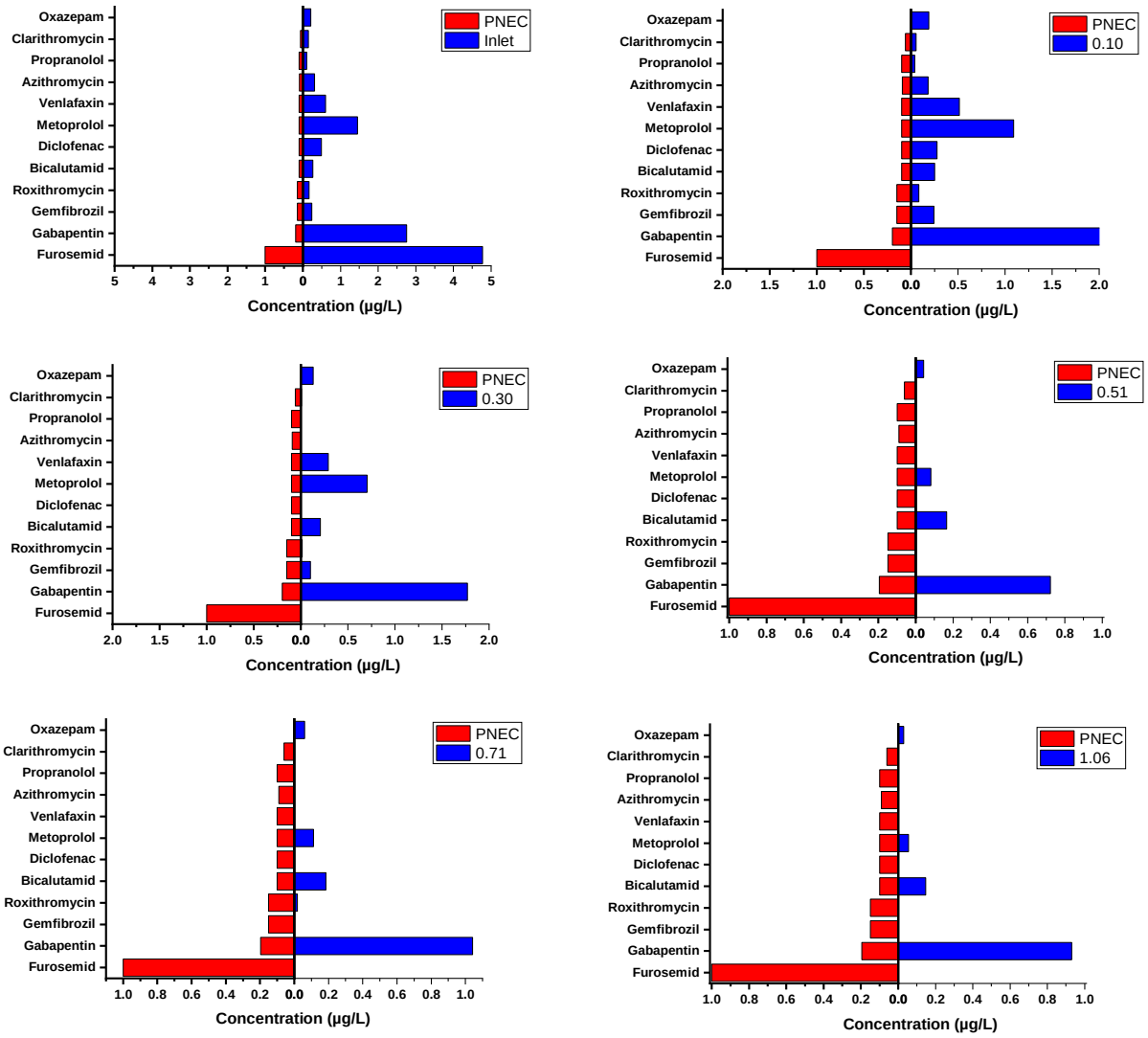

Figure 5. Residual concentrations of pharmaceuticals *vs* PNEC in different ozone doses

Conclusion on removal of pharmaceuticals by ozonation

All considered compounds except Oxazepam, Bicalutamid and Gabapentin were successfully removed at Hillerød WWTP with a specific ozone dose of 0.5 mg O_3 / mg DOC.

4.3.3 Results of the ozonation pilot concerning ozonation products

Ozonation at low ozone dose does not mineralize the compounds but rather transforms them to other compounds (ozonation products). *N*-oxides are major ozonation products formed during the reactions of ozone with tertiary amines (such as several pharmaceuticals are) (Von Sonntag and Von Gunten, 2012, De Witte *et al.*, 2009; Hörsing *et al.*, 2012; Lajeunesse *et al.*, 2013; Zimmermann *et al.*, 2011a). Hence, in this study the formation of several known transformation products in dependence of specific ozone dose was measured. The formation of tramadol *N*-oxide which is an ozonation product of tramadol is shown in **Figure 6**. Similar formation of tramadol N-oxide were described in Kharel *et al.*, (2020).The formation of 5 other *N*-oxides is shown in the appendix (Appendix: Figure 21).

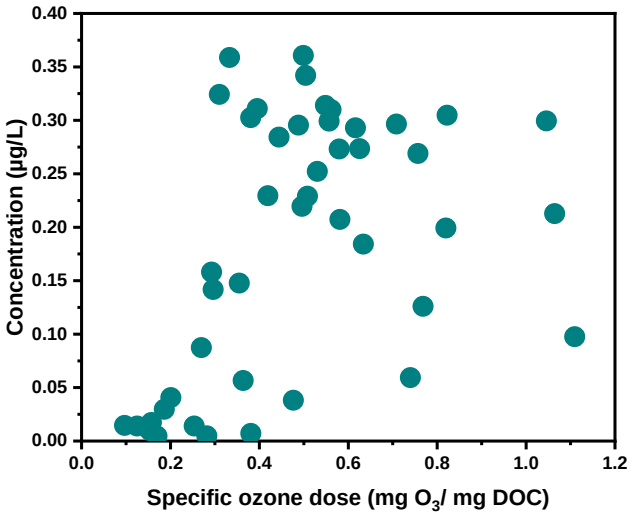

Figure 6. Formation of Tramadol *N*-oxide in relation to specific ozone dose

Conclusion on ozonation products:

Ozonation products are formed from a multitude of compounds. – A maximum formation should be expected around the specific ozone dose for removing the respective parent compounds.

4.3.4 Possibilities for process control for ozonation

Several ozonation experiments were carried out to test the removal of micropollutants in the ozonation pilot plant at Hillerød wastewater treatment plant. DOC is an essential parameter that needs to be considered during an ozonation of wastewater as ozone is highly reactive with the background DOC of the effluent wastewater (Buffle *et al.*, 2006). Hence, the normalized ozone with DOC (specific ozone dose) are often used to describe ozonation for the removal of micropollutants (Lee *et al.*, 2013). Further, nitrite consumes ozone considerably (when present in effluent wastewater) as it is also highly reactive with ozone (Lee *et al.*, 2013).

As DOC cannot easily be measured online (so far), other surrogate parameter can be used to monitor the ozone dose for micropollutants removal. One such example is Ultraviolet absorption at 254 nm (UV254) (Stapf *et al.*, 2016). Often UV254 signal decreases after ozonation. The relation of relative difference in UV254 (ΔUV_{254} = ($UV_{254,\ influent}$ – $UV_{254,\ effluent}$) / $UV_{254,\ influent}$) to specific ozone dose in shown in **Figure 7**. Relative UV254 differences increase with increasing specific ozone dose (specific ozone dose is not corrected for nitrite).

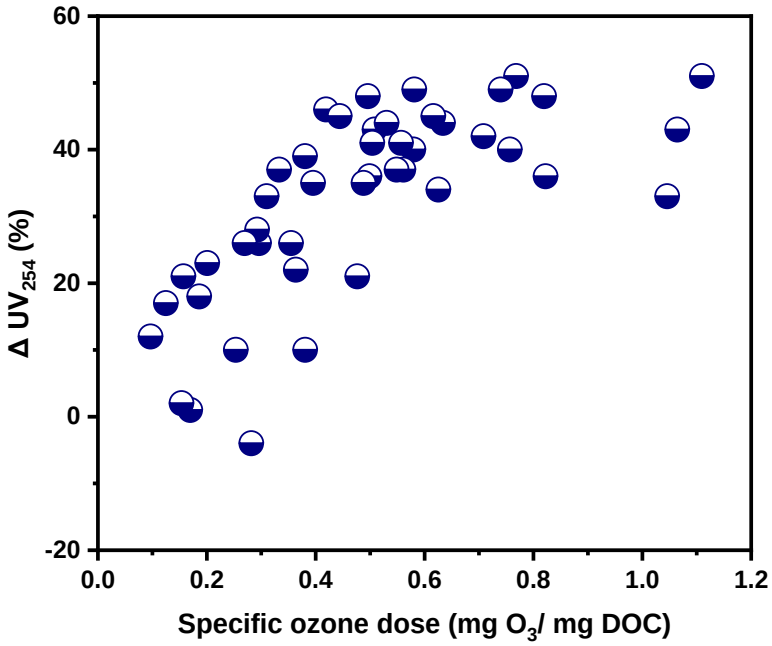

Figure 7. Δ UV254 relationship with specific ozone dose

The removal of four pharmaceuticals in relation to specific ozone dose and difference in UV254 is shown as an example in **Figure 8**. In this experiment nitrite was not measured subsequently the specific ozone dose is not corrected for nitrite. The large variation observed is possibly due to presence of nitrite. Carbamazepine and diclofenac are considered to be among the most reactive substance to ozone. Hence, their removal above 90% can be achieved at around delta UV254 of 25% (specific ozone dose of 0.2 mgO_3/mg DOC. However, venlafaxine and metoprolol are less reactive to ozone (compared to carbamazepine) and thus 90% removal of venlafaxine can only be achieved at around delta UV254 of 40% and for metoprolol at around delta UV254 of 50%. The removal of other 21 APIs in relation to specific ozone dose and UV254 differences is shown in appendix (Figure 15, Figure 16, Figure 17 and Figure 18).

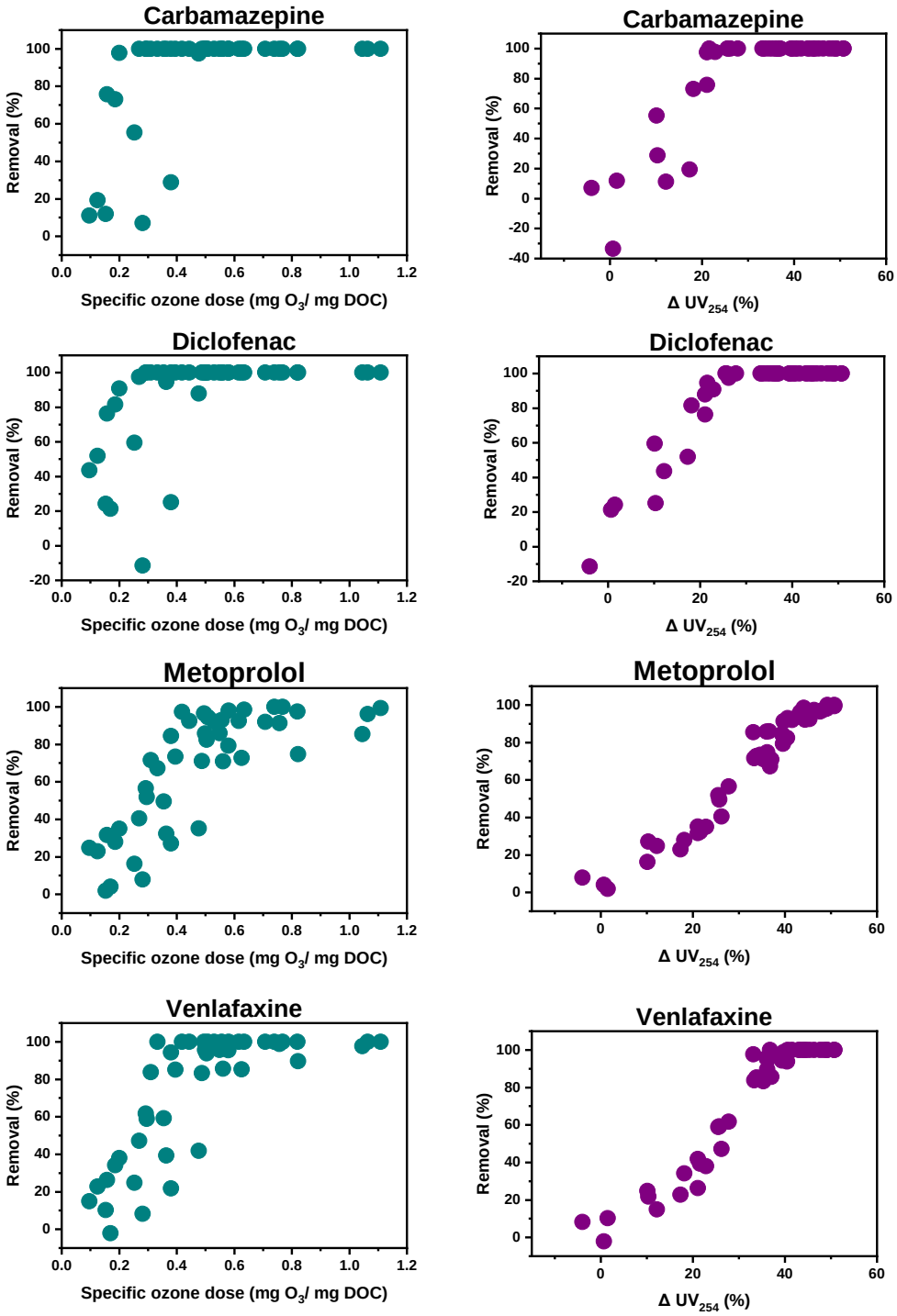

Figure 8. Removal of 4 pharmaceuticals in dependent with specific ozone dose vs delta UV254

Similar to pharmaceuticals, pharmaceuticals metabolites are removed with increasing ozone dose. The removal of one such transformation product di-hydroxy carbamazepine removal is shown in **Figure 9**. The removal of this transformation product were also reported in Kharel et. al., (2021). To achieve 90% removal of di-hydroxy carbamazepine the delta UV254 is needed to be above 50%. The removal of other metabolites in relation to specific ozone dose and UV254 differences is shown in appendices (Appendix: Figure 19 and Figure 20).

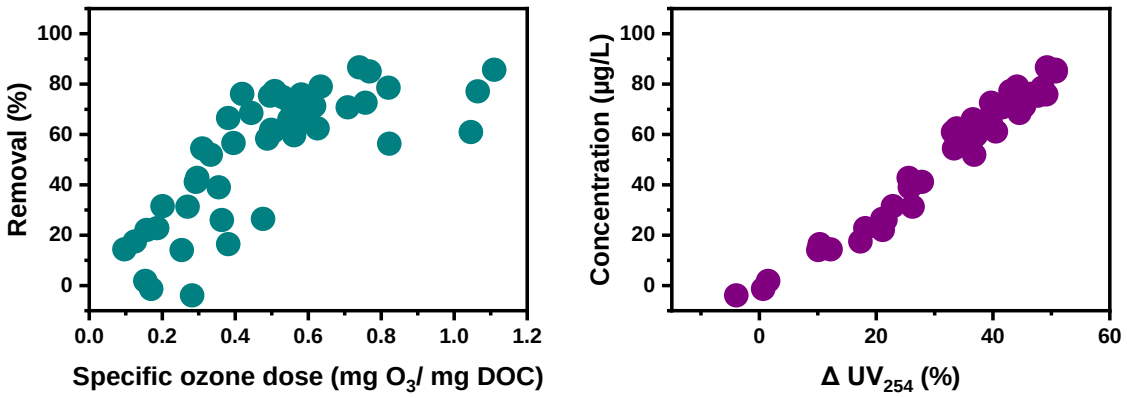

Figure 9. Removal of the carbamazepine ozonation product di-hydroxy carbamazepine in relation to specific ozone dose and delta UV254.

The formation of transformation products in relation to the specific ozone dose did not have any clear trend in the Hillerød dataset as shown in **Figure 6**. However, there is a clear trend visible in the concentration vs delta UV254 plot (**Figure 10**). Tramadol *N*-oxide concentrations increased up to delta UV254 of 38% and then start to decrease. Similarly, the trend visible for 5 other *N*-oxides is shown in appendix (Appendix: Figure 21). This behavior is clearly indicating the formation of the respective N-oxides with increasing ozone dose, followed by further reactions of the *N*-oxides at further elevated ozone doses.

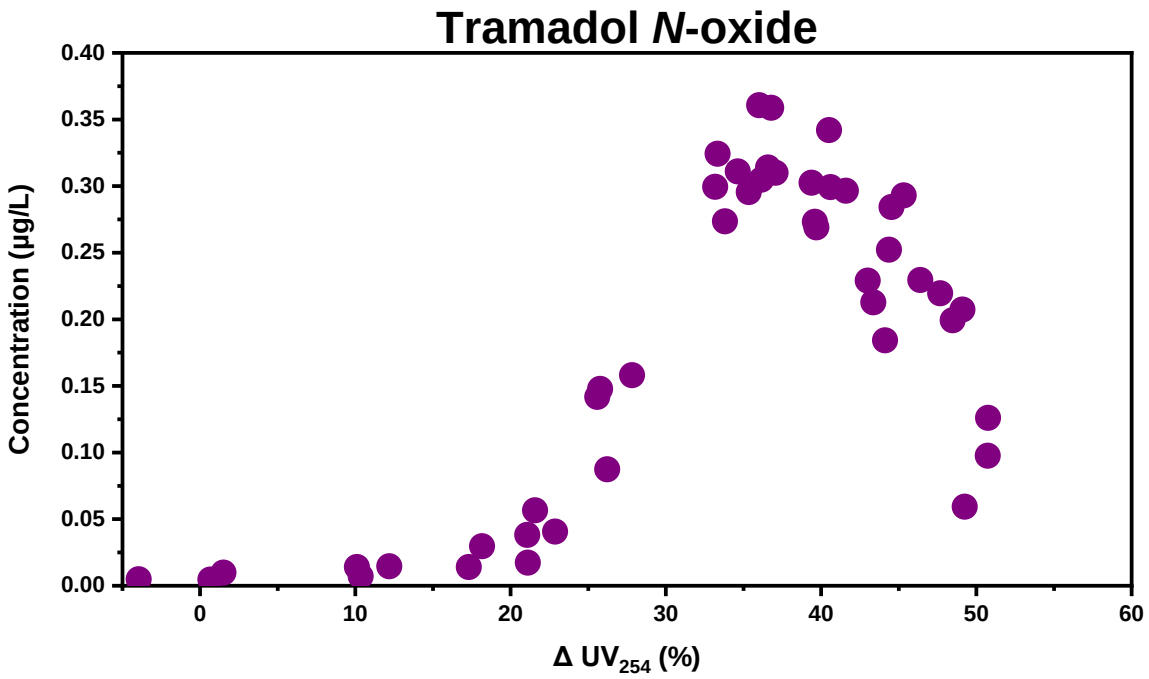

Figure 10. Formation and removal of Tramadol *N*-oxide in relation to delta UV254

Conclusion on ozone process control assessments:

In principle the performance of the ozone reactor can be controlled by offline UV measurements that the WWTP operator can conduct.

4.4 Pilots with granulated activated carbon (GAC)

4.4.1 Set up of GAC Pilots at HFORS for operation on site

In the pilot plant at the HCR Syd two GAC filters of about 1.8 meter height were installed. One for treatment of water directly from the HCR Syd effluent, and the other as ozonation post treatment. The filters were filled with GAC BRENNSORB 1240 from the company Brenntag.

Due to its mesoporosity, BRENNSORB 1240 is designed for the removal of organic contaminants such as pesticides, chlorinated and aromatic solvents, oils, colour bodies, phenols, tannin, taste and odour producing compounds and trihalomethane precursors (humic acids). It can be used for the removal of chlorine, ozone, hydrogen peroxide, permanganate and other oxidants.

BRENNSORB 1240 fulfils the standard UNI ISO EN 12915 and is recommended for different applications such as potable water production, process water, condensate stream purification, and wastewater treatment. When exhausted BRENNSORB 1240 can be reactivated.

Figure 11. GAC pilot plant filters with samplings points at WWTP in Hillerød.

Flow through the columns was initially set by a pressure drop over the columns of 0.3 meter. During the test this meant a flow through the columns of starting with 1 800 litres per hour (equalling a flow of 24 bed volumes/day) falling down to 600 litres per hour at the end of the experiment. This is corresponding to a contact time (EBCT) of 30 minutes in the start up to 90 minutes in the end of the experiment. No Backwash was tested to remove the sludge layer.

A faulty bypass of the Dyna Disc filters at the end of the pilot period caused significant input of suspended solids into the influent to the GAC filter resulting in a capacity as low as 120 litre/hour even at adjusted extra pressure loss up to a total of 0.6 meter.

4.4.2 Results on Pharmaceuticals removal by GAC pilots

Over the period of April 14 – May 4 the 53 PNEC-regulated compounds (x-ray contrast media and APIs) have been measured in the outflow of the GAC-stand-alone treatment (14 samples) and the outflow of the combined O_3-GAC treatment (24 samples including different applied O_3 dosages). Only 4 of the compounds have been detected in any of those samples in negligible concentrations, far from approaching the PNEC thresholds. Because of a relative short operational period (approx. 1 700 bed volumes), the results of the pilot-scale GAC filters will not be used to estimate lifetime and thus economy of this approach However principle results from the fresh GAC are documented in **Table 6**.

Table 6. Compound detection in the outflow of the pilot-scale GAC filters

Compound name	Outflow of GAC treatment. (Number of samples = 14)	Outflow of combined O_3-GAC treatment. (Number of samples = 24)
49 of the 53 monitored compounds (see **Table 7**)	Not detected/ quantitative removal	Not detected/ quantitative removal
Iohexol	10 samples with average concentration of 0.55 (±0.33) µg/L; 3 samples <0.02 µg/L	18 samples with average concentration of 0.29 (±0.29) µg/L; 5 samples <0.02 µg/L
Iomeprol	11 samples <0.02 µg/L	18 samples <0.02 µg/L
Iopromide	10 samples <0.02 µg/L	18 samples <0.02 µg/L
Erythromycin	All samples with a trace of << 0.01 µg/L	All samples with a trace of << 0.01 µg/L

Iohexol, Iomeprol and Iopromide (**Table 6**) have been detected in the outflow of the GAC because of their low affinity to GAC (x-ray contrast media). On the other hand, the trace levels of Erythromycin have been detected because of an exceptionally low limit of detection of this compound in the mass spectrometric method.

Results on removal of transformation products in GAC pilots after ozonation

Six *N*-oxides of pharmaceuticals (ozonation products), i.e., Erythromycin *N*-oxide, Venlafaxine *N*-oxide, Azithromycin *N*-oxide, Clarithromycin- *N*-oxide, Tramdol *N*-oxide and Citalopram *N*-oxide were measured after ozonation with concentrations between 0.00005 and 0.126 µg/L (**Figure 6**). The fresh GAC pilots were efficiently able to remove all *N*-oxides measured after ozonation to values below the limits of quantification.

Conclusions on GAC Pilots:

The GAC pilots were only run over a short time period. Over this short time period with fresh GAC they were able to remove all compounds surviving the ozone treatment or being formed in the ozone treatment that were measured.

4.4.3 Offline (laboratory) GAC columns

Set up of offline GAC columns

The GAC pilot filters described above have not been exploited over commercially relevant period. To obtain knowledge on longer GAC exploitation potential, a smaller scale experiment has been performed in the AU for 6 months (February – August 2021). The granules from the same GAC batch (0.4-1.7 mm *Brennsorb 1240*) were tightly packed in a 180 mL (40cm height) cylindric glass column, which has been continuously operated pumping wastewater from

bottom to top, allowing its contact with the GAC for 25 min. The applied 3.6 mL/min flowrate corresponded to those in the pilot with 25.6 bed volumes (BV)/day, resulting in 4 500 BVs over the total period of the experiment.

Results of GAC columns (capacities)

The wastewater applied in the laboratory-scale GAC filtration was taken in batches in the outlet of Hillerød WWTP CAS in time intervals of approx. 3–4 weeks. Prior to feeding the GAC filter, the wastewater was stored in 5°C. In the beginning of the experiment the outflow of the GAC filter was collected at time intervals of approximately 2 samples per week, with decreasing frequency resulting in 1 sample per 2 weeks at the end of period (**Figure 12**). The GAC column inflow and outflow have been measured for the 53 compounds and 12 API transformation products.

For the first 230 BVs of the GAC exploitation none of the monitored compounds were detected in the outflow of the GAC (besides trace levels of Erythromycin). By the middle of the experiment (~2 200 BVs) – 8 compounds, and by the end of the experiment – 16 compounds have been detected in the GAC outflow. The CAC performance for each compound was expressed in the removal percentage of the corresponding compound. **Figure 12** graphically illustrates examples of change of the GAC performance in time.

Table 7. GAC performance for removal of the compounds reported in CWPharma (No. 1-36) and compounds that were measured only in CWPharma2 (No. 37-53).

		LOD	LOQ	Average effluent 2021 April 14 -May 4 (n=45)	Standard deviation from the average conc.	PNEC	Is tertiary wastewater treatment needed to meet PNEC	Min. further removal required to treat the effluent	Max GAC exploitation for meeting the required % removal
	Compound name	µg/L					No/Yes	%	BV
1	Atenolol	0.01	0.02	0.14	0.01	128	No		
2	Azithromycin	0.05	0.3	0.28	0.05	0.09	**Yes**	68	> 4 500
3	Benzotriazole	0.02	0.05	8.05	1.83	19	No		
4	Candesartan	< 0.01	0.01			0.12	No		
5	Carbamazepine	0.01	0.02	0.10	0.02	0.50	No		
6	Ciprofloxacin	0.5	1			0.09	No		
7	Citalopram	< 0.01	0.01	0.16	0.02	0.51	No		
8	**Clarithromycin**	< 0.01	< 0.01	0.15	0.01	0.06	**Yes**	60	3 200
9	Clindamycin	0.01	0.03			0.014	No		
10	Diatrizoic acid	< 0.01	< 0.01			100 000	No		
11	Diclofenac	0.05	0.1	0.45	0.05	0.10	**Yes**	78	> 4 500
12	Eprosartan	0.05	0.1			100	No		
13	Erythromycin	< 0.01	< 0.01	0.07	0.01	0.20	No		
14	**Gabapentin**	0.01	0.02	3.07	0.51	0.196	**Yes**	94	> 1 500 (< 3 000)*
15	Ibuprofen	0.2	0.5			4	No		
16	Iohexol	0.01	0.02	7.88	4.02	1 000	No		
17	Iomeprol	0.01	0.02	0.86	0.52	1 000	No		
18	Iopamidol	0.01	0.02	0.86	0.52	1 000	No		
19	Iopromide	0.01	0.02			1 000	No		
20	Irbesartan	< 0.01	0.01	0.37	0.09	700	No		
21	Losartan	0.01	0.2	0.35	0.10	331	No		
22	Metoprolol	0.01	0.02	1.36	0.10	0.10	**Yes**	93	> 4 500
23	Mycophenolic acid	0.02	0.05			0.1	No		
24	Olmesartan	0.05	0.1				No		
25	Oxazepam	0.02	0.05	0.18	0.02	0.0019	**Yes**	99	> 4 500
26	Phenazone	0.03	0.05			0.276	No		
27	Propranolol	0.01	0.02	0.09	0.01	0.10	No		
28	Roxithromycin	0.05	0.1	0.16	0.02	0.150	**Yes**	4	> 4 500
29	Sotalol	0.03	0.05			13	No		
30	Sulfadiazine	< 0.01	0.01	0.03	0.01	4.6	No		
31	Sulfamethizole	< 0.01	0.02	0.35	0.08	2.54	No		
32	Sulfamethoxazole	0.01	0.02	0.05	0.01	0.12	No		
33	Trimethoprim	< 0.01	< 0.01	0.13	0.01	10	No		
34	Tramadol	0.05	0.5	0.90	0.08	5	No		
35	Valsartan	0.02	0.05	0.12	0.07	560	No		
36	**Venlaflaxine**	0.01	0.03	0.63	0.07	0.1	**Yes**	84	4 500
37	Amoxicillin	0.02	0.05			0.078	No		
38	Ampicillin	0.02	0.05			0.0005	No		
39	Bicalutamide	0.10	0.30	0.25	0.03	0.10	**Yes**	60	> 4 500
40	Ceftazidime	0.30	1.00			0.13	No		
41	Codeine	0.05	0.10			0.060	No		
42	Estrone	0.10	0.30			0.00016	No		
43	Furosemide	1.00	2.00	4.30	0.70	1	**Yes**	77	> 4 500
44	Gemfibrozil	0.10	0.20	0.25	0.03	0.15	**Yes**	40	> 4 500
45	Lidocaine	0.01	0.02	0.30	0.06	0.00261	**Yes**	99	> 4 500
46	Mefenamic acid	0.01	0.05	0.02	0.00	0.001	**Yes**	95	> 4 500
47	Miconazole	0.20	0.50			0.01	No		
48	Propyphenazone	0.02	0.07			0.0086	No		
49	Ranitidine	0.05	0.10	0.03	0.15	0.001	**Yes**	97	> 4 500
50	Rosuvastatin	0.02	0.05	0.09	0.02	0.001	**Yes**	99	> 4 500
51	Sertraline	0.03	0.05	0.07	0.02	0.00052	**Yes**	99	> 4 500
52	Simvastatin	0.30	0.70			0.0002	No		
53	Sulfapyridine	0.20	0.50	0.76	0.08	0.000122	**Yes**	99	> 4 500

*Result of the Gabapentin is provided as an interval because of unstable concentration of the compound in the CAS effluent (explained further in the text).

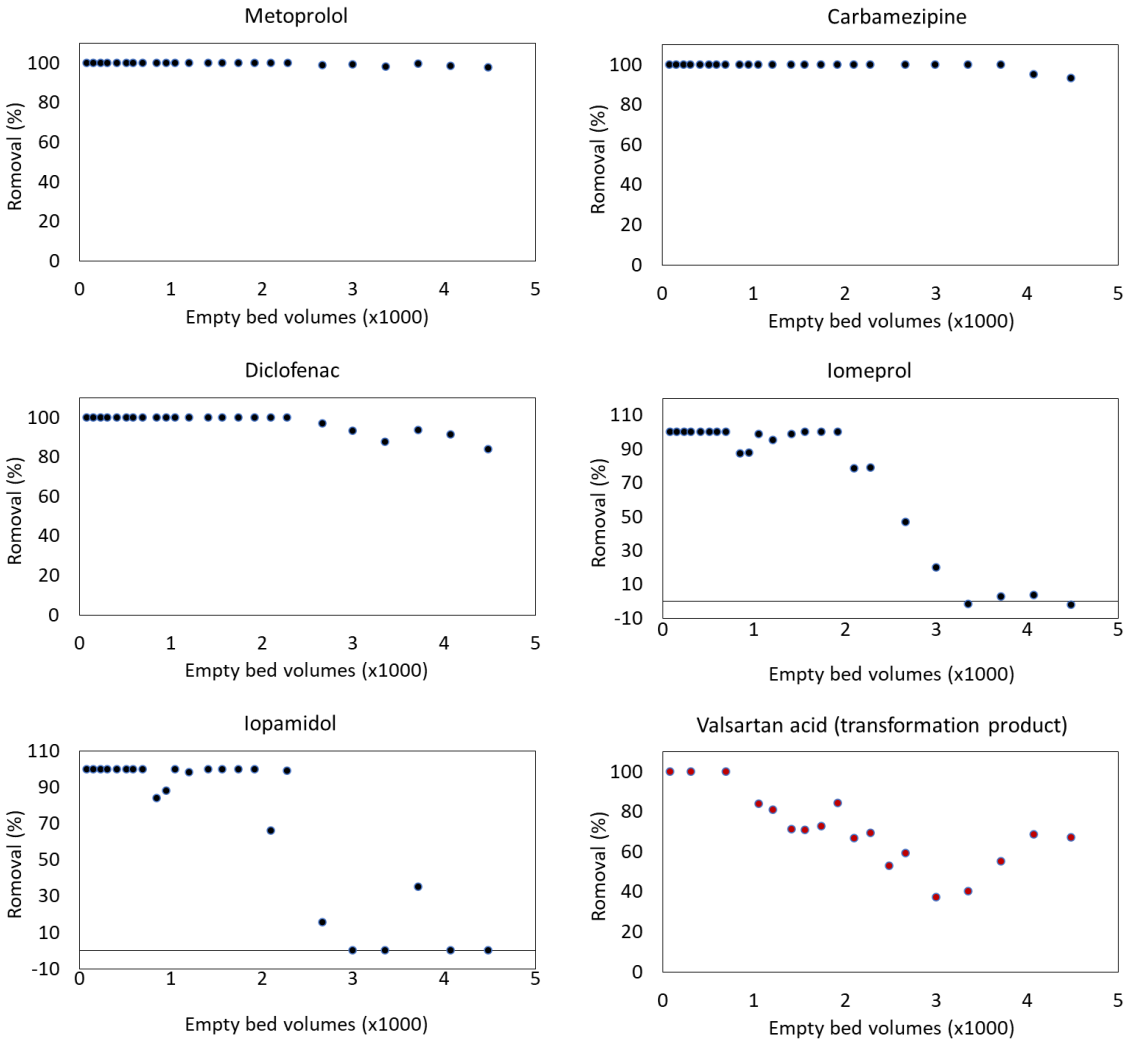

Figure 12. Selected examples of GAC performance for several compounds in time. APIs (Metoprolol, Carbamezepine, Diclofenac) X-ray contrast media (Iomeprol, Iopamidol) and API transformation product (Valsartan acid).

Conclusion on GAC-Laboratory columns:

At the beginning the removal in the laboratory GAC columns was quantitative, and mirrored thus the behavior in the big pilots.

While a multitude of compounds behaved like Metoprolol, Carbamazepine and Diclofenac and revealed quantitative removal over the whole period, other compounds were less well retained over time.

X-ray contrast media (Iomeprol, Iopamidol) and few API transformation products start to show lower removal after 2000 BV. However, the x-ray contrast media have relatively high PNEC requirements that are fulfilled already after the CAS, whereas API transformation products have no PNEC values imposed. The PNEC-relevant removal of all the 53 compounds is provided in **Table 7.**

Especially the target values for Clarithromycin, Gabapentin and Venlafaxine are difficult to reach with the GAC column after treatment of 4 500 BV.

4.5 Assessment of the ozone/GAC combination in respect to the discussed demands

As Oxazepam, Bicalutamide and Gabapentin cannot efficiently be controlled by ozonation alone and Clarithomycin, Gabapentin and Venlaflaxine cannot be efficiently be controlled by GAC alone it was calculated whether the combination would be efficient.

The combined O_3-GAC treatment has not been experimentally performed in the lab. However, the CAS outflow in Hillerød WWTP after applying different O_3 dosages has been experimentally tested in pilot-scale ozonation experiment. **Table 8** illustrates Clarithromycin concentrations in the CAS outflow in Hillerød WWTP when different O_3 dosages were applied, the minimal further required compound removal to meet the PNEC threshold and the corresponding BV exploitation of GAC. Clarithromycin is selected for illustration as it is one of the three compounds identified as limiting the GAC exploitation lifetime in **Table 7**. The other two of the three identified compounds had either just reached the required limit with the applied 4 500 BV (Venlafaxine) or had statistical uncertainty due to unstable concentration in the CAS outlet (Gabapentin) (**Figure 13**).

Table 8. Illustration of GAC filter lifetime exploitation for Clarithomycin with ozonation pretreatment

Specific ozone dose (mg O_3/mgDOC) *	Corresponding average effluent April 14 -May 4, 2021 (µg/L)	PNEC (µg/L)	Min. further removal required to reach the PNEC (x%)	GAC exploitation for the x% of removal (BV)
0	0.15	0.06	60	3 200 (observed)
0.15	0.09		33	> 4 500 (extrapolated)
0.25	0.07		14	> 4 500 (extrapolated)
0.35	0.04		none	GAC not required

*Designed value

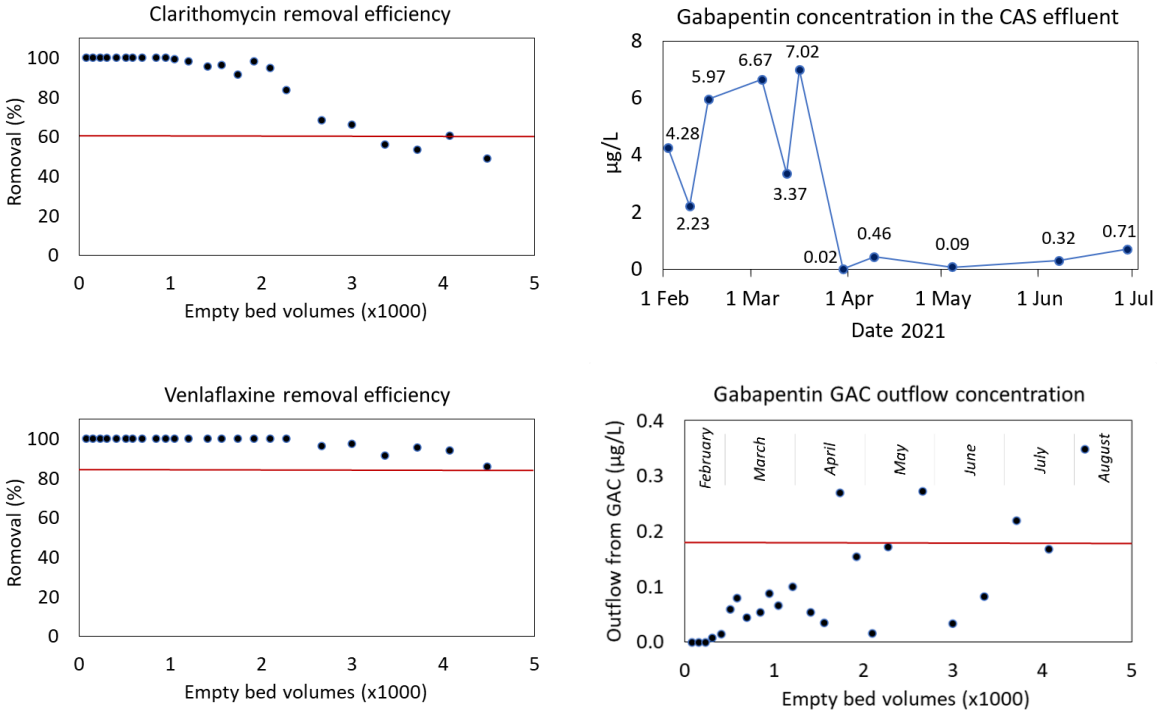

Figure 13. Removal efficiency of three compounds identified as limiting for > 4,500 BV GAC exploitation. The red line indicates required removal efficiency to meet the PNEC threshold (for

Clarithromycin and Venlafaxine) and required PNEC threshold concentration (for Gabapentin). For the thresholds refer to **Table 7**.

Conclusions on ozone GAC combination:

While all compounds including Oxazepam, Bicalutamide, Clarithromycin, and Venlafaxine can efficiently be removed by the ozone GAC combination, Gabapentin cannot be removed to an extent as required by the original PNEC assessments (**Table 3**).

4.6 Biotests based assessment of efficiency of ozone and GAC pilot at Hillerød WWTP

Ecotoxicological assessment

Overview on conducted ecotoxicological tests

Three ecotoxicological tests were performed in laboratories in Germany (UBA) and Poland (IOS). The used test systems covered the proposed range of ecotoxicological endpoints suggested from the CWPharma1 project, i.e., mutagenicity, estrogenic effects and bioluminescence inhibition. All tests were performed with enriched samples based on extracts from a solid phase extraction (SPE) using dimethyl sulfoxide (DMSO) as solvent.

Sampling and SPE procedure

During the two sampling campaigns for Hillerød the sampling points (**Figure 4**)

- influent O_3 (S1)
- effluent O_3 (S2)
- O_3 + GAC (S3)
- GAC stand alone (S4)

have been sampled with corresponding grab samples. Sample volumes covered each two technical replicates. More technical details are in appendix Page 43.

Results of ecotoxicity tests

Mutagenicity with Ames (YG7108, +/-S9)

Samples from Hillerød 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 20. Accordingly, secondary effluents of WWTP Hillerød as well as ozonation and post treatment effluents, respectively, showed no mutagenic effects regarding alkylating agents.

Estrogenicity with YES

No estrogenic potential was detected in all samples (four sampling points from two sampling campaigns) at WWTP Hillerød.

Aliivibrio fischeri bioluminescence inhibition test

The most important parameter determined in the Microtox procedure is the percentage inhibition effect (PI) of *Aliivibrio fischeri* bioluminescence (%), which is converted into EC_{50} -t values and Toxicity Units (TU) using special Microtox Omni software algorithms. These both parameters were used to assess the toxicity degree of samples taken from individual stages of

wastewater treatment and the impact of the ozonation process and other treatment stages on the ecotoxicological safety of the aquatic environment. Classification of toxicity to bacteria *Aliivibrio fischeri* (**Table 9**) was made based on the criteria proposed by Persoone *et al.* (2003). Anyhow, the assessment of sample toxicity according to the Persoone classification only serves for comparability of different samples, as classification of wastewater regarding EC_{50} values is originally to be used for native samples without enrichment. The more concentrated the sample, the higher is the toxicity.

Table 9. Toxicity classification according to Persoone et al. (2003) used for result interpretation. PI = percentage inhibition effect, TU = toxicity unit.

Toxicity class	Toxicity level	PI	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	PI 100% in at least one test	10.0 – 100.0	<25%
Class V	Very high acute toxicity	PI 100% in all tests	>100.0	-

Generally speaking, these tests were conducted to reveal differences due to processes in the treatment train, thus the samples were up-concentrated considerably.- Thus the detected effects can not be read as any of the water is actually toxic.

On the basis of EC_{50} values, toxicity levels of samples from the WWTP Hillerød varied between minimum 24 % and maximum 76 %, while most of the samples varied between 45 % and 70 %, which is "acute toxic" (class III, **Figure 14**). This toxicity classification further holds for the evalution of the inhibition effect (Appendix, Figure 23). However, it is emphasized that acute toxicity is due to the very high enrichment factor of 100 and does not indicate that the treated wastewater itself has any toxicity. As the purpose of such tests using enriched samples is to identify differences between the different treatment stage (i.e., impact of ozonation or impact of post-treatment, the absolute values themselves cannot be used to draw any conclusions on wastewater toxicity. The highest bioluminescence inhibition effect to *A. fischeri*, expressed as lowest EC_{50} value, was detected in samples at the influent of the ozonation. Based on this study it can be concluded that the ozonation process reduced the level of toxicity towards tested bioluminescent bacteria. Statistical analyses have confirmed this effect (see statistical analysis section in the appendix, Table 13 - 15) and a statistically significant difference between samples before and after the ozonation process were found. No significant difference was found between the effluent of the combined treatment ozonation and GAC and the stand-alone GAC-filter. See appendix Table 16 for all available data of bioluminescence inhibition test.

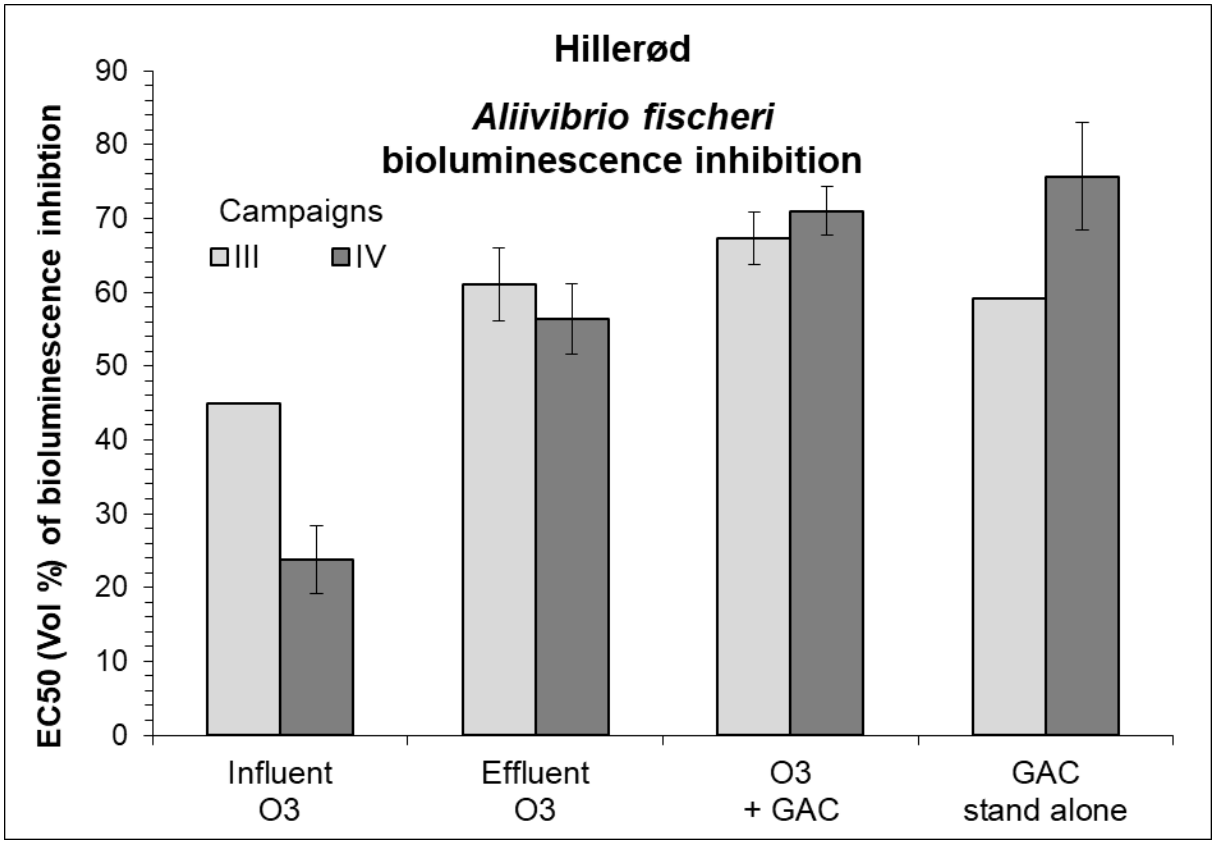

Figure 14. Mean EC50 value (Vol %, ± SD) to *Aliivibrio fischeri* bioluminescence at different sampling points at WWTP Hillerød for an enrichment factor of 100.

Conclusions from the ecotoxicological assessment:

Only the bioluminescence of *A. fischeri* was affected by samples from WWTP Hillerød and a beneficial impact of the ozonation process was found for the inhibition of *Aliivibrio fischeri* bioluminescence. Interestingly, the relatively fresh GAC-filter used as ozonation post-treatment did not cause a significant impact on bioluminescence inhibition. However, a trend for lower toxicity, i.e. a higher EC50-value, due to the GAC filter can be seen for the sampling campaign IV.In turn, and opposing to results from most other plants, no effects have been determined regarding estrogenicity and mutagenicity, neither before, nor after the ozonation process. Accordingly, no assumptions regarding the impact of each single treatment stage can be done with respect to those two bioassays.

4.7 PNEC uncertainty based impact on dimensioning of pharmaceutical removal

PNEC Values have been established in chemicals risk assessment (ECHA 2008). PNEC values are calculated by using the lowest observed effect concentration (LOEC) that is relevant for the ecosystem under discussion followed by assessing the uncertainties of the observed determination.

The uncertainties are compensated with uncertainty factors with low uncertainty factor (e.g. 10) typically used for long-term studies with mesocosm systems with several trophic levels and high uncertainty factors (e.g., 1000) used for short time single species test. Compare (ECHA, 2008).

As risk assessment PNEC values are used in relation to be predicted (or measured) environmental concentrations (PEC/PNEC).

PEC/PNEC considerations have been relatively successful in identifying seriously toxic compounds in the REACH process, sorting compounds with very high PEC/PNEC values such as 1000 as problematic for which use should be restricted from those for which environmental impact in improbable PEC/PNEC 0.001.

Usually the database for PEC/PNEC assessment is not precise enough to discriminate between, e.g., 5 and 1.

PEC/PNEC assessment can be used also for assessing concrete emissions but not too high precision should be expected. However, generally speaking PNEC values include a high uncertainty and high comparability is not given: a comparison between two databases in CW Pharma 2 revealed differences of several orders of magnitude:

As especially Gabapentin is causing issues for the design of the API removal part of the Hillerød plant, CW Pharma 2 dug a bit deeper into the data of the Danish assessment. The PNEC of 0.196 µg/L was given in the Danish assessment based on Orias & Perrodin, 2013. These authors indeed give this number, though not as a result of experimental data as such but based on an ECOSAR approach. ECOSAR is a software tool that partially models, partially links to experimental data. Putting the CAS number of Gabapentin [60142-96-3] into the current version of ECOSAR (ECOSAR 2.0 downloaded 17th November 2021) gives lowest effect levels for Gabapentin as 243 mg/L (for daphnids)(**Table 11**). Applying an assessment factor of 1000 as usual for the situation and as Orias & Perrodin, 2013 did, results in a PNEC of 243 µg/L and not of 0.196 µg/L as tablised by Orias & Perrodin, 2013. Assessing whether there was a technical error in Orias & Perrodin, 2013 or whether the dataset behind ECOSAR has been updated is close to impossible to find out at this stage. However, the PNEC resulting from the current version of ECOSAR (243 µg/L) and the one determined by CWPharma (100 µg/L) based on experimental data is very close. The revised PNEC and the resulting new suggested target value for Gabapentin is contained in **Table 10**.

Table 10. Numerical comparison of PNEC values from the Danish and the CWPharma database

PNEC value	DK PNEC	Ref. A CWPHARMA PNEC values	Factor of difference
Pharmaceutical	Fresh water [µg/l] DK	Fresh water [µg/l] CWPharma	
17α-Ethinylestradiol	0.000 075	0.000 408	5
17β-Estradiol	0.000 1	0.000 032 3	3
α-Estradiol		0.000 853	
Estriol (E3)		0.000 75	
Estrone (E1)		0.000 008	
Sertraline	0.000 52	1.07	2 058
Ciprofloxacin	0.005	0.005 1	1
Clarithromycin	0.06	0.003 91	15
Amoxicillin	0.078		
Azithromycin	0.09		
Erythromycin	0.04	0.083 5	2
Diclofenac	0.1	0.085 2	1
Bicalutamide	0.1		
Ofloxacin	0.1	0.020 4	5
Venlafaxine	0.1	3.22	32
Sulfamethoxazole	0.12	0.043 8	3
Candesartan	0.12	0.421	4
Atorvastatin	0.2	2.1	11
Doxycycline	0.3	0.003 91	77
Citalopram	0.51	15.4	30
Oxazepam		0.81	
Amlodipine	1	0.099 5	10
Gabapentin	0.196*	100	1000
Trimethoprim	1	508	508
Allopurinol	2.33	100	43
Sulfadiazine	4.6	0.135	34
Sulfamethizole	12		
Bisoprolol	35.6	8	4
Metoprolol	75	4.38	17
Atenolol	128	194	2

*Value most probably faulty or based on outdated data - to be replaced by CWPharma 2019 value

Table 11. excerpt of data for Gabapentin from ECOSAR 2.0

Organism	Duration	End Point	Concentration (mg/L)	Max Log Kow	Flags
Fish	96h	LC50	53851.61	5	• Chemical may not be soluble enough to measure this predicted effect. If the effect level exceeds the water solubility by 10X, typically no effects at saturation (NES) are reported
Daphnid	48h	LC50	4338.24	5	
Green Algae	96h	EC50	7771	6.4	• Chemical may not be soluble enough to measure this predicted effect. If the effect level exceeds the water solubility by 10X, typically no effects at saturation (NES) are reported
Fish		ChV	10350.03	8	• Chemical may not be soluble enough to measure this predicted effect. If the effect level exceeds the water solubility by 10X, typically no effects at saturation (NES) are reported
Daphnid		ChV	243.47	8	
Green Algae		ChV	1944.95	8	

Table 12. Revised target values based on PNECs for assessing Hillerøds extension for API removal

API suggested for a control program to document cleaning efficiency of API at HCR Syd	PNEC fresh water [µg/l]	Detections-limits in [µg/l]	IN HCR Syd mean [µg/l]	OUT HCR Syd mean [µg/l]	Proposed target values in µg/l
Azithromycin	0.09	0.01	0.104	0.128	0.09
Metoprolol	75	0.01	1.263	1.36	75
Diclofenac	0.1	0.01	0.811	0.451	0.1
Venlaflaxine	0.1	0.01	0.502	0.634	0.1
Sulfadiazine	4.6	0.01	0.062	0.029	4.6
Trimethoprim	10	0.01	0.165	0.137	10
					Target values
Erythromycin	0.02	0.003	0.019	0.068	0.02
Amoxillin	0.078	0.05	<0.05	<0.05	0.078
Sulfamethoxazole	0.12	0.01	0.195	0.047	0.12
Gabapentin	100	0.03	23.12	3.073	100
Bicalutamide	1	0.01	0.273	0.251	1
Sulfamethizole	2.54	0.01	1.207	0.353	2.54
Citalopram	0.51	0.003	0.226	0.163	0.51
Indicator compounds					No demands
17α-Ethinylestradiol	0.000075	0.0002	<0.02*	<0.0004*	Measurement
17β-Estradiol	0.0001	0.002	0.010*	<0.002*	Measurement
Iohexol	1 000 000	0.05	114	7.88	Measurement

*Very uncertain values

Conclusions from PNEC considerations:

Applying the PNEC value, that is based on the current ECOSAR and the independent CW Pharma ecotox assessment as new target values to the ozone/GAC combination results in a CW Pharma based design of a pharmaceutical removal wastewater treatment plant that can without trouble comply to all target values.

Generally it needs to be considered that PNEC values and even more PEC/PNEC considerations are considerably less well established as classical engineering approaches. As long as the high uncertainties both in the original toxicity data as well as the assessment or uncertainty factors prevail, can PEC/PNEC assessments be used to support motivations to implement new technologies. To base design values on such assessments is a fragile matter and should be avoided as long as authorities have not established quality assured normative values.

5 Summary and recommendations

- **Use of PNEC assessments**
  PNEC assessments are excellent tools for range finding assessments on whether a chemical might give problems in the environment. They are derived by assessing a multitude of toxicity data and identifying the lowest findable effect concentration e.g., an EC 50 of 10 µg/L. These data are then assessed on how well the test system is supposed to represent the ecosystem, and thus divided by uncertainty. A simple one species test over a shorter time period (e.g. 2 days) would be calculated with a factor 1000 while a test performed with a whole ecosystem would be put in with uncertainty 1. In the example the EC 50 obtained with a single species test would be calculated with uncertainty factor 1000 leading to a PNEC of 0.01 µg/L.

- **Gabapentin issue**
  Gabapentin concentrations changed over the sampling period. While they were around 10 µg/L during winter 2020/21 they were only 10% of that after April 2021. Analysing, whether this was due to season or ending of the corona lockdown in Denmark or other reasons is out of scope for this project.

  In the literature there is only very few investigations of the toxicity of this API. Based on faulty data it was assumed the PNEC for Gabapentin is 0.196 µg/l during the pilot plant investigation. It turned out that a more correct PNEC would be 100-243 µg/L thus the problems discussed in chapter 3 are no longer relevant.

- **Resume and basic design of API removal at Hillerød WWTP**
  A combination of ozone (at a specific ozone dose of 0.5 mg ozone/mg DOC) with a contact time of minutes with GAC with a contact time of 30 min is suitable for HFors, ozone or GAC alone have a risk on not being able to reach the target values. An estimate in the dimensioning of a full scale treatment gave the following results: Ozone reaction tanks are somewhat dependent on the engineering of the manufacturer. The CW Pharma guideline (2020) suggested to work with 20-30 mins residence time, while during the pilot in this project run successfully with 7 mins. As Hillerød WWTP has a dry weather flow of 16.000 m^3/d this would require an ozone reaction tank with 78 m^3 – 333 m^3 volume. If also the treatment of stormwater was planned under the same conditions a volume of 233-1000 m^3 should be taken into account. As the DOC in the effluent water is around 10 mg/L, the ozone generator needs to have a capacity of 3.3 kg/h is needed under dry weather conditions.
  As the residence time in the GAC filters is around 30 mins, the GAC filters should have a volume of 333 m^3 to treat the effluent under dry weather conditions. – As it should be taken into account that GAC needs replacing/exchanging under operation, it would be wise to plan for 350 m^3 GAC volume. Assuming the GAC can treat 25 000 BV (industry standard) the GAC would need replacing every 520 days.

6 Impact to the CWPharma guideline

The recommendations and procedures as described in the CW Pharma Guideline (2020) were very useful in pratice. Especially the succession of fitness check and feasibility study were successfully conducted in a very short period at Hillerød. These gave a good basis for the detailed planning that is currently ongoing at Hfors. The only challenge observed was the tendency of the Danish authorities to focus on ecotoxicologically reasoned "target concentrations", while the older Swiss and German approaches fokus on "removal". Even though the target concentrations approach is logical in the political system it provides a lot of practical issues, which are discussed in this report. In summary:

- Due to great uncertainties on the PNEC values the use of PEC/PNEC values has to be carefully evaluated before implementing these values as demands on pharmaceutical discharge concentrations, that has to be used in the process design. Removal of pharmaceuticals in the at Hillerød WWTP as well as generally on WWTPs in the Baltic Sea Region is feasible and compound specific removal of 90-99% are achievable.

Another obstacle that was observed at Hillerød during the pilot testings was: GAC filters used as polishing to wastewater treatment with sludge need an efficient protection against sludge in case of malfunctioning of sludge settler as within the project gradual clogging was observed during operation with a disk filter and especially during a floating sludge event which corrupted all GAC operation. – This can conventionally be overcome by installing backflush operations. This is already discussed in the CW Pharma Guideline (2020).

References

Asner. R, "Projekt „RISK-IDENT“ - Bewertung bislang nicht identifizierter anthropogener Spurenstoffe im aquatischen System. (In English: Project "RISK-IDENT" - Assessment of previously unidentified anthropogenic trace substances in the aquatic system)," Augsburg, 2013.

BEK nr 1625 af 19/12/2017. Bekendtgørelse om fastlæggelse af miljømål for vandløb, søer, overgangsvande, kystvande og grundvand Miljø- og Fødevareministeriet

Bourgin, M., Beck, B., Boehler, M., Borowska, E., Fleiner, J., Salhi, E., Teichler, R., von Gunten, U., Siegrist, H. and McArdell, C.S., 2018. Evaluation of a full-scale wastewater treatment plant upgraded with ozonation and biological post-treatments: Abatement of micropollutants, formation of transformation products and oxidation by-products. Water research, 129, pp.486-498.

Buffle, M.O., Schumacher, J., Meylan, S., Jekel, M. and Von Gunten, U., 2006. Ozonation and advanced oxidation of wastewater: effect of O3 dose, pH, DOM and HO•-scavengers on ozone decomposition and HO• generation. Ozone: Science and Engineering, 28(4), pp.247-259.

Buus Kjær, K.; Nielsen, U., 2018. Fokusstoffer på Biofos' renseanlæg Miljøskadelige stoffer i fokus for oplandskommunernes regulering Rapport udarbejdet af DHI juni 2018

CW Pharma., ecotox assessments. 2019 https://helda.helsinki.fi/handle/10138/317151

CW Pharma. Guideline for advanced API removal GoA3.4: Optimization and control of advanced treatment, 2020 (https://zenodo.org/record/4305935#.YrWDNOxByUk)

De Witte, B., Van Langenhove, H., Hemelsoet, K., Demeestere, K., De Wispelaere, P., Van Speybroeck, V. and Dewulf, J., 2009. Levofloxacin ozonation in water: rate determining process parameters and reaction pathway elucidation. Chemosphere, 76(5), pp.683-689.

ECHA (European Chemicals Agency), 2008, Guidance on information requirements and chemical safety assessment Chapter R.10: Characterisation of dose [concentration]-response for environment (seen at https://echa.europa.eu/documents/10162/13632/information_requirements_r10_en.pdf/bb902be7-a503-4ab7-9036-d866b8ddce69 16th November 2021.)

Ekengren, Ö.; Filipsson, S., Baresel, C., Karlsson, J., Winberg von Friesen, L., Blomqvist, S., Hasselgren, M., Grosch, J., Lazic, A., Stapel, H., Fassbender, M., Feldthusen, M., Hellman, J., Nordin, A., 2020 The municipal wastewater treatment plant of the future - A water reuse facility IVL Swedish Environmental Research Institute, juni 2020

Fransen Krog, G.; Borksted, B.; Gade Holm, A.; Mose Pedersen, B.; Nielsen, U.; Møller Jensen, L., NOVANA- Screening for humane lægemidler i vandmiljøet. Miljøministeriet, Naturstyrelsen juli 2015.

Guideline for advanced API removal. GoA3.4: Optimization and control of advanced treatment. December 2020. “Clear Waters from Pharmaceuticals” (CWPharma).

Henning H.E., Putna-Nimane I., Kalinowski R, et. al., "Pharmaceuticals in the Baltic Sea Region – emissions, consumption and environmental risks," Länsstyrelsen Östergötland, Linköping, Sweden, 2020Hörsing, M., Kosjek, T., Andersen, H.R., Heath, E. and Ledin, A., 2012. Fate of citalopram during water treatment with O3, ClO2, UV and fenton oxidation. Chemosphere, 89(2), pp.129-135.

Kharel, S., Stapf, M., Miehe, U., Ekblad, M., Cimbritz, M., Falås, P., Nilsson, J., Sehlén, R. and Bester, K., 2020. Ozone dose dependent formation and removal of ozonation products of

pharmaceuticals in pilot and full-scale municipal wastewater treatment plants. Science of the Total Environment, 731, p.139064.

Kharel, S., Stapf, M., Miehe, U., Ekblad, M., Cimbritz, M., Falås, P., Nilsson, J., Sehlén, R., Bregendahl, J. and Bester, K., 2021. Removal of pharmaceutical metabolites in wastewater ozonation including their fate in different post-treatments. Science of the Total Environment, 759, p.143989.

Koncentrationer (AMK) for lægemiddelstoffer. Notat udarbejdet af DHI. Juni 2013.

Lajeunesse, A., Blais, M., Barbeau, B., Sauvé, S. and Gagnon, C., 2013. Ozone oxidation of antidepressants in wastewater–Treatment evaluation and characterization of new by-products by LC-QToFMS. Chemistry central journal, 7(1), pp.1-11.

Lee, Y.; Gerrity, D.; Lee, M.; Bogeat, A. E.; Salhi, E.; Gamage, S.; Trenholm, R. A.; Wert, E. C.; Snyder, S. A.; Von Gunten, U., Prediction of micropollutant elimination during ozonation of municipal wastewater effluents: use of kinetic and water specific information. Environmental Science & Technology 2013, 47, (11), 5872-5881.

Mose Pedersen, B.; Nielsen U., og Halling-Sørensen B., 2007. Begrænsning af humane lægemiddelrester og antibiotikaresistens. DHI 2007. Miljøprojekt nr. 1189, 2007.

Nielsen, U.; Rasmussen, D., Hastrup, A.C., Slothuus. T., Forslag til administrationsgrundlag for lægemiddelstoffer i hospitalsspildevand. Anbefalede maksimale koncentrationer ved tilslutning til kloak. Input til KL's Arbejdsgruppe omkring hospitalsspildevand.

Orias, F. & Perrodin Y., Characterisation of the ecotoxicity of hospital effluents: A review, Science of the Total Environment 454–455 (2013) pp 250–276.

Schaefer A., "Proposals for Acute and Chronic Quality Standards," Swiss Ecotox Centre, 2016.

Stapf, M., Miehe, U. and Jekel, M., 2016. Application of online UV absorption measurements for ozone process control in secondary effluent with variable nitrite concentration. Water Research, 104, pp.111-118.

Stuer-Lauridsen, F.; Halling Sørensen B., Overgaard, S., 2011. Miljøvurdering af speciallægemidler i spildevand fra sygehuse + appendixer. Naturstyrelsen 2011.

Persoone, G., Marsalek, B., Blinova, I., Törökne, A., Zarina, D., Manusadzianas, L., Nalecz-Jawecki, G., Tofan, L., Stepanova, N., Tothova, L., Kolar, B., 2003. A practical and user-friendly toxicity classification system with microbiotests for natural waters and wastewaters, Environ Toxicol., 18, 395-402. doi: 10.1002/tox.10141

Von Sonntag, C. and Von Gunten, U., 2012. Chemistry of ozone in water and wastewater treatment. IWA publishing.

Zimmermann, S.G., Schmukat, A., Schulz, M., Benner, J., Gunten, U.V. and Ternes, T.A., 2012. Kinetic and mechanistic investigations of the oxidation of tramadol by ferrate and ozone. Environmental science & technology, 46(2), pp.876-884.

Appendix

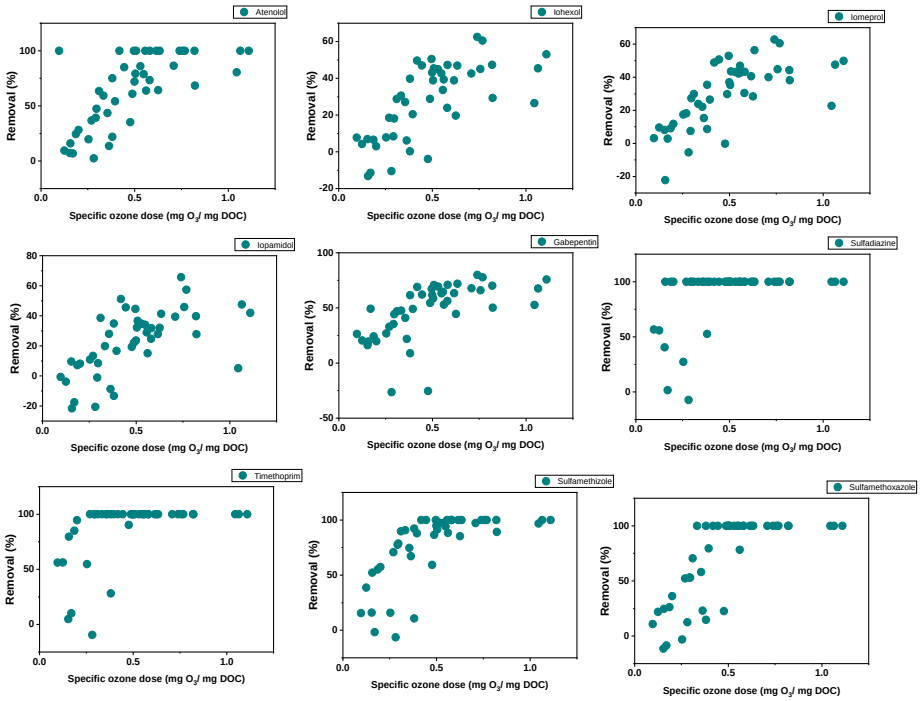

Figure 15. Removal of pharmaceuticals vs specific ozone dose (I)

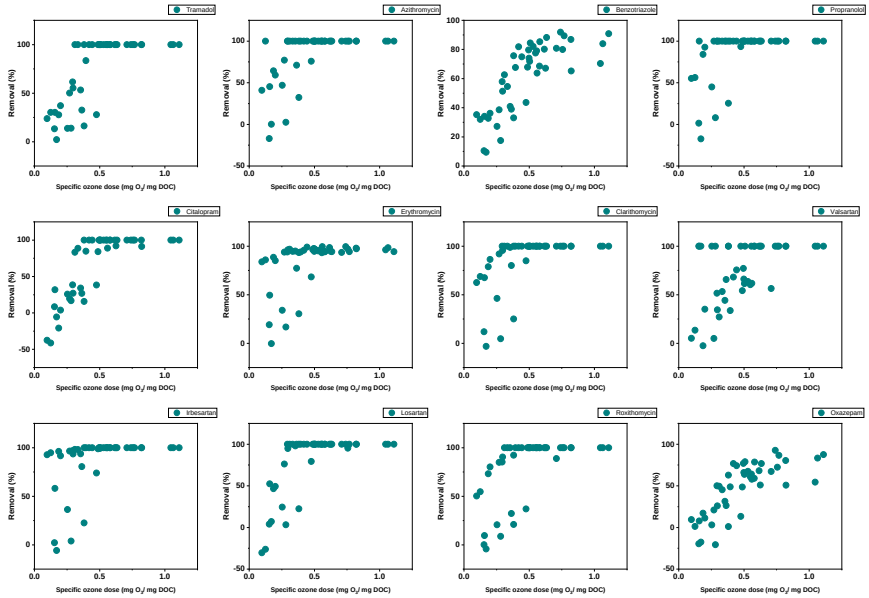

Figure 16. Removal of pharmaceuticals vs specific ozone dose (II)

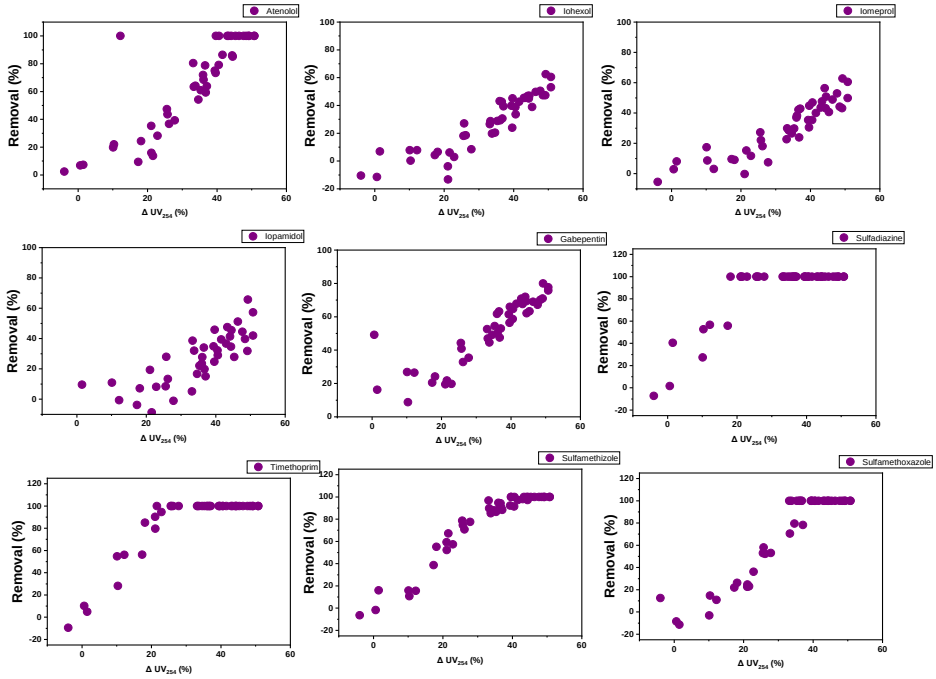

Figure 17. Removal of pharmaceuticals vs delta UV254 (I)

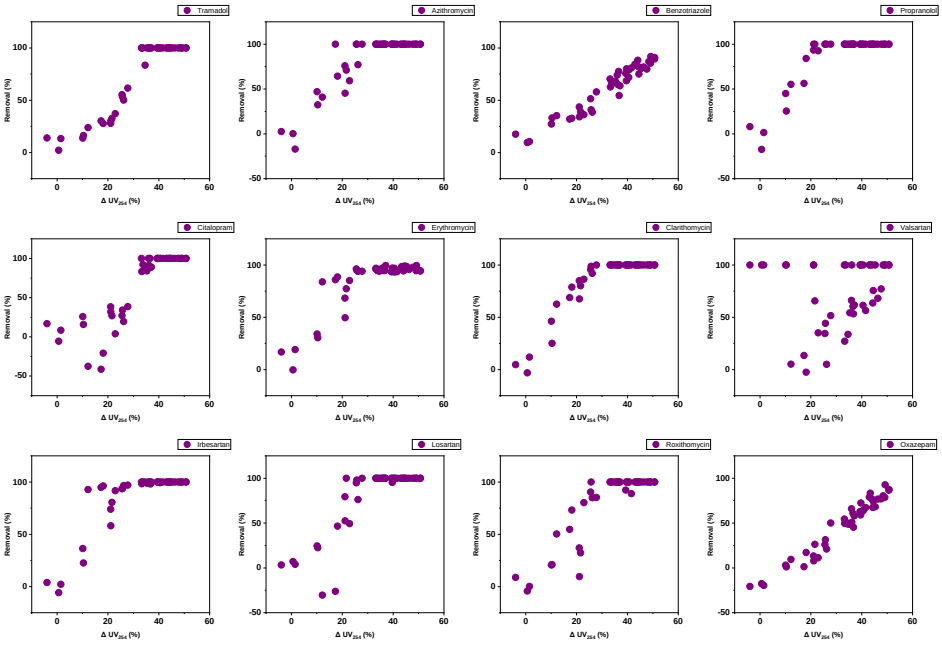

Figure 18. Removal of pharmaceuticals vs delta UV254 (II)

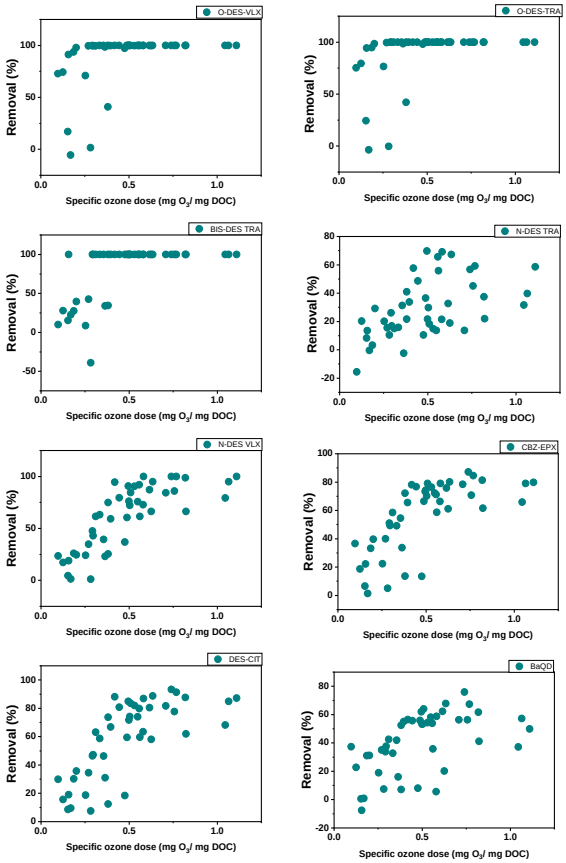

Figure 19. Removal of pharmaceuticals metabolites vs specific ozone dose

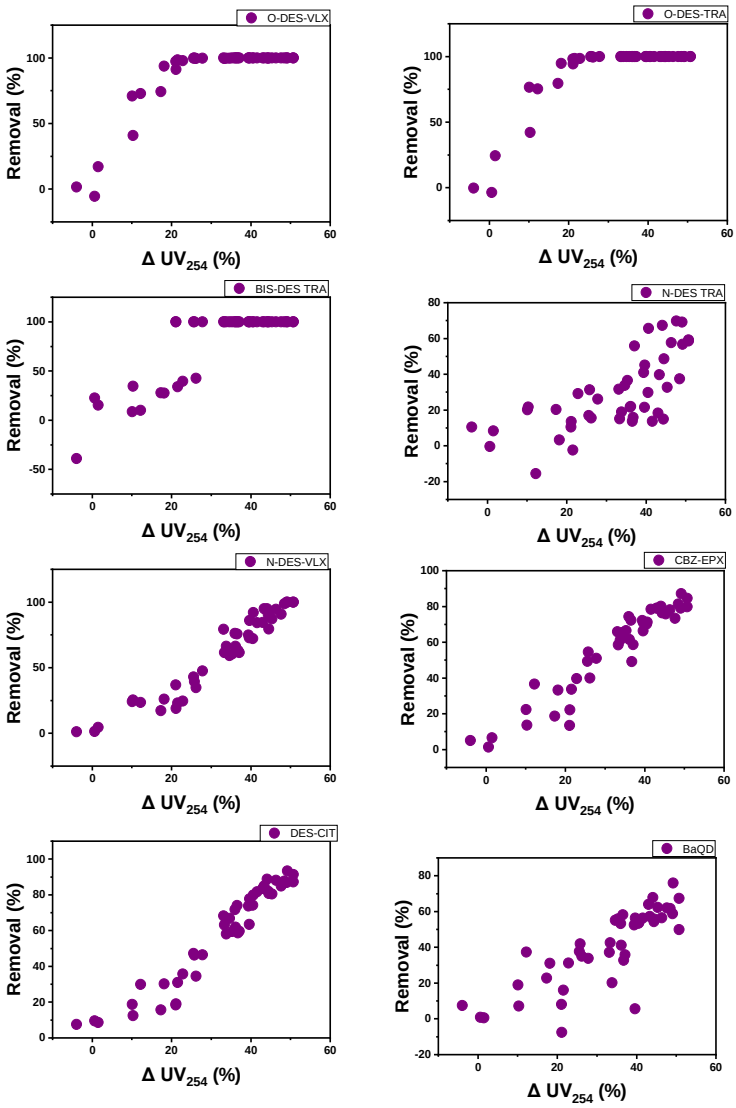

Figure 20. Removal of pharmaceuticals metabolites vs delta UV254

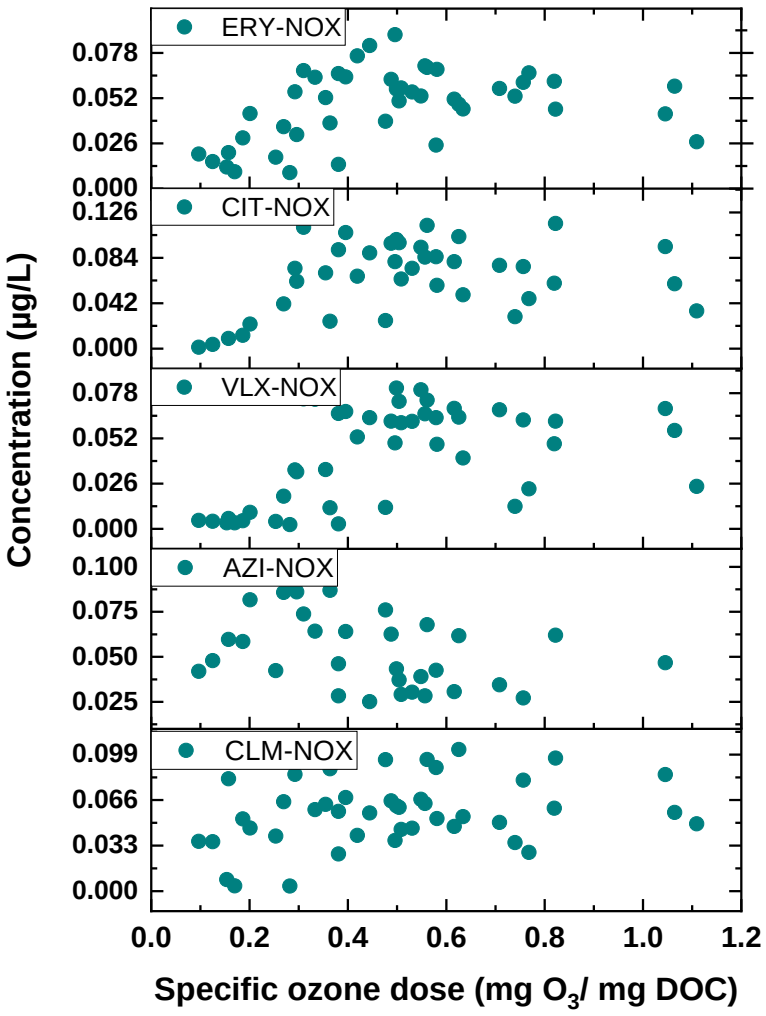

Figure 21. Formation of ozonation products of pharmaceuticals (ERY-NOX: Erythromycin N-oxide, CIT-NOX: Citalopram N-oxide, VLX-NOX: Venlafaxine N-oxide, AZI-NOX: Azithromycin N-oxide, CLM-NOX: Clarithromycin N-oxide) in relation to specific ozone dose in Hillerød WWTP

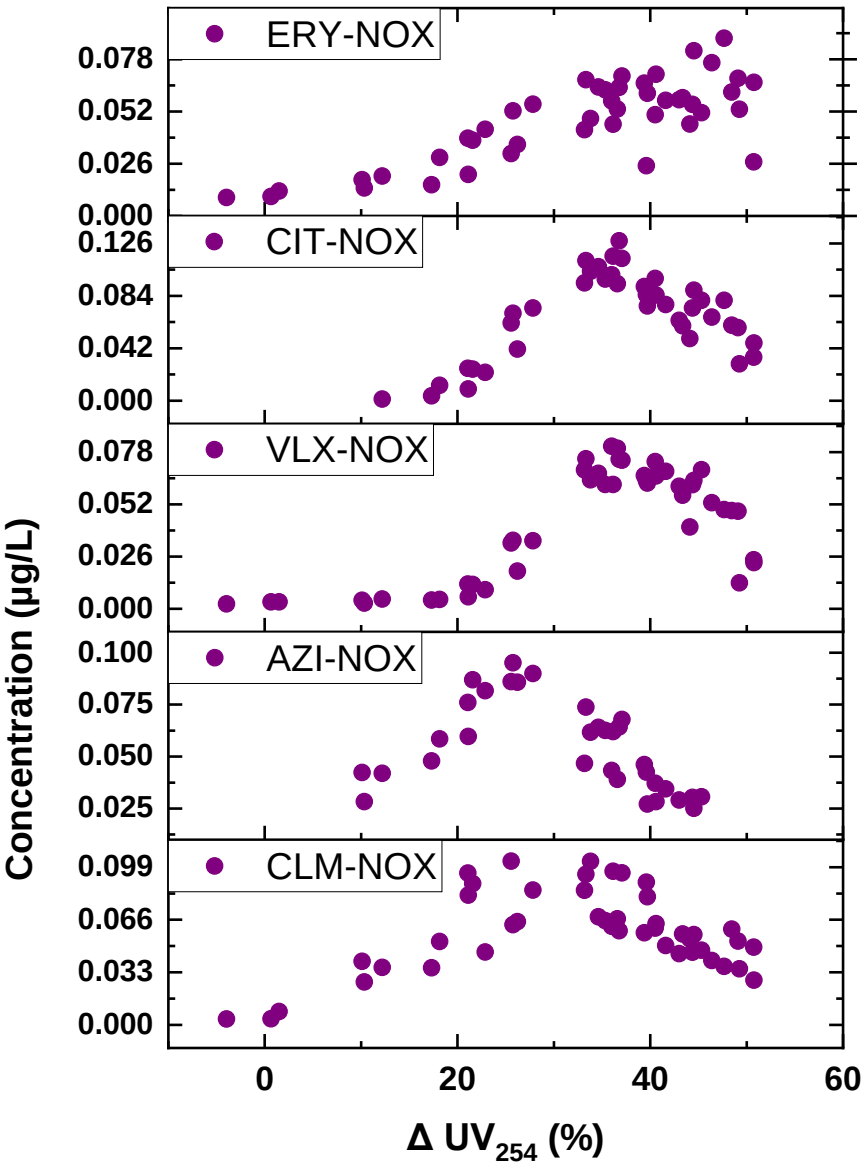

Figure 22. Formation of ozonation products (ERY-NOX: Erythromycin N-oxide, CIT-NOX: Citalopram N-oxide, VLX-NOX: Venlafaxine N-oxide, AZI-NOX: Azithromycin N-oxide, CLM-NOX: Clarithromycin N-oxide) in relation to delta UV254

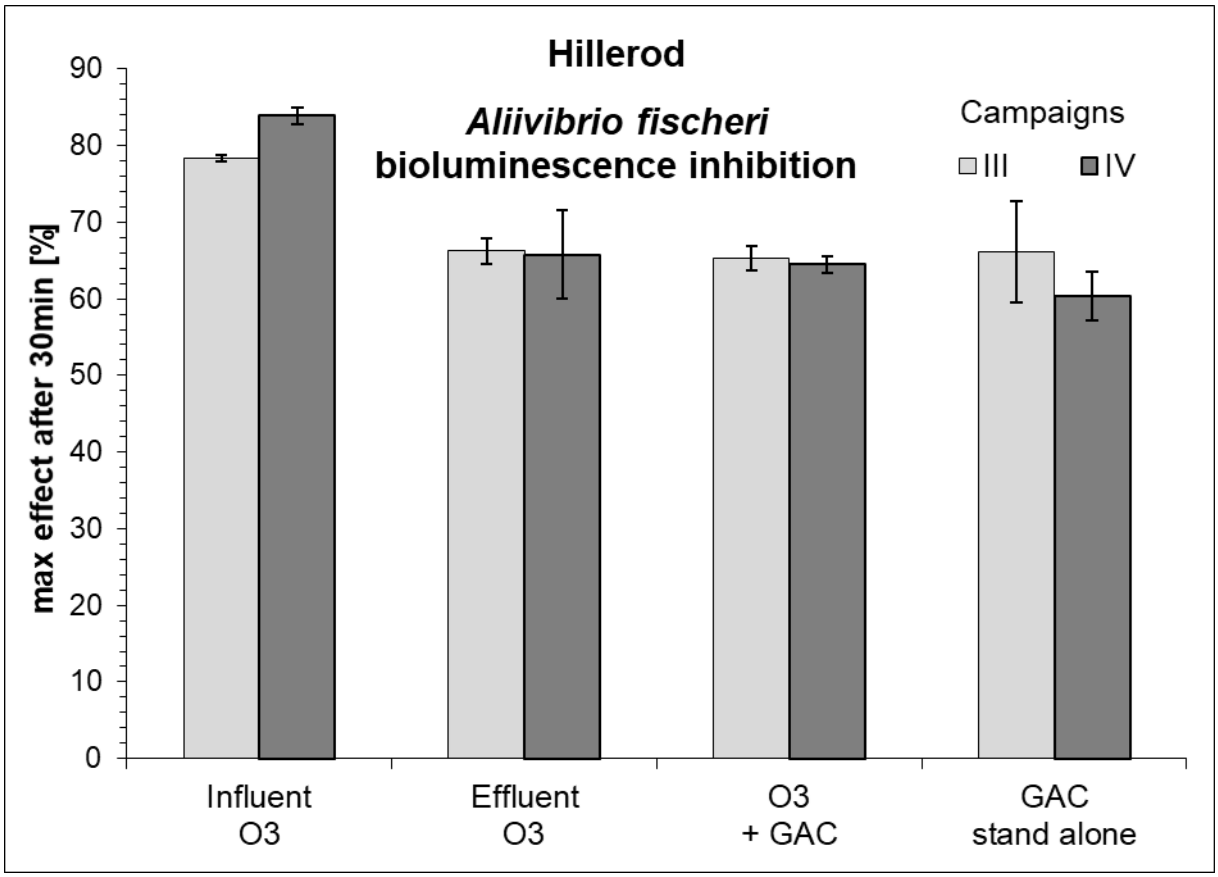

Figure 23. Mean maximum inhibition effect of *Aliivibrio fischeri* luminescence at different sampling points at WWTP Hillerød.

Statistical analysis of bioluminescence inhibition test

The ANOVA table composes the variance of max % effect into two components: between group component and within group component (Table 13). The F – ratio, which in this case equals 51.625, 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.

Table 13. Statistical analysis of *Aliivibrio fischeri* test results from WWTP Hillerød samples: analysis of variance ANOVA

Source of variance	SS (sum of square)	df	MS (Mean square)	F - ratio	P - value
Between groups	1885.06	3	628.355	51.62	0.0000
Within groups	340.803	28	12.1715		
Total (Corr.)	2225.87	31			

Table 14 and Table 15 show results of a multiple comparison procedure to determine which means was significantly different from which others. In the Table 14, two 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.

Table 15 showed the estimated difference between each pair of means. An asterisk (*) marks the three 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 zero.

Table 14. Statistical analysis of *Aliivibrio fischeri* test results from WWTP Hillerød samples: post hoc Multiple Range Test

	Count	*Mean*	*Homogeneous Groups*
S4	8	61.7975	X
S2	8	62.9463	X
S3	8	64.5787	X
S1	8	80.685	X

Table 15. Statistical analysis of Aliivibrio fischeri test results from WWTP Hillerød samples: Least Significant Differences (LSD) according Fisher's procedure

Contrast	*Difference*	*LSD*
S1 - S2	17.7387*	3.57322
S1 - S3	16.1063*	3.57322
S1 - S4	18.8875*	3.57322
S2 - S3	-1.6325	3.57322
S2 - S4	1.14875	3.57322
S3 - S4	2.78125	3.57322

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

Table 16. Toxic effect of wastewaters samples from WWTP Hillerød (fEF=100) at differ sampling points on the luminescent properties of *Aliivibrio fischeri* after 5, 15 and 30 minutes of exposition: percentage inhibition effects [PI, %], EC50-t values and toxicity units (TU), SD = Standard deviation.

Sampling campaign	Sample	fEF=100		PI (%) after time (fEF=100)			
		EC_{50} (%)	TU	Repetition 1	Repetition 2	Mean	SD
Time 5 min							
HIL III	HIL-C3-S1_1	52.03	1.922	74.58	73.88	74.23	0.49
	HIL-C3-S1_2	40.44	2.473	73.29	72.32	72.81	0.69
	HIL-C3-S2_1	57.62	1.736	66.11	67.07	66.59	0.68
	HIL-C3-S2_2	70.74	1.116	61.39	63.49	62.44	1.48
	HIL-C3-S3_1	58.53	1.035	67.74	68.00	67.87	0.18
	HIL-C3-S3_2	78.70	1.271	58.75	59.96	59.36	0.86
	HIL-C3-S4_1	62.46	1.601	68.78	61.13	64.96	5.41
	HIL-C3-S4_2	68.21	1.466	59.55	60.00	59.78	0.32
HIL IV	HIL-C4-S1_1	28.00	3.571	77.63	78.16	77.90	0.37
	HIL-C4-S1_2	39.55	2.528	75.45	76.72	76.09	0.90
	HIL-C4-S2_1	50.77	1.970	64.40	67.80	66.10	2.40
	HIL-C4-S2_2	62.73	1.594	57.82	58.24	58.03	0.30
	HIL-C4-S3_1	64.26	1.556	66.30	66.97	66.64	0.47
	HIL-C4-S3_2	83.38	1.143	60.19	60.76	60.48	0.40
	HIL-C4-S4_1	65.95	1.516	63.75	62.75	63.25	0.71
	HIL-C4-S4_2	83.80	1.193	56.67	57.49	57.08	0.58

(continues next page)

Table 16 (Continues)

Time 15 min							
	HIL-C3-S1_1	48.82	2.049	76.60	76.24	76.42	0.25
	HIL-C3-S1_2	37.05	2.699	75.20	73.71	74.46	1.05
	HIL-C3-S2_1	58.47	1.710	65.02	66.37	65.70	0.95
HIL III	HIL-C3-S2_2	68.26	1.465	63.08	63.57	63.33	0.35
	HIL-C3-S3_1	63.60	1.572	66.08	66.37	66.23	0.21
	HIL-C3-S3_2	76.25	1.311	59.72	61.63	60.68	1.35
	HIL-C3-S4_1	58.12	1.721	71.06	63.77	67.42	5.15
	HIL-C3-S4_2	66.07	1.514	59.47	59.69	59.58	0.16
	HIL-C4-S1_1	23.11	4.328	80.38	80.78	80.58	0.28
	HIL-C4-S1_2	32.18	3.034	79.65	80.30	79.98	0.46
	HIL-C4-S2_1	55.12	1.814	61.83	65.73	63.78	2.76
HIL IV	HIL-C4-S2_2	66.15	1.583	58.74	56.69	57.72	1.45
	HIL-C4-S3_1	68.71	1.455	65.25	65.01	65.13	0.17
	HIL-C4-S3_2	81.66	1.225	61.70	61.70	61.70	0.00
	HIL-C4-S4_1	65.56	1.525	62.94	62.93	62.94	0.01
	HIL-C4-S4_2	88.30	1.132	53.86	55.45	54.66	1.12
Time 30 min							
	HIL-C3-S1_1	44.88	2.228	78.63	78.04	78.34	0.42
	HIL-C3-S1_2	44.95	2.224	78.09	76.72	77.41	0.97
	HIL-C3-S2_1	64.53	1.550	63.22	65.07	64.15	1.31
HIL III	HIL-C3-S2_2	57.55	1.738	65.52	67.37	66.45	1.31
	HIL-C3-S3_1	64.79	1.544	66.02	66.37	66.20	0.25
	HIL-C3-S3_2	69.85	1.432	62.57	64.15	63.36	1.12
	HIL-C3-S4_1	59.16	1.690	70.84	63.29	67.07	5.34
	HIL-C3-S4_2	59.14	1.691	60.11	61.41	60.76	0.92
	HIL-C4-S1_1	20.55	4.865	82.65	83.12	82.89	0.33
	HIL-C4-S1_2	27.03	3.700	83.54	84.69	84.12	0.81
	HIL-C4-S2_1	52.94	1.889	62.35	66.87	64.61	3.20
HIL IV	HIL-C4-S2_2	59.74	1.674	54.51	58.66	56.59	2.93
	HIL-C4-S3_1	68.64	1.457	64.80	65.29	65.05	0.35
	HIL-C4-S3_2	73.28	1.365	63.70	63.73	63.72	0.02
	HIL-C4-S4_1	70.58	1.417	62.54	61.19	61.87	0.95
	HIL-C4-S4_2	80.81	1.237	56.92	58.08	57.50	0.82

In order to minimize contamination due to sample handling, each sample was collected in a single 10-liter HDPE canister. All 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.

Sample logistics was a crucial part of the sampling campaigns as the SPE was conducted centralized by partner UBA (German environmental protection agency) in Berlin. The SPE process was conducted centralized to avoid impacts on ecotoxicity results. As a result, samples 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). Extracts from SPE were stored at -18°C and shipped from partner UBA to Partner IOS insulated between cooling packs at below 0°C.

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 (10 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 CWPharma2, sample logistics and handling were optimized in a way that the sample filtration and extraction step could be completed within 72 h after sampling.

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 and divided into glass bottles. Extraction was performed by the automatic SPE-unit, 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 and evaporated completely with a gentle nitrogen gas stream (TurboVap II, Biotage). The extracts were reconstituted in 1 ml of dimethyl sulfoxide (DMSO). Afterwards, all extracts reconstituted in DMSO 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, some of the ecotoxicity test systems cannot

directly use the extract but require an aqueous dilution of an extract. Each used final EF in the respective test system is given in each section.

Description of ecotoxicity tests

Mutagenicity with Ames fluctuations test (YG 7108, +/-S9)

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.

The Ames-test with YG 7108 (+/-S9) was conducted according to ISO 113501[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 final EF of 20. 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 (technical) replicates each samples 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.

Estrogenicity with YES test

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.

The YES-test was conducted according to ISO 19040-3[2]. 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). 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

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

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

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).

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).

1000-fold enriched SPE extracts were diluted with redistilled water (fEF of 100 + 18.1%) in order to achieve the targeted final enrichment factor (fEF) of 100 in the test. The SPE extract with an EF of 100 + 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: 100, 50, 25, 12.5 and 6.25) 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 EC-50 value at 5, 15 and 30 minutes after sample introduction. In addition, % effect (PI) of bioluminescence inhibition and Toxicity Units (TU) were presented. 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 PI above 50%, an EC-50 value below 100 and a TU above 1.0 indicates toxicity of the samples. Classification of toxicity to bacteria *Aliivibrio fischeri* was made based on the criteria proposed by Persoone *et al*. (2003).