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Removal of micropollutants by application of multiple point ozonation and powder activated carbon

MUDP Report

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Miljøteknologisk Udviklings- og Demonstrationsprogram

Projektet, som er beskrevet i denne rapport, er støttet af Miljøteknologisk Udviklings- og Demonstrationsprogram, MUDP, som er et program under Miljøministeriet, der støtter udvikling, test og demonstration af miljøteknologi.

MUDP investerer i udvikling af fremtidens miljøteknologi til gavn for klima og miljø i Danmark og globalt, samtidig med at dansk vækst og beskæftigelse styrkes. Programmet understøtter dels den bredere miljødagsorden, herunder rent vand, ren luft og sikker kemi, men understøtter også regeringens målsætninger inden for klima, biodiversitet og cirkulær økonomi.

Det er MUDP's bestyrelse, som beslutter, hvilke projekter der skal modtage tilskud. Bestyrelsen betjenes af MUDP-sekretariatet i Miljøstyrelsen.

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Denne slutrapport er godkendt af MUDP sekretariatet, men det er alene rapportens forfatter/projektlederen, som er ansvarlige for indholdet.

Contents

Preface

This development and demonstration project is conducted by SAMN Forsyning A/S, Aalborg University, KD Maskinfabrik, SUEZ Water A/S and COWI A/S. The project is partly founded by Miljøteknologisk Udviklings- og Demonstrationsprogram (MUDP), however a large part of the economy comes from SUEZ Water A/S and a smaller part from the rest of the partners. The project is published by The Danish Environmental Protection Agency.

The steering committee for this MUDP project consists of:
- SUEZ Water A/S
- COWI A/S
- Aalborg University
- SAMN Forsyning A/S
- KD Maskinfabrik A/S

The advisory group for this MUDP project consists of:
- Herning Vand A/S
- Aarhus Vand A/S
- DANVA
- Hjørring Vandselskab A/S
- VandCenter Syd A/S

The project started in January 2018 and ended in February 2021. Experiments were performed in the period November 2018 to the end of April 2020.

This main focus of this project is to examine and demonstrate the effect of two innovative ways of using well-known technologies for organic micropollutant removal at wastewater treatment plants and show that treatment of organic micropollutants can be implemented at small, medium or large wastewater treatment plant in a cost effective way. These tested technologies are not widespread in Denmark but are used in Switzerland among others. Ozonation which is normally used as a tertiary treatment is injected into mixed liquor activated sludge (aeration tank) combined with tertiary treatment.
Process of adsorption on powder activated carbon (PAC) in a pulsed sludge-blanket reactor in Pulsazur™ is used to eliminate dissolved organic matter and micro-pollutants in advanced treatment. Filtration using granular activated carbon is also normally used as a tertiary treatment. In this project the effect of dosing powdered activated carbon in activated sludge process tank is investigated. The effect on the “conventional activated sludge” treatment is also evaluated.

By accepting the economic support from the MUDP program, the steering committee have committed to communicate the results from the project. Which have been done by articles in technical magazines, national magazines, theme days, webinars and others. In 2021 two presentations and one poster about the project will be held at the IWA conference.

The detail discussion of the test results is presented in:

Appendix 1. Effect of ozonation and PAC conditions on micropollutant removal
Appendix 2. Removal of Micropollutant from sludge liquid and solid phases
Appendix 3. Physical-chemical sludge characterization
Appendix 4. Sludge characterization by light microscopy and 16S rRNA sequencing

Sammenfatning, dansk

I de seneste år har der været et øget fokus på mikroforureninger i renset spildevand. I Schweiz er der indført lovgivning, som kræver, at der implementeres rensning for mikroforureninger på større kommunale renseanlæg, samt renseanlæg, med udløb til søer som fungerer som drikkevandsreservoir eller recipienter med lav fortynding af det rensede spildevand. I Schweiz benytter de fleste centraliserede renseanlæg ozonering og/eller aktivt kul som en tertiær behandling til fjernelse af mikroforurenende stoffer.

Ved udgivelsestidspunktet for denne rapport, er det endnu ikke defineret i Danmark, om behandlingen skal udføres lokalt (decentralt) på hospitaler eller centraliseres i kommunale renseanlæg. Indtil videre på Herlev Hospital behandles spildevandet lokalt for fjernelse af mikroforureninger i et dedikeret renseanlæg tilknyttet hospitalet ved hjælp af ozonering og GAC-filtre (Granular Activated Carbon filtre). Det er det samme for Nyt Aalborg Universitets Hospital, hvor hospitalets spildevand vil behandles ved en kombination af PAC-MBR og tertiær ozonering.

I dette projekt anvendes ozon og aktivt kul innovativt. Ozonering tilføres både i procestanken og som en tertiær behandling, bedre kendt som fler-punkts ozonering. I efterfølgende eksperimenter tilsættes pulveriseret aktivt kul (PAC) direkte til procestanken, og der udføres ingen tertiær behandling.

Teknologierne er testet på Brædstrup Renseanlæg, Samn Forsyning. Renseanlægget består af to adskilte biologiske behandlingslinjer. Der er fælles ristefunktion og sand- og fedtfang for de to linjer, men i udløbet fra sand- og fedtfanget deles spildevandsstrømmen til de to proceslinjer. Indløbet for de to linjer er dermed de samme, og ydeevnen af de testede teknologier kan direkte sammenlignes med konventionel aktiv slambehandling.

Dosering af 3.98 mgO3/l i procestanken og 7.2 mgO3/l som tertiær behandling viste de bedste resultater for ozoneksperimenterne. Ved anvendelse af denne konfiguration blev der opnået en gennemsnitlig fjernelse af 93% af mikroforurening og koncentrationer af alle målte mikroforureninger var under PNEC (Predicted No Effect Concentration) efter ozonering. Økotoksiciteten af det ozon-behandlede spildevand var lig med eller lavere sammenlignet med det konventionelt behandlede spildevand. Desuden blev en forbedret fjernelse af antibiotikaresistente bakterier observeret. Ozoneringen ændrede det mikrobielle samfund i den eksperimentelle linje, men uden tab af kritiske mikroorganismer. Ozoneringen effekt på forbedret SVI og dermed slammets bundfældningsegenskaber blev tydeliggjort.
For et 100.000 PE renseanlæg, der behandler 8.300.000 m³/år, kan fler-punkts ozonering implementeres og drives for 26-42 DKK/år pr. indbygger afhængigt af den anvendte ozondosis.

Dosering af 5 mg PAC/l eller 10 mg PAC/l i procestanken forårsagede en øget fjernelse af mikroforurenende stoffer sammenlignet med konventionel behandling. Koncentrationen for visse mikroforureninger var over PNEC, og yderligere behandling ses derved nødvendig for at nå ikke skadelige koncentrationer for alle mikroforureningerne.
For et 100.000 PE renseanlæg, der behandler 8.300.000 m³/år, kan PAC-dosering i procestanken implementeres og betjenes for 23-31 DKK/år pr. indbygger afhængigt af den anvendte PAC-dosis.

Summary, English

The focus on micropollutants in treated wastewater have increased in the recent years. Switzerland have implemented legalisation which requires micropollutant removal at larger municipal centralised wastewater treatment plant (WWTP) or WWTPs that discharge to lakes with drinking water interest or recipient with small dilution of the treated wastewater. In Switzerland, most centralised WWTPs are using ozonation and/or activated carbon as a tertiary treatment for micropollutant removal.

In Denmark it is not defined yet if the treatment should be performed locally at hospitals or centralized in municipal WWTPs at the time of this report. So far, at Herlev Hospital the wastewater is treated locally for removal of micropollutants in a dedicated WWTP to hospital wastewater by ozonation and GAC filters. It is the same for NAU hospital, where the hospital wastewater is going to be treated by combination of PAC-MBR and tertiary ozonation.

In this project ozone and activated carbon are used in an innovative way. Ozonation is applied in the process tank and as a tertiary treatment called multiple point ozonation. In subsequent experiments powdered activated carbon (PAC) is added to the process tank, and no tertiary treatment is performed.

The technologies are tested at Brædstrup WWTP in Samn Forsyning. This WWTPs has two separated biological treatment lines. The two lines separate in the outlet of the sand- and grease trap. Inlet measurements for the two lines are thereby the same, and the performance of the tested technologies can be directly compared to conventional activated sludge treatment.

Dosing 3.98 mgO_3/l in mixed liquor in process tank and 7.2 mgO_3/l as tertiary treatment showed the best results for the ozonation conditions. Using this configuration an average removal of 78% after mixed liquor ozonation and 93 % after of micropollutants were obtained, and concentrations of all measured micropollutants were below PNEC (predicted no effect concentration) after ozonation. The ecotoxicity of the ozonated treated wastewater was equal or lower compared to conventional treated wastewater and it improved removal of antibiotic resistant bacteria. The ozonation changed the microbial community in the experimental line, but without loss of any critical microorganisms. The ozonation improved the SVI and thereby the settleability of the sludge. For a 100,000 PE WWTP treating 8,300,000 m³/year multipoint ozonation can be implemented and operated for 26-42 DKK/year per inhabitant depending on the applied ozone dosage.

Dosing 5 mg PAC/l or 10 mg PAC/l in the process tank caused an increased micropollutant removal compared to conventional treatment by 20-30%. The concentration for some micropollutants were above PNEC, and further treatment is thereby necessary to reach safe concentrations for all micropollutants. For a 100,000 PE WWTP treating 8,300,000 m³/year PAC dosing in the process tank can be implemented and operated for 23-31 DKK/year per inhabitant depending on the applied PAC dosage.

1. Introduction

Micropollutants are seen in many different forms and can affect the life on earth in many and severe ways. Released micropollutants to the aquatic or freshwater environment can affect the organisms living here.

An example of micropollutants which affects the aquatic environment is xenobiotics and pharmaceuticals transported to the aquatic environment through sewage and wastewater treatment plants (WWTP). Different groups of chemicals have different effects. One example is endocrine disrupting chemicals where oestrogenic substances affect the reproductivity of male fish and cause them to evolve female characteristics (Walker, et al., 2012). Laboratory studies have shown that fathead minnows affected by the antidepressant sertraline showed less anxiolytic behaviour than fathead minnows not affected. The fish affected by sertraline spend less time hiding during light hours than fish not affected (Valenti, et al., 2012). A lot of different studies worldwide have shown the presence of pharmaceuticals, for example psychoactive drugs, antihistamines, β-blockers, antibiotics, anti-inflammatories and synthetic hormones, in aquatic organisms (Huerta, et al., 2018).

Another important pollutant which is lead to the aquatic environment through wastewater (among others) is antibiotics and antibiotic resistant bacteria and genes. Discharge of antibiotics to the aquatic environment enhance the selection of antibiotic resistant bacteria and spreading of antibiotic resistance.

Control of antibiotic resistant bacteria is very important since infections by antibiotic resistant bacteria already cause 25.000 deaths a year in Europe and 700.000 deaths a year globally. If no actions are taken, antibiotic resistance might cause millions of deaths globally, and more deaths than cancer in 2050. The economic will also be affected by the antibiotic resistant bacteria, and in some simulations, the effect on the future economic is larger than the economic crisis in 2008-2009 and costs trillions of dollars (European Commission, 2017).

One way antibiotic resistant bacteria and genes are transferred is through the environment. The environment hosts a large pool of antibiotic resistant bacteria and antibiotic resistant genes. Humans and animals affect the pool of antibiotic resistant bacteria and genes, and humans are affected by the pool in the environment (Bengtsson-Palme, et al., 2018). In “A European One Health Action Plan against Antimicrobial Resistance (AMR)” this interaction is described by the “One Health” principle which is described as “*a term used to describe a principle which recognises that human and animal health are interconnected, that diseases are transmitted from humans to animals and vice versa and must therefore be tackled in both. The One Health approach also encompasses the environment, another link between humans and animals and likewise a potential source of new resistant microorganisms. This term is globally recognised, having been widely used in the EU and in the 2016 United Nations Political Declaration on AMR”* (European Commission, 2017). Discharging of antibiotic resistant bacteria to the environment is therefore important to human health.

Antibiotic resistant bacteria and genes are among others transferred between hosts (animals, humans, environment and others) through sewage, WWTP and water bodies. However untreated sewage released into water bodies poses a bigger risk, since treatment in WWTP often lower the abundance of resistant genes and the total bacterial abundance (Bengtsson-Palme, et al., 2018).

Pharmaceuticals including Antibiotics are used in hospitals and in private households. At Herlev Hospital the wastewater is treated to remove pharmaceuticals, endocrine disrupters and antibiotic resistant bacteria. Other hospitals in Denmark discharge their wastewater to municipal WWTP where it is treated along with household wastewater. The WWTP aren't designed to remove pharmaceuticals and xenobiotics, nevertheless some substances are removed to a large extent while other substances pass through the WWTP with none or limited degradation.

In other European countries for example Switzerland recently legalisation requires that larger wastewater treatment plants treat the wastewater to remove micropollutants, including 10 pharmaceuticals and 2 corrosion inhibitors. The goal is to have an average removal of the 12 micropollutants higher than 80% over the whole WWTP. This treatment is primarily performed as tertiary ozonation or a tertiary filtration with granular activated carbon.

In this project, the known technologies to remove micropollutants from the wastewater (ozonation and activated carbon adsorption) are used in a new innovative way. The processes are implemented in the conventional biological treatment processes at Brædstrup WWTP. The new way to use these technologies are thereby tested in full scale. The experiment prove that these known technologies can be used in this new innovative way, that the technologies can be implemented in an existing wastewater treatment plant without need for big rebuilding and that it is possible to treat the water at a low cost.

Brædstrup WWTP has been found as an ideal plant for these tests since the plant has two separate biological treatment lines. At Brædstrup WWTP, all wastewater is treated in a screen and a sand- and grease chamber, and afterwards the wastewater is directed in to two separate biological treatment lines, which have separate sludge recirculation systems. Experiments can therefore be performed in one line while the other line acts as a reference line which allow direct comparison of the effect of the different tested technologies. The inlet concentration of the different micropollutants to the two lines will be the same, since they share the same inlet. There is no hospital in the catchment area to Brædstrup WWTP. Therefore, the WWTP will be affected by pharmaceuticals and xenobiotics which are present in normal household wastewater. Brædstrup WWTP is shown in FIGURE 1. The experimental line and reference line split in the outlet of the sand- and grease trap.

FIGURE 1. Show Brædstrup WWTP. The experimental line and reference line split in the outlet of the sand- and grease trap.

Implementation of these known technologies in an innovative way in an existing WWTP should prove that it is possible to treat municipal wastewater, at WWTP, and remove micropollutants

to a level below predicted no effect concentration (PNEC) at a low cost. The effect of the conventional activated sludge process and effects to the sludge will be investigated too.

Key figures for Brædstrup WWTP are given in TABLE 1. If the wastewater flow is below 800 m³/h the wastewater is distributed evenly to the two biological treatment lines. If the wastewater flow exceeds 800 m³/h, the inlet to the reference line (line 2) remains at 400 m³/h and the inlet to the experimental line (line 1) will increase to 700 m³/h reaching the plant's maximum hydraulic capacity at 1,100 m³/h.

TABLE 1. Brædstrup WWTP key figures.

Parameter	Unit	Value
Capacity	PE	16,000
Annual flow average	m^3/year	1,300,000
Daily flow average	m^3/d	3,500
Q max dry weather	m^3/h	360
Q max rain weather	m^3/h	1,100
Sludge production	ton/year	1,500

The outlet concentrations from both lines fulfil the discharge requirements. Average outlet concentrations in the period October 2018 to February 2019 shows very similar outlet concentrations from the two lines for most parameters, however the outlet concentration of Total-N and NH_4-N is higher in line 1. For further information see Appendix 1, chapter 6.

2. Objectives

Multiple point ozonation has been tested successfully at both laboratory and pilot scales in SIPIBEL TRIUMPH Project in 2013-2015. In this innovative approach, the remaining technical challenges are the final adjustment of ozone dosing points, dosing amounts and certain details of the injection systems of ozone.

In present project, the overall objective is to demonstrate the removal efficiency of multiple point ozonation and compare it with Powder Activated Carbon (PAC) addition to process tank at full scale, to reach PNEC values at the outlet of WWTP.

Project's main objectives include:

- To demonstrate the positive nexus in applying ozone into Conventional Activated Sludge: improve SVI, control bulking problem in secondary clarifier, disinfection by tertiary ozonation and micropollutant removal in sludge
- To test, optimize and commercialize the technical design of the solution in full scale.
- To assess whether ozone injection in activated sludge can reduce micropollutant concentrations in both the liquid and solid phases of the sludge
- To estimate Total Cost of Ownership (TOC) for both multiple point ozonation and Power Activated Carbon (PAC) addition to process tank.

New important knowledge on how the ozone should be applied into mixed liquor by development of dosing equipment, injectors and monitoring equipment are gathered from the project and the effects on the effluent quality are demonstrated.

This project is an opportunity to:

- Test and compare the efficiency of both micropollutant treatment processes on a new array of pharma components.
- Measure on a large-scale project, the impact of ozone on the biological treatment and the impact of ozone injection on sludge
- Demonstrate new and cost-effective solutions that can remove micropollutant in any small, medium or large wastewater treatment plant with a reasonable cost.

Based on the full-scale test results from biological process monitoring program, ozone dosing injection equipment, PAC injection to process tank, the outcome are a fully documented solution adapted to Nordic conditions.

In this project, not only these two technologies are tested and demonstrated, but also the need for services and maintenance are documented and total costs of ownership demonstrated.

After positive demonstration, the multiple point ozone injection can be a "Proof of Concept" and are documented with clients, universities and operators in the industry.

2.1 Project key performance indicators

- Process characterization:
  - Micropollutant removal from water and sludge
  - Enhanced Sludge Volume Index (SVI)
  - Physical-chemical sludge characterization
  - Toxicity effects and Disinfection
- Total Cost of Ownership of the micropollutant removal.

3. Baseline tests

To assess which micropollutants are present in treated wastewater discharged from Brædstrup WWTP to environment, a pre-screening of 365 compounds was conducted.

Pre-screening results showed 36 compounds have relatively high concentration in outlet of the plant which have been chosen to analyse during the experimental phases. These compounds appear in various list from different studies among others:

- AMK-list (BIOFOS' Arbejdsgruppe omkring hospitalsspildevand Opdaterede Anbefalede Maksimale Koncentrationer (AMK) for lægemiddelstoffer. 2015)
- This list includes recommended maximum concentrations for pharmaceuticals.
- The Switzerland list of indicators: In Switzerland larger WWTPs (> 80,000 PE) or WWTPs which discharge to lakes or rivers used for drinking water is required to perform micropollutant removal by ozonation or filtration using activated carbon. To evaluate the removal efficiency five compounds from a predefined list holding 12 compounds (10 pharmaceuticals and 2 corrosion inhibitors) must be measured. For each sampling, the arithmetic mean of the removal efficiencies for the 5 compounds should be above 80 %.
- MERMISS-list: Danish MUDP project investigating pharmaceutical removal in wastewater using MBBR technology and ozone.

Information about PNEC and degradability are thereby known for most of the chosen compounds.
The chosen 36 compounds are presented in TABLE 2. As seen from the table, the chosen compounds originate from many different categories of pharmaceuticals for example analgesic (painkiller), antidepressant and anti-infective. Pharmaceuticals are divided into groups using the ATC system. This system divides the different compounds into groups depending on which organ system the pharmaceutical affect for example nervous system.

TABLE 2. Pharmaceuticals and other micropollutants investigated at Brædstrup. ATC codes for the different compounds are given in Appendix 1.

	ATC Family	Compound	PNEC [ng/l]	PNEC Reference*
Alimentary tract and metabolism	Steroid medicament	Prednisolone	2.000	AMK 2013
Cardiovascular system	Lipid modifying agents	Atorvastatin	200	AMK 2013
	Cardiac therapy	Ibuprofen	4.000	AMK 2015
	Agents acting on the renin-angiotensin system	Candesartan	120	AMK 2015
	Agents acting on the renin-angiotensin system	Losartan	1.000.000	FASS
	Beta-blocking agent	Metoprolol	7.300	FASS
	Beta-blocking agent	Propranolol	100	AMK 2015

	ATC Family	Compound	PNEC [ng/l]	PNEC Reference*
Antiinfectives for systemic use	*Antibiotics*	Azithromycin	19	Recommendations for 2nd watch list, EU
		Cefalexin	-	-
		Ciprofloxacin	89	AMK 2015
		Clarithromycin	120	Recommendations for 2nd watch list, EU
		Erythromycin	200	Recommendations for 2nd watch list, EU
		Ofloxacin	100	AMK 2015
		Sulfamethoxazole	120	AMK 2015
		Sulfapyridine	-	-
		Trimethoprim	-	-
Antineoplastic and immunomodulating agents	Antiandrogens	Bicalutamide	100	AMK 2015
	Antimetabolite	Capecitabine	200	AMK 2015
	Anti-estrogens	Fulvestrant		-
	Immunosuppresant	Mycophenolic acid	100	AMK 2015
Musculo-skeletal system	Analgesic	Diclofenac	50	Recommendations for 2nd watch list, EU
	Analgesic	Naproxen	6.400	AMK 2015
Nervous system	Antiepileptic	Carbamazepine	500	AMK 2015
	Antiepileptic	Gabapentin	-	-
	Analgesic	Tramadol	2.300	AMK 2013
	Analgesic	Paracetamol	9.200	AMK 2015
	Antidepressant	Sertraline	0,52	AMK 2015
	Antidepressant	Venlafaxine	100	AMK 2015
	Antidepressant	Citalopram	8.000	AMK 2015
	Psycholeptic	Zopiclone	-	-
Antiparasitic products, insecticides and repellents	Antiprotozoals	Metronidazole	12.500	FASS
Antiparasitic products, insecticides and repellents	Neonicotinoid insecticide	Imidacloprid	8,3	Recommendations for 2nd watch list, EU
Contrast media (x-ray)	Contrast media (X-ray)	Iomeprol	1.000.000	FASS
	Contrast media (X-ray)	Iohexol	1.000.000	FASS
Corrosion inhibitors		(methyl) 1H-Benzotriazole	-	-
		∑ 4+5-Methyl-benzotriazole	-	-
		Dimethylbenzotriazole	-	-

	ATC Family	Compound	PNEC [ng/l]	PNEC Reference*
Perfluorinated Substances		PFOA	-	-
		PFOS	-	-

* Local Government Denmark (KL): Proposal for administrative basis for pharmaceuticals in hospital wastewater, recommended maximum concentration for connection to sewer, Input for KL working group concerning hospital wastewater. Prepared by DHI, June 2013, (in Danish); AMK (2015). BIOFOS' Arbejdsgruppe omkring hospitalsspildevand Opdaterede Anbefalede Maksimale Koncentrationer (AMK) for lægemiddelstoffer. 2015; the Swedish Farmaceutiska Specialiteter - www.fass.se; European Union updated watch list (Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List, EUR 29173).

The baseline test has been conducted in September-December 2018 in 3 campaigns, in 9 sampling days. In each campaign, the concentrations of selected compounds have been measured in influent of the plant and effluents of line 1 and line 2, and in each sludge, recirculation lines, in both solid and liquid phase of the samples. The reason was to evaluate the performance of conventional activated sludge in both line 1 and line 2 comprehensively, in different weather conditions and flow and pollution loads.

3.1 Removal of pharmaceuticals from water

At Brædstrup WWTP the inlet concentrations of the different compounds fluctuate. The top three most abundant pharmaceuticals in the influent of the WWTP was paracetamol (painkiller, 7.100-160.000 ng/l), gabapentin (antiepileptic, 18,000-50,000 ng/l) and ibuprofen (painkiller/antiinflammatory, 9,000-39,000 ng/l). Although, Brædstrup WWTP does not receive wastewater from hospitals, the two x-ray contrast media, iohexol and iomeprol, are found in the range 6,400-24,000 ng/l and 250-25,000 ng/l, respectively. These compounds are mainly expected to be present in wastewater from hospitals.

During the baseline test 31 of the 36 chosen compounds were measured above Limit of Quantification (LQ). The measured inlet concentrations are given in FIGURE 2. This figure shows the minimum measured concentration, quartile 25, median, quartile 75 and maximum concentration.

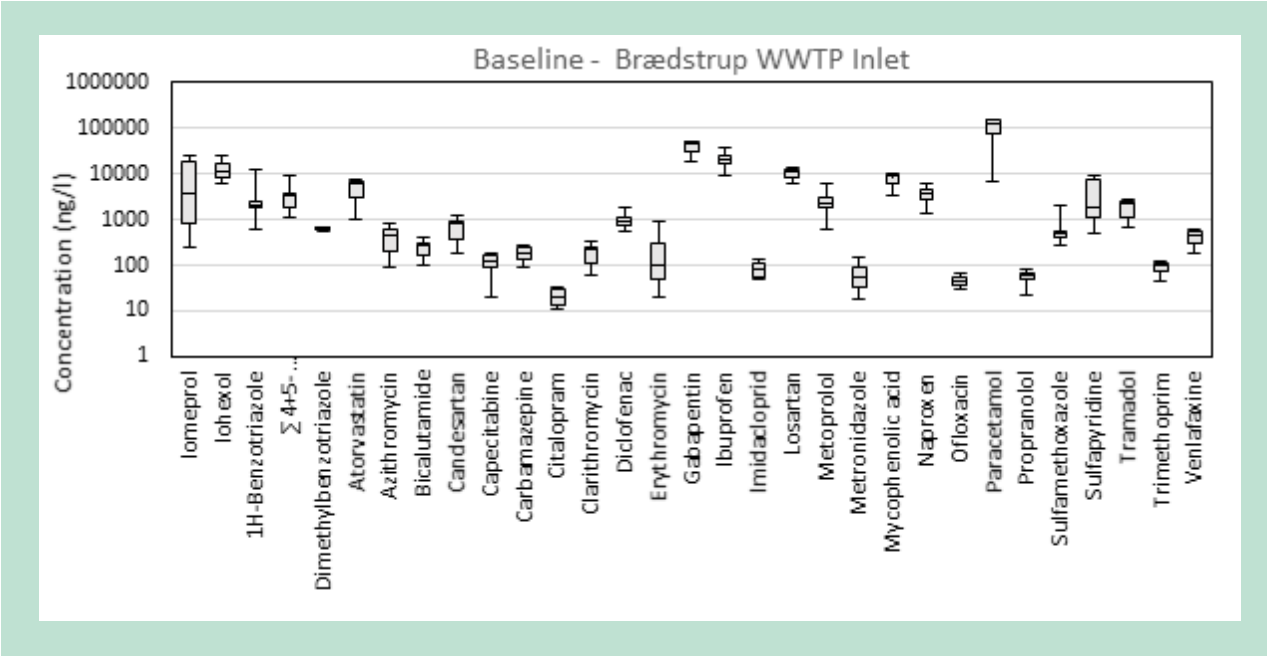

FIGURE 2. Average (based on 9 samples) inlet concentration of the 31 pharmaceuticals quantified above their limit of quantification (LQ) in liquid phase for the baseline samples. It shows the minimum, quartile 25, median, quartile 75 and maximum concentration. Notice logarithmic scale.

The distribution of the different ”pharmaceutical groups” for example analgesic and antiepileptic in the inlet and outlets of Brædstrup WWTP are shown in FIGURE 3. The differences between inlet distribution and outlets distribution are the removals in the activated sludge plants. From this, it is found that for example Paracetamol is almost completely degraded.

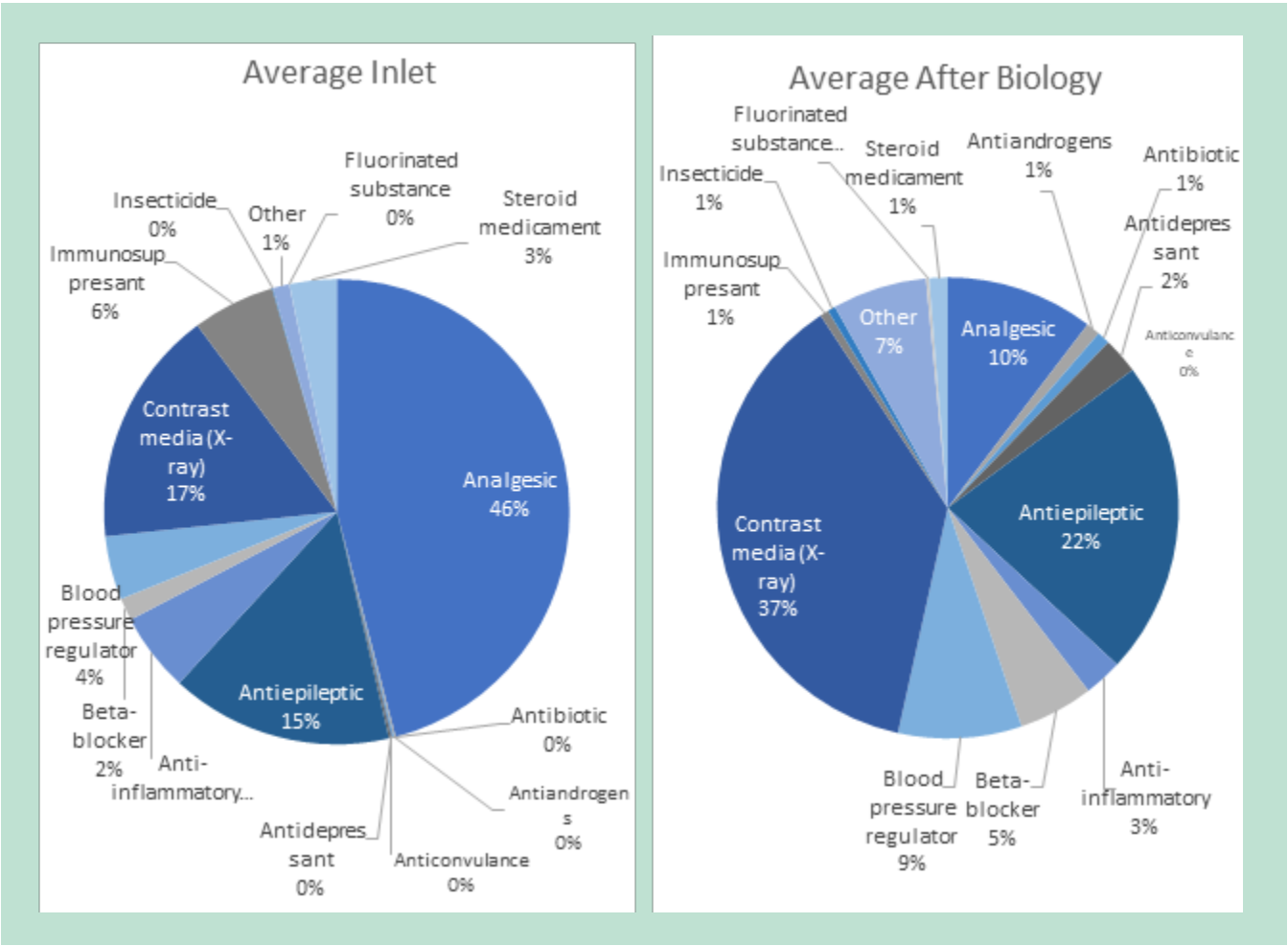

FIGURE 3. Percentage distribution of the different ATC groups/substance families of the overall concentration at Brædstrup WWTP inlet and outlets after conventional activated sludge (CAS).

The removal efficiencies for the different compounds in the two lines at Brædstrup WWTP are shown in FIGURE 4. The outlet concentrations from both lines are presented in Appendix 1.

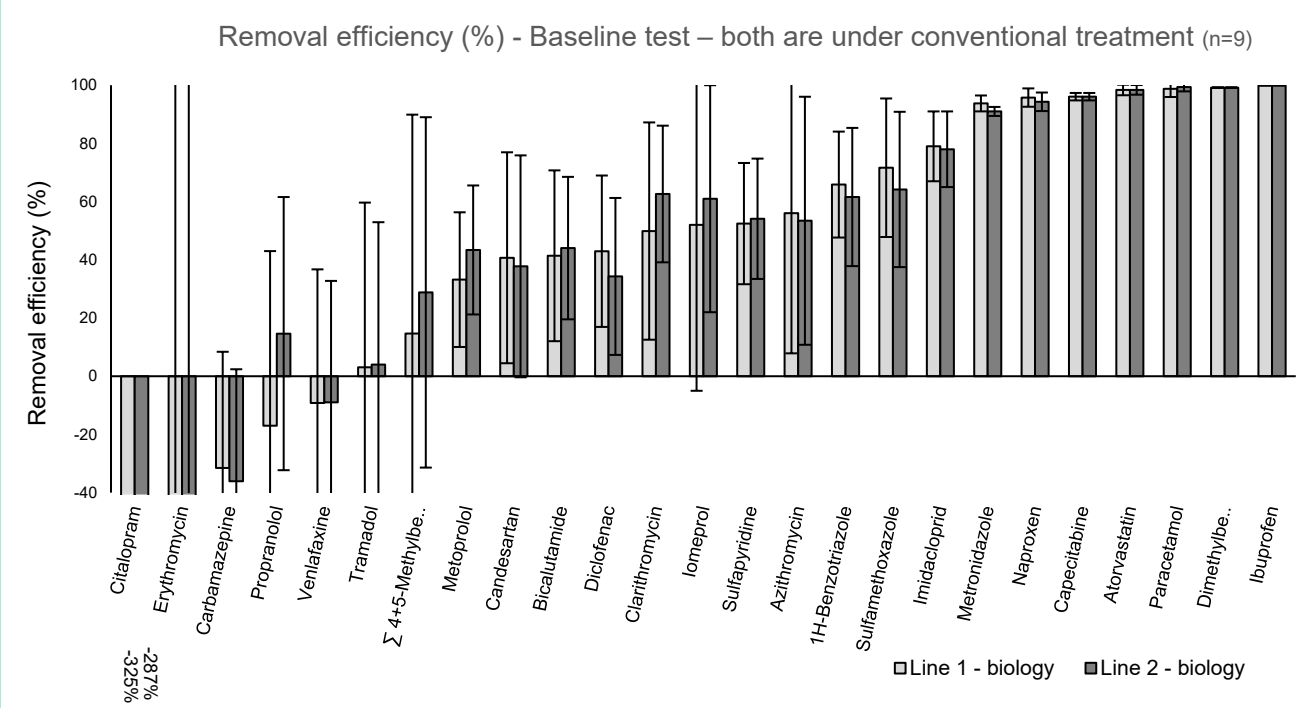

FIGURE 4. Removal efficiency (Rw %) for the 36 pharmaceuticals investigated in the Baseline test. Compounds where neither positive or negative removal efficiency are given, did not fulfil the rules of calculation or the concentration was below Limit of Quantification at all time. The five compounds with a negative removal are from left to right: citalopram, erythromycin carbamazepine, propranolol and venlafaxine.

For some compounds the removal efficiency fluctuates significantly. For example, Bicalutamide was in Line 1 relatively removed (46%) one day, while it showed negative reduction (-23%) the next day. While Line 2 showed 43% removal and -8% on the same correspondingly days. Largely, the removal difference among different compounds in WWTPs could be ascribed to numerous factors such as retention time, inlet concentrations, micropollutant properties and operational conditions.

Negative eliminations might be caused by transformation of different metabolites or conjugates into the parent compounds during biological treatment. When human use different pharmaceuticals, the pharmaceuticals might be excreted as conjugates, meaning that an extra molecule is attached to the pharmaceuticals. During the biological process at the WWTP this extra molecule can be removed from the “original” pharmaceutical. The metabolite might not be detected in the inlet sample, but the parent pharmaceutical is detected in the outlet sample leading to negative removal. It could be also an analytical problem. Besides, the water quality is not the same between the WWTP inlet and outlet and it can affect micropollutant analysis (dissolved organic matter, other unknown micropollutants with a similar retention time in liquid chromatography or similar mass for mass spectrometry). There is also the release of micropollutants adsorbed onto or inside faeces and other solids. Another hypothesis is a modification of water quality (pH, temperature, redox potential) which can release micropollutants which are for example complexed with metals, etc...For further explanation se Appendix 1.

To make sure that two lines are comparable, and thereby make sure that the effect of mixed liquor ozonation and PAC dosing can be evaluated, the removal efficiencies in the two lines

are compared, see FIGURE 5. The comparison is made because the lines do not have the same configuration.

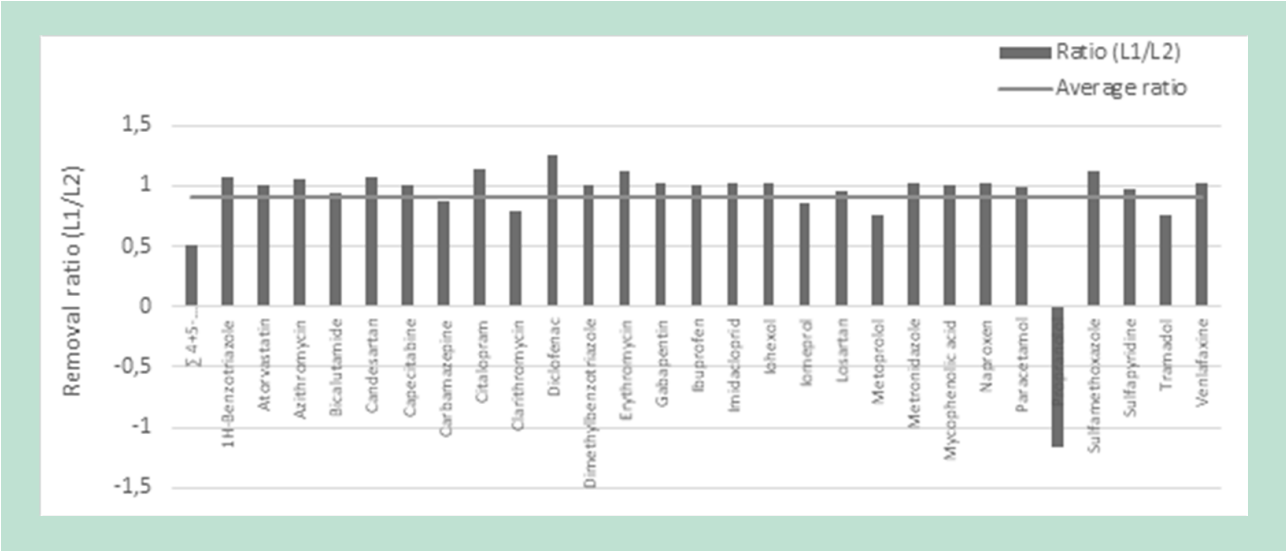

FIGURE 5. The removal efficiency ratio between Brædstrup WWTP Line 1 and Line 2 for the 29 calculable removal efficiencies. The average ratio is 0.9 illustrated by the orange line.

A ratio of 1 shows equal removal efficiency in the two lines. The average ratio is 0.9 supporting similarity across both lines. The negative ratio for propranolol is because it was negatively removed in line 1 (-17%) but positively removed in line 2 (15%). This negative result affects the average, for remaining compounds the removal efficiencies are similar. However, the two lines are well suitable for evaluating the efficiency of the ozonation and PAC dosing.

During the baseline tests 6 compounds were measured above PNEC in the outlets from the reference line and the experimental line. These consist of two antibiotics, one anti-inflammatory, one antidepressant, one antiandrogen and a blood-pressure regulator (Azithromycin, Sulfamethoxazole, Diclofenac, Venlafaxine, Bicalutamide and Candesartan) (FIGURE 6). Additional 5 compounds were measured in the inlet at concentrations above PNEC. The concentrations of these compounds were reduced to levels below PNEC during normal biological treatment. For further information see Appendix 1.

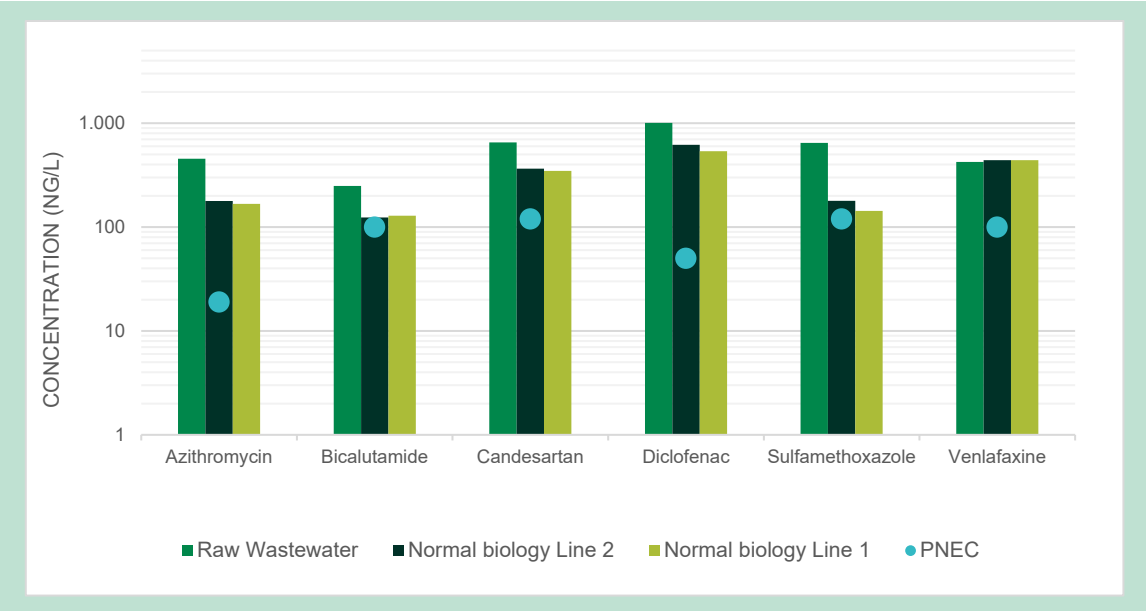

Figure 6 Average inlet concentration and outlet concentration from Brædstrup WWTP (n=9) of those 6 micropollutants above PNEC (Blue dots) in the fall of 2018 (Baseline). Note logarithmic scale.

3.2 Micropollutants in sludge liquid and solid phases

In order to study the micropollutant concentration in sludge, in each campaign, sludge samplings have been taken from activated sludge recirculation pipes of each line. In addition to other analysis, 30 compounds were analysed in the WWTP inlet, liquid phase and 11 compounds in solid phase (capecitabine, carbamazepine, citalopram, clarithromycin, diclofenac, erythromycin, ibuprofen, metoprolol, naproxen, propranolol and tramadol).
Of the 30 micropollutants studied during Baseline tests, 10 were not detected in the liquid phase of the sludge for both lines (dimethylbenzotriazole, capecitabine, cefalexin, ciprofloxacin, ibuprofen, imidacloprid, metronidazole, ofloxacin, prednisolone and zopiclone). On the other hand, almost all 11 compounds are mainly found in the solid phase.

The comparison of sludge analysis result in line 1 and line 2 shows that the two lines have similar micropollutants levels in the solid phase of the sludge. The calculation of the concentration ratio of the two lines for each micropollutant, as well as for the average concentration and the sum of the concentrations, confirms this observation. For further detail see Appendix 2.

4. Multiple point ozonation

Multiple point ozonation lies on the combination of conventional activated sludge and multi-point ozone injection at low dose. Ozone can be injected in 4 different points as shown in FIGURE 7:

- Ozone injection after pre-treatment and before biological treatment
- Ozone injection in activated sludge mixed liquor in aerobic tank
- Ozone injection in return sludge flow
- Ozone injection after secondary clarifier

The combination of two or more ozone injection points allows optimal micropollution treatment and induces positive synergistic effects. The biological treatment is optimized to maximize the removal of biodegradable micro-pollutant. Ozone oxidizes biological recalcitrant substances in both soluble and particulate phases into more assailable substances.

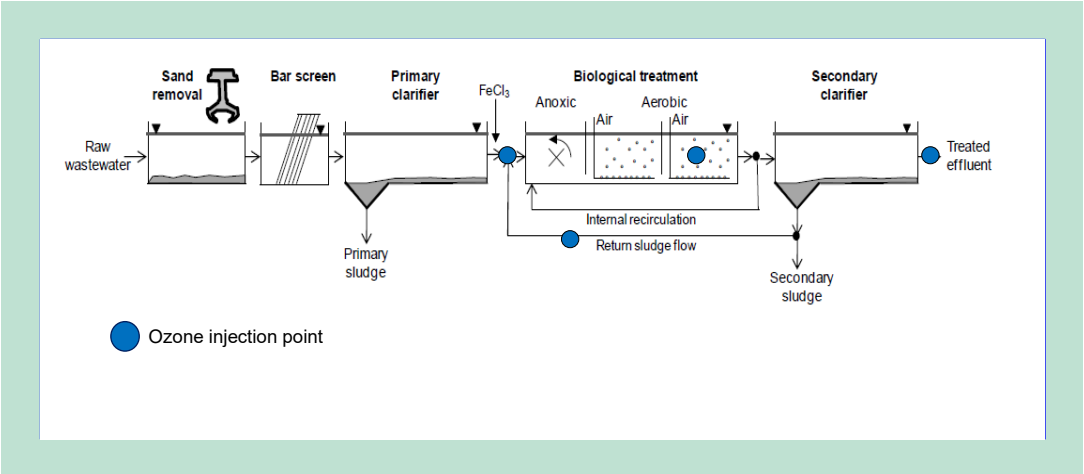

FIGURE 7 Possible points of ozone injection to conventional wastewater treatment plant in multi points ozonation solution

Configuration of the ozone dosing points into the biological treatment, or into the polished effluent, shall be defined according to the quality requirements on nutrients and to required micropollutant removal.

4.1 General description of experiments

In order to scope the whole experimental program, comprehensive study of the Brædstrup WWTP has been conducted. The performance of the plant for one year and analysis statistic of the biological key parameters have been deeply evaluated.

Evaluation of the design and performance of the plant showed the combination of ozone injection in activated sludge mixed liquor in aeration tank and ozone injection after secondary clarifier is the best suited to Brædstrup WWTP line 1.
According to baseline test results, total ozone dose of 11 mg/l has been chosen to remove the list of 36 selected compounds below PNEC values.

In order to evaluate the performance of multiple points ozonation, testing of two different ozonation strategies were conducted. In first strategy, low ozone dose in mixed liquor and high ozone dose after secondary clarifier to remove micropollutant to below PNEC values.
Each ozonation strategy should last for 3 months to assess the effect of ozonation in mixed liquor ozonation and sludge parameters.
The ozonation in the biological reactor was conducted with a venture ejector in full-scale. This use of the ozonation technology is innovative and is demonstrated in full scale in this study.
The tertiary ozonation was conducted on a smaller part of the wastewater flow (10 m3/h), since this technology is well tested and established in other countries.
The first ozonation condition conducted of 3.98 mg O_3/l dosing in the biological reactor and dosing of 7.20 mg/l as a tertiary treatment. The results of the first strategy could help us to define the ozone dose in two points ozonation in second ozonation strategy.
The second ozonation condition conducted of 7.20 mg O_3/l dosing in the biological reactor and dosing of 3.98 mg O_3/l as a tertiary treatment.

A schematic drawing of the experimental layout is presented in FIGURE 8. In the experimental line, a wastewater sub-stream is pumped from one ring in the process tank through an ozone injector and into another ring in the process tank. The aeration tanks shown in the schematic drawing is thereby to different zones/rings in the process tank.

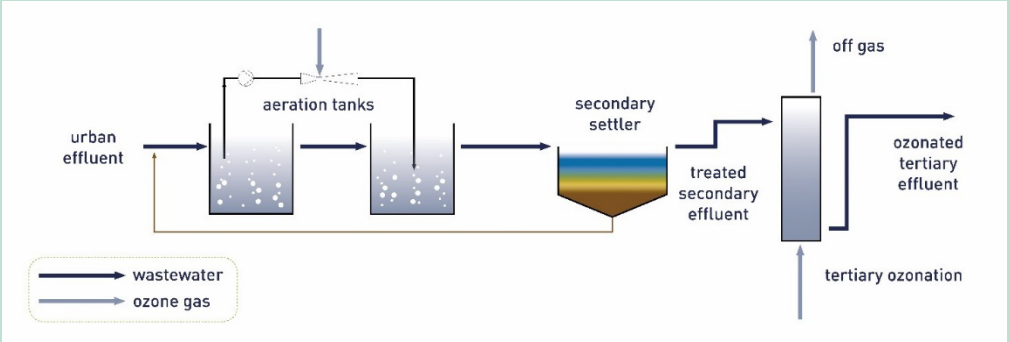

FIGURE 8. Schematic drawing of the experimental layout in Brædstrup WWTP.

FIGURE 9. 2 points ozone injection - point 1: mixed liquor ozonation by venture ejector, Point 2: tertiary ozonation in ozone contact tank

4.2 Degradation of micropollutants by multiple points ozonation

4.2.1 Removal of pharmaceuticals from water

During the ozonation condition 1, 30 compounds were included in the survey. Samples were taken at the inlet, after integrated ozonation and after tertiary ozonation in the experimental line, and in the outlet of the reference line. After tertiary ozonation 4 compounds were measured above detection limit. For further details see Appendix 1.

During the ozonation condition 2, 32 compounds were included in the survey, since the per fluorinated substances PFOA and PFOS were added to the survey. PFOA and PFOS was added to the analysis program since these substances are included in EU watchlist as they are classified as carcinogenic, harmful to reproduction and acute toxic. Furthermore, the substance is persistent and possible bioaccumulating (Nicolajsen & Tsitonaki, 2016). After tertiary ozonation 10 compounds were measured above detection limit.

Calculation of removal efficiencies, when concentrations are near the limit of quantification, is subject to considerable uncertainties. To give confidence in the calculated removal efficiencies, certain rules have been used to decide whether removal efficiencies is calculable or not. These rules are presented in Appendix 1. The removal efficiency is calculated for 21 compounds in the first condition and 20 compounds in the second condition.

For the both ozonation conditions, the average removal efficiencies for each pharmaceutical are calculated. Removal efficiencies for the individual pharmaceuticals is used to calculate the average removal efficiencies for all pharmaceuticals. Average removal efficiency for all pharmaceuticals during the first and second ozonation conditions are presented in TABLE 3.

TABLE 3. Average removal efficiency of pharmaceuticals in the two ozonation conditions.

Ozonation campaign	Removal efficiency reference line [%]	Removal efficiency mixed liquor ozonation [%]	Removal efficiency multipoint ozonation [%]
1.condition mixed liquor ozone dosage 3.98 mg/l Tertiary ozone dosage 7.20 mg/l	63	78	93
2. condition mixed liquor ozone dosage 7.20mg/l Tertiary ozone dosage 3.98 mg/l	65	79	86

From table 3 it is seen that the average removal efficiency in the experimental line is considerably higher than the removal efficiency in the reference line, showing the effect of the ozonation. The configuration in the first ozonation condition provided the highest removal efficiency in the outlet. The higher integrated ozone dosage during the second condition slightly increased the removal efficiency in the step.
The removal efficiencies for the individual pharmaceuticals during the ozonation conditions are shown in figure 10 and FIGURE 11.

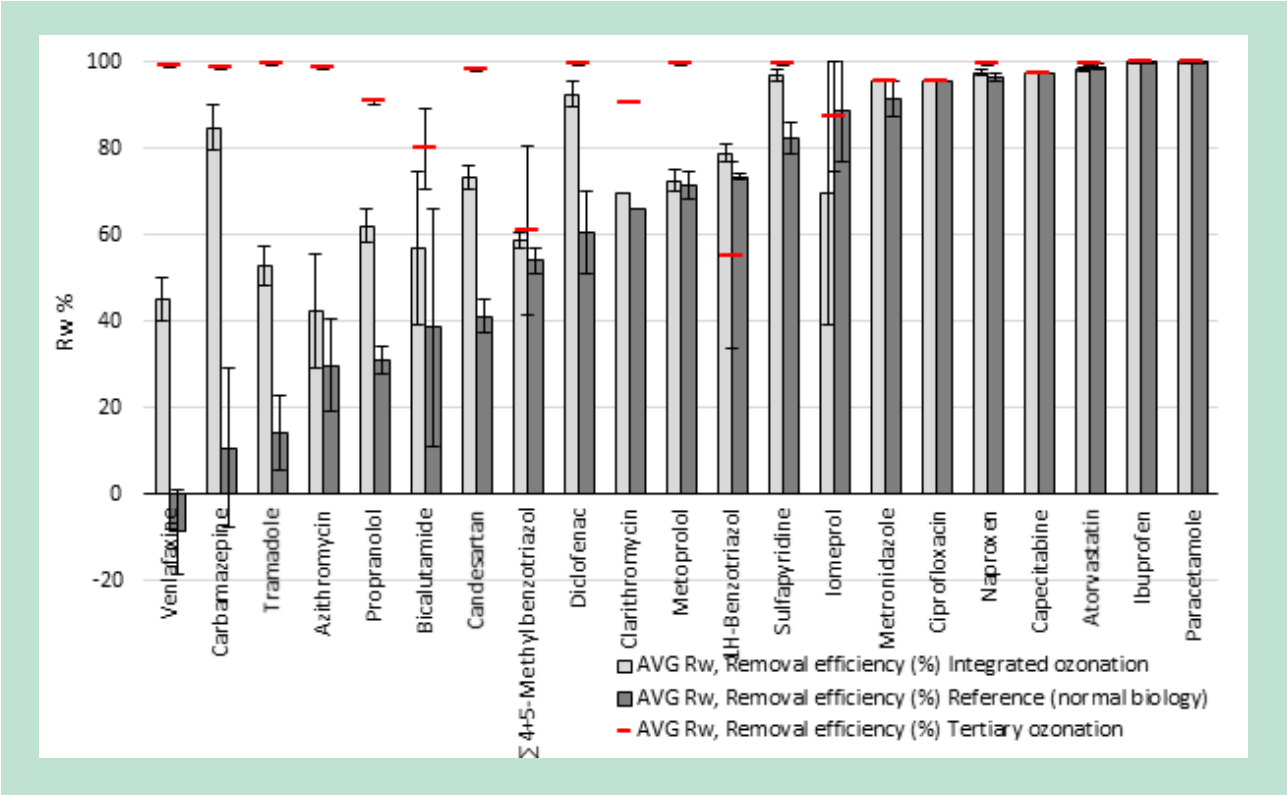

FIGURE 10. Compound specific removal efficiency (Rw %) in percentage with standard deviations for 21/30 compounds that fulfilled the rules for removal percentage calculation during the first condition of multiple point ozonation (based on 3 samples in Endway campaign). Results shown against reference line with no interference within the biology.

From FIGURE 10 it is seen that the multiple point ozonation caused high removal efficiencies for some of the hard-biodegradable substances such as Venlafaxine, tramadol and carbamazepine. ∑ 4+5-Methylbenzotriazol a corrosion inhibitor did not seem to be affected by neither of the ozonation points.

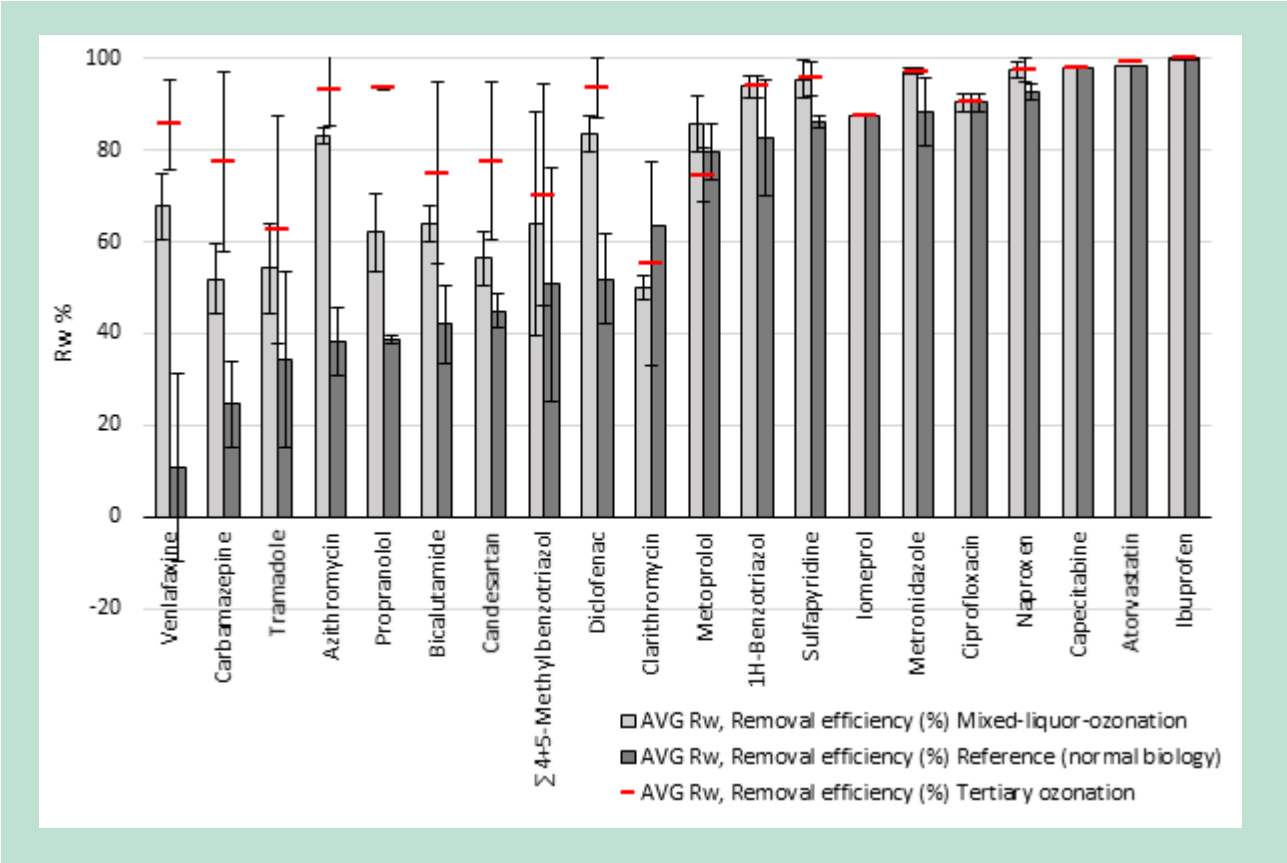

FIGURE 11. Compound specific removal efficiency (Rw %) in percentage with standard deviations for 20/32 compounds that fulfilled the rules for removal percentage calculation during the second condition of multiple point ozonation (based on 3 samples). Results shown against reference line with no interference within the biology.

As described above the highest removal efficiency after multipoint ozonation was achieved during the first ozonation condition. The removal efficiency of some pharmaceuticals after mixed liquor ozonation increased during the second condition, for example azithromycin (antibiotic) the removal efficiency increased from 42% to 83%.

During the first ozonation condition, the concentration of 5 compounds were above PNEC in the inlet and the outlet from the reference line. While all these compounds were below limit of quantification in the experimental line. The results are presented in FIGURE 12

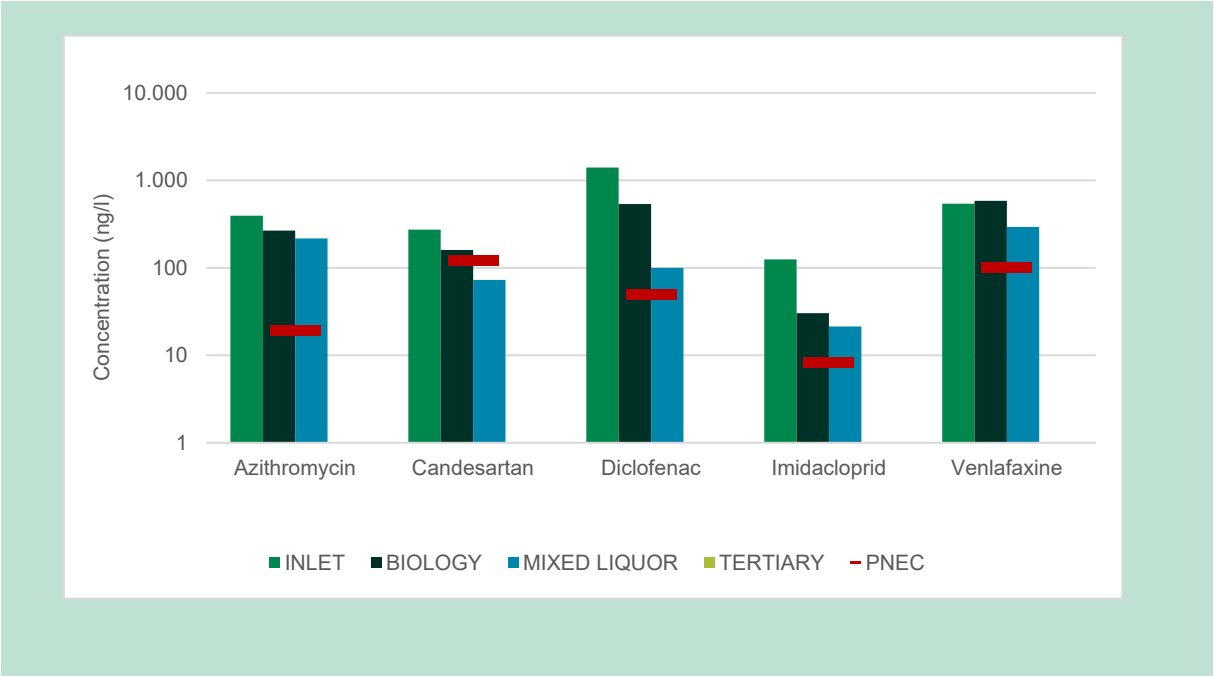

FIGURE 12. Average inlet concentration, outlet concentration from reference line (biology), and outlet concentration after mixed liquor ozonation and multiple point ozonation (Tertiary) in the experimental line from Brædstrup WWTP (based on 3 samples) of those 5 micropollutants above PNEC (red line) during first ozonation condition. Note logarithmic scale!

As it shows in FIGURE 12, the outlet concentration after mixed liquor ozonation is less than outlet of reference line, and even for Candestraten, this value is below PNEC. This prove that even by very low mixed liquor ozone dose, good removal is achieved.

During the second ozonation condition, 6 compounds were measured above PNEC in the inlet of Brædstrup WWTP. Three compounds were above PNEC in the outlet from the reference line. None were above PNEC in the outlet from the experimental line but two compounds (Diclofenac and Venlafaxine) were measured above limit of quantification. Further details are given in Appendix 1.

4.2.2 Removal of pharmaceuticals from sludge liquid and solid phases

An ozone dose of 4 mgO3/L in mixed liquor in process tank reduced micropollutants concentrations in the liquid and solid phases of the sludge (removal of 45% and 16%, respectively), for a total removal of 38%. An increase to a dose of 7 mgO3/L in mixed liquor ozonation had no additional effect on micropollutants concentrations in the liquid phase. In contrast, removal of 64% was achieved in solid phase. These results indicate that a dose of 4 mgO3/L is sufficient to treat only micropollutants in wastewater. To treat micropollutants in wastewater and produced sludge, a dose of at least 7 mgO3/L is required.

The removal for the different quantified micropollutants as well as the average removal and the removal for the sum of the concentrations for both ozone doses in comparison to the reference line are shown in FIGURE 13 for the solid phase of the sludge. In contrast to the liquid phase, it can be observed that an increase of the mixed liquor ozone dose could significantly increase the removal of micropollutants, with an average removal of 1% with 4 mgO_3/L to 61% with 7 mgO_3/L, and a removal of 16% to 64% for the sum of the concentrations, respectively.

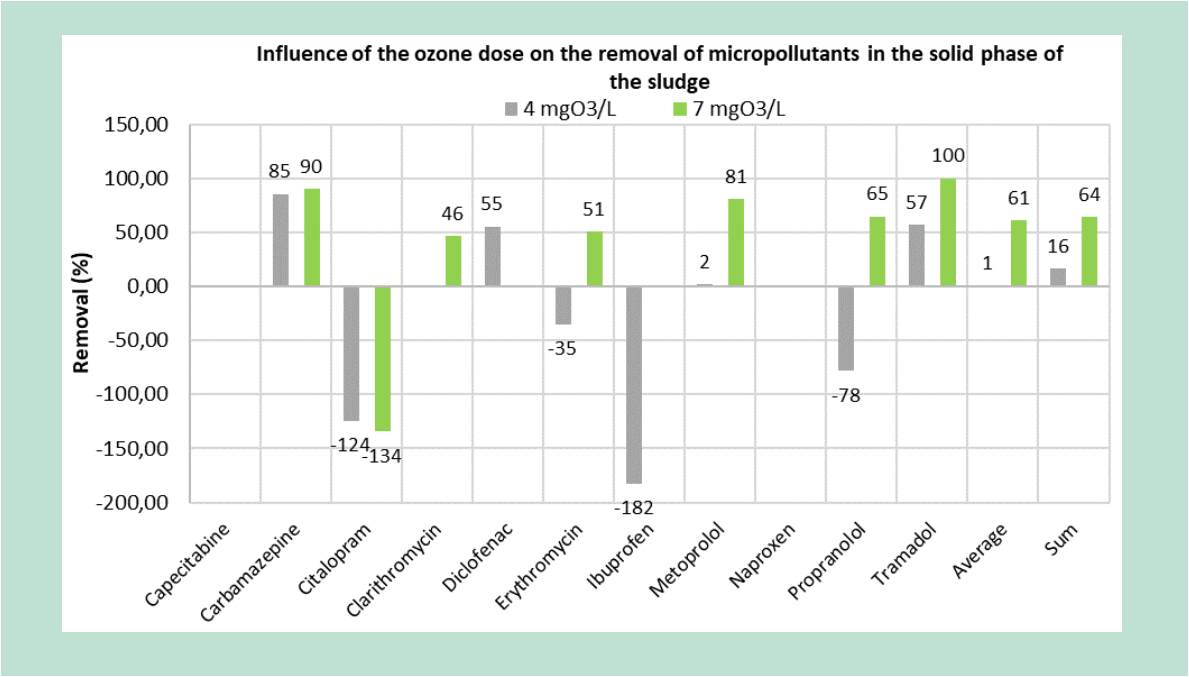

FIGURE 13. Influence of the ozone dose on the removal of micropollutants in the solid phase of the sludge compare to reference line.

The results show that a low dose of ozone is not sufficient to oxidize the micropollutants in the sludge due to the high competition in the liquid phase, the transferred ozone is rapidly consumed by the micropollutants and dissolved organic matter. As the ozone dose increases, there is enough transferred ozone to react with both the water phase and solid phase micropollutants. Further details are given in Appendix 1.

4.2.3 Ecotoxicological effects

One concern regarding the ozonation is that some pharmaceuticals or micropollutants might be degraded to intermediate substances and not fully mineralised. Similar to metabolites that are not measured in the inlet but measured after biological treatment (see section 3) intermediate products might not be measured in the outlet samples, and the measured removal efficiencies might indicate better outlet quality than actually present. The toxicity of some of these intermediate products might be equal to the parent compound, for example metformin (the most often prescribed antidiabetic drug worldwide) which is primarily degraded to guanylurea by activated sludge (Markiewicz, et al., Primary degradation of antidiabetic drugs, 2017a), have comparable toxicities (Markiewicz, et al., Ultimate biodegradability and ecotoxicity of orally administered antidiabetic drugs, 2017b).

To investigate whether toxic intermediate compounds are produced during multiple points ozonation, ecotoxicity studies were conducted to qualify and quantify the impact of multiple points ozonation compared to the reference line effluent. Based on these findings ozonation has not shown any negative effects. The ecotoxicological studies were only done at the end of the condition 1 of ozonation.

9 different ecotoxicological test were performed to investigate different ecotoxicological effects. These tests are described in table 4.

TABLE 4. The different ecotoxicological tests performed after the ozonation condition 1.

Test	Impact/description
Chronic reproductive test (Vibrio fischeri)	Investigation of how population growth is affected.
Growth inhibition of unicellular green algae (Scenedesmus)	Investigation of how Photosynthesis and growth is affected.
Fish embryo/egg test (Danio rerio)	Investigation of how fish early life stages are affected.
ER Calux	Investigation of Estrogen activity of human receptors – thereby indication whether the wastewater causes estrogenic endocrine disruption.
PXR-Calux	General Xenobiotic effect Investigation of "activity" of PXR which is active in the production of enzymes involved in metabolism and transport of xenobiotic from the body/cell.
Ames test	Mutagenic activity Comparing quantity of mutations in cells affected by wastewater (treated and untreated) and cells not affected.
SOS chromotest	Genotoxicity in E coli Investigation of activity of the SOS response which is active during DNA repair. Activity of the SOS response thereby indicate DNA damage.
A-YES	Human estrogen receptor. A human reporter gene is incorporate into a yeast. The activity of the receptor gene is investigated thereby showing if the wastewater causes estrogenic endocrine disruption.
A-YAS	Human androgen receptor A human reporter gene is incorporate into a yeast. The activity of the receptor gene is investigated thereby showing if the wastewater causes androgenic endocrine disruption.

Chronic reproductive tests, Growth inhibition of unicellular green algae and Fish embryo/egg tests are performed in-vivo – investigation of the effect on whole living organisms or cells. The remaining tests are performed in-vitro – test tubes experiment for example human reporter gene incorporated into a yeast.

The results from the ecotoxicological test are presented in TABLE 5. This table gives the summarized results from the different tests, for example four different Ames tests were conducted.

TABLE 5. Results from ecotoxicological tests.

Eco-toxic effects	In-vivo tests			In-Vitro tests					
	Green algae inhabitation (LID: G A)	Vibrio Fischeri (EC50 ml/l)	Fish embryo (LID:G E)	ER Calux (Estrogens ng 17b Estradiol)	PXR Calux (Xenobiotic)	SOS chromotest	Ames test (mutagenic activity)	A-YES (Estrogenic activity) ngEEQ/L	A-YAS (Androgenic activity) ngEEQ/L
Inlet	1	>800	1	Very High Risk	High risk	Low/ medium genotoxicity	Medium mutagenic activity	20	140
Outlet reference	1	>800	1	High Risk	High risk	Medium/ high genotoxicity	Medium/high mutagenic activity	0.25	0.37
Multiple point ozonation	1	>800	1	No risk	No Risk	Medium genotoxicity	Very low mutagenic activity	0.17	0.57

From TABLE 5 it is seen that no differences were seen for the In-vivo tests. For the In-Vitro tests the outlet from the experimental line shows significantly lower ecotoxicology than the inlet except for SOS chromotest. The outlet from the experimental line shows lower ecotoxicology than the reference line except for the androgenic activity.
The ecotoxicology tests there by shows that the ozonation at Brædstrup WWTP don't produce toxic intermediate compounds, and the different pharmaceuticals and micropollutants are degraded to other less toxic compounds or fully degraded.

4.2.4 Removal of antibiotic resistant bacteria and genes for antibiotic resistance

As described in section 1 antibiotic resistant bacteria are of general concern worldwide. During the project the disinfectant effect against antibiotic resistant bacteria and removal of genes for antibiotic resistant were tested.

Five antibiotics were selected based on their coverage of different antibiotic classes: sulfamethoxazole (sulfamides), trimethoprim (diaminopyrimidines), ciprofloxacin (fluoroquinolones), ofloxacin (fluoroquinolone) and the Extended Spectra Beta Lactamase resistance, ESBL (β-lactam family - cephalosporine resistance).

The number of cfu/ml (coli forming units per millilitre) were measured at Brædstrup WWTP by heterotrophic plate count. Samples were taken at the Inlet, outlet of the reference line, and outlet after multiple point ozonation.

The removal of the chosen antibiotic resistant bacteria is shown in TABLE 6. The removal is given as log-removal. A log-removal of 1 is equivalent to 10 times reduction, log-removal of 2 is equivalent to 100 times reduction, log-removal of 3 is equivalent to 1,000 times reduction and so on.

TABLE 6. Log-Removal of the 5 chosen antibiotic resistant bacteria, during first (July 8th, 2019) and second (October 16th, 2019) ozonation condition (HPC – heterotopic plate count).

Antibiotic resistant bacteria	Total HPC	CIP-HPC	TMP-HPC	OFL-HPC	SMX-HPC
July 8th, 2019 - Ozonation condition 1					
Inlet / Outlet line 1 - after mixed liquor O_3	3.6	2.8	3.8	3.3	3.8
Inlet / Tertiary Ozonation	4.0	2.9	3.9	3.4	3.9
Inlet / Outlet line 2	2.8	2.5	3.1	2.8	3.0
October 16th, 2019 - Ozonation condition 2					
Inlet / Outlet line 1 - after mixed liquor O_3	2.6	2.3	3.7	2.4	2.6
Inlet / Tertiary Ozonation	2.9	1.6	2.6	2.6	2.6
Inlet / Outlet line 2	2.6	2.6	2.9	2.4	2.9

From TABLE 6 it is seen that the conventional biological treatment reduces the number of all antibiotic resistant bacteria. During the first ozonation condition both mixed liquor and multipoint ozonation caused an additional removal of antibiotic resistant bacteria. During the second ozonation campaign no supplementary removal was observed by the multiple points ozonation.

The measured concentrations of the chosen antibiotic resistant bacteria (cfu/ml) are shown in FIGURE 14.

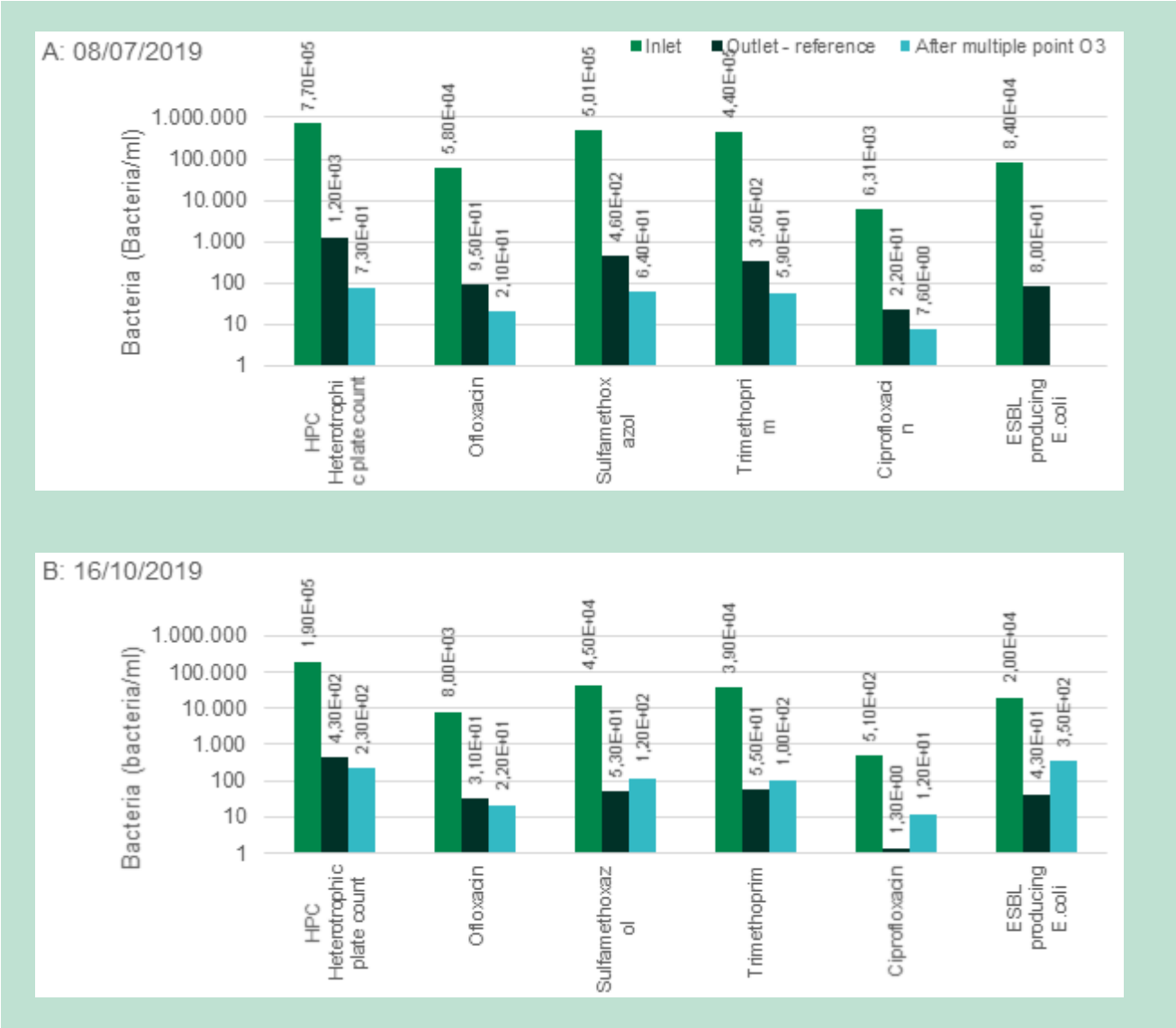

FIGURE 14. Concentration (cfu/ml) of 5 different **antibiotic** resistant bacteria at the inlet, outlet reference line and outlet after multiple point ozonation, and total heterotrophic plate count. A: ozonation condition 1, B: ozonation condition 2

For all the targeted antibiotics, the proportion of antibiotic resistant bacteria in October were about two-fold lower than in July, suggesting the possible variations in ARB abundance, depending on the collection flows and multiple sources of antibiotic resistance.

The ozonation showed no significant removal of genes for antibiotic resistance compared to the reference line. See Appendix 1 for further details.

4.3 Effect on wastewater treatment plant performance from multi point ozonation

The effluent quality was monitored weekly during the baseline, ozonation and PAC conditions. There was not observed any change (reduction or improvement) of bio-logical performance in the experimental line compared to the control line, as effluent concentrations of TN, P and COD were similar between the lines and all under the threshold limit values. This is supported by analyses of BioP capacity, oxygen up-take rate, and nitrification and denitrification rate,

showing no clear and systematic differences between the lines before and after the onset of ozonation. However, reduction of SVI and DSVI was observed along with a change in microbial community composition. See Appendix 1 and Appendix 3 for further details.

4.3.1 Sludge characteristics

The effect of mixed liquor ozonation on sludge characteristics were examined in different ways including physical- and chemical characterization, biological performance, microscopy analysis, and DNA sequencing. For further information see Appendix 3 and Appendix 4.

4.3.2 Sludge settling properties

To test the sludge settling properties the SVI (sludge volume index) were measured before ozonation, in all duration of the project. Measurements were performed weekly at Brædstrup WWTP and at a lower frequency at Aalborg University. At Aalborg University, SVI and DSVI (diluted SVI) were measured 3 times during the baseline campaigns, 2 times during the first ozonation condition, 1 during the second ozonation condition and 2 time during the PAC conditions.

Before the ozonation conditions, the SVI were similar in the experimental and control line and SVI were varying in the same way during the year in the two process lines.

During the first ozonation conditions, the SVI in the experimental line drops and differs clearly from the control line, showing better settling properties in the experimental line.
During the second ozonation condition the SVI of two lines becomes more similar, as a result of reduced SVI in the control line. The SVI in the experimental line is steady at approximately 50 mL/ g TS during the second condition, showing good settling properties.

The reason for improved settling properties in the control line is unsure, but it isn't caused by mixing of the sludge, which is proven by the DNA sequencing, see section 4.3.7.

In general, both multiple point ozone conditions show improved settling properties. When ozonation stopped, the SVI in the experimental line increases showing that the improved settling properties caused by mixed liquor ozonation is reversible.

Weekly measurements of SVI in the experimental and control line are seen in FIGURE 15. These measurements are performed at Brædstrup WWTP. Measurements performed at Aalborg University show the same tendency.

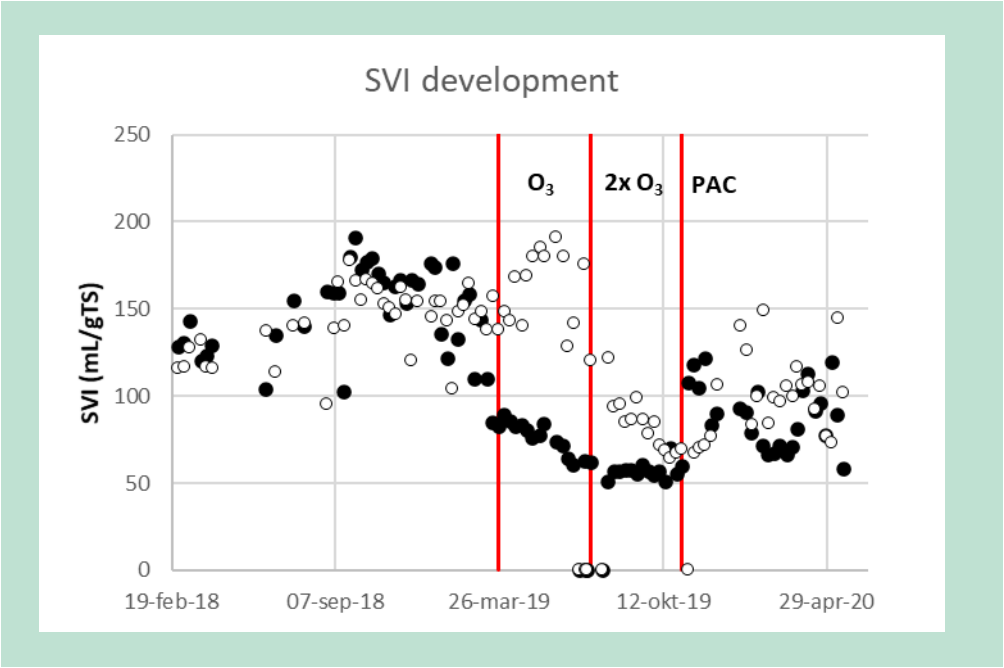

FIGURE 15. Development in SVI of Line 1 (experimental line, ●) and Line 2 (reference line,○) measured weekly at Brædstrup WWTP during the baseline campaigns, ozonation and PAC conditions

4.3.3 Sludge dewaterability

Sludge dewaterability have been tested using Capillary Suction Time (CST). CST is a simple and precise measure of the rate at which water is released from a sludge matrix. Sludges that release water quickly have a low CST and vice versa.
The capillary suction time (CST) was measured at Aalborg University. Sludge in Line 1 (experimental) and Line 2 (reference) had similar CST values, and no effect was observed during the ozonation conditions. Therefore, CST measurements couldn't reveal any differences in sludge dewaterability caused by mixed liquor ozonation. All measurements of CST are low (less than 20 seconds) making it hard to determine CST changes.

Further information is given in Appendix 3.

4.3.4 Sludge floc properties

The sludge floc properties were investigated in different ways including – residual turbidity, deflocculation potential, shear sensitivity and floc diameter.

The amount of non-flocculated matter in the aqueous phase of the sludge was quantified measuring the residual turbidity. The test was performed by measuring the turbidity of sample which had been centrifuged to remove the flocculated matter from the sample. The test showed no significant difference between the experimental and control line, and thereby mixed liquor ozonation wasn't seen to affect the degree of flocculation.
The deflocculation potential was larger for flocs in the experimental line compared to the control line, showing a higher floc strength for sludge in the control line. This was seen before and during the mixed liquor ozonation. Thereby mixed liquor ozonation wasn't seen to affect the floc strength.
The difference is probably caused by the different aeration systems in the two lines. The reference line is aerated by surface aeration while the experimental line is aerated by diffusors in the bottom of the tank. The sludge in the control line is thereby exposed to more physical stress than sludge in the experimental line, which might improve floc strength since the flocs

that isn't broken by the aeration system will have a higher tendency to settle in the clarifier and survive in the plant.

No differences in shear sensitivity was seen between the two lines, as the rate of release of matter from the flocs was similar.

Further information is given in Appendix 3.

4.3.5 Sludge biological performance

Oxygen uptake rate (OUR), denitrification rate, nitrification rate and BioP capacity was examined to evaluate the biological performance of the sludge.

No systematic variation between the experimental and control line was observed before and during the ozonation campaigns. Hence neither positive or negative effects of mixed liquor ozonation on these biological parameters were seen during the project.
Seasonal variations were seen for OUR while BioP capacity was at the same level during the project. Denitrification and nitrification rate varied too during the project, but not in the same pattern as OUR. The OUR is shown in FIGURE 16

No systematic difference between concentration of Tot-N and COD in the effluent from experimental and reference line was seen, supporting that no effect of mixed liquor ozonation on the biological performance was observed.

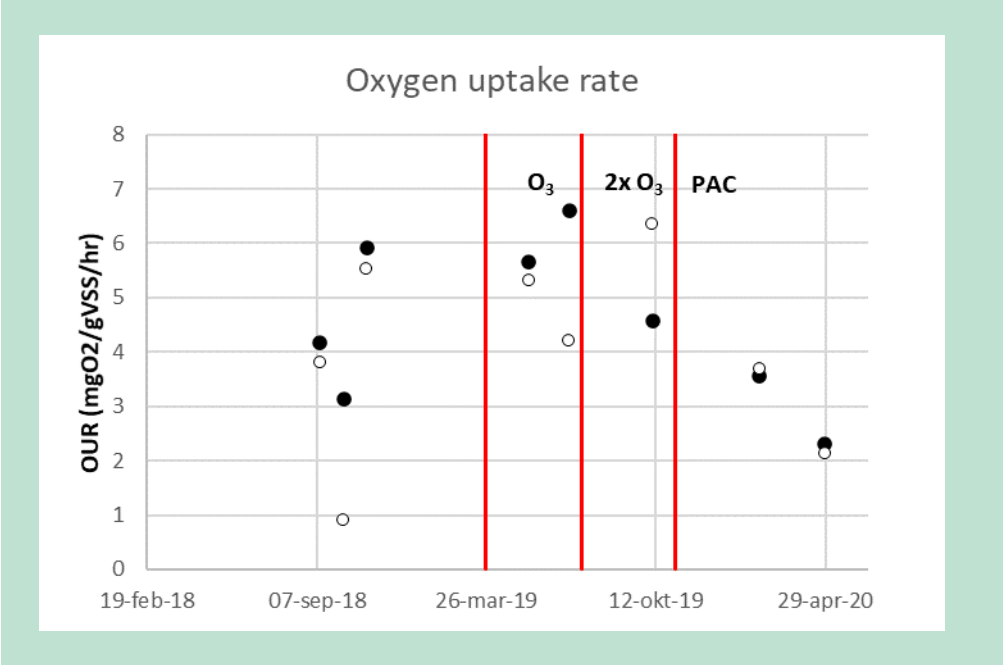

FIGURE 16. Oxygen uptake rate (OUR) of Line 1 (experimental line ●) and Line 2 (reference line ○) sludge samples during baseline campaigns, ozonation and PAC treatment.

Further information is given in Appendix 3.

4.3.6 Microbial community characterization

Through light microscopy analysis the sludge was characterized in respect of filament index, animals, free cells, compactness and firmness.

Before the ozonation both lines showed similar characteristics, with fluctuation looking at compactness and firmness.

During the second ozonation condition (7.2 mgO3/l mixed liquor ozone dose) the lines differentiated in respect to the number of animals, compactness and firmness. The number of animals decreased and only a few or none animals were observed. The animals consume the free cells, and therefore reduced number of animals could cause an increased number of free cells. This didn't happen as the number of free cells stayed at the same level as before ozonation.

The firmness and compactness of the flocs in the experimental line increased during the second ozonation condition.

Another effect of ozonation was removal of attached growth to filamentous bacteria. The removal of attached growth is seen in FIGURE 17.

Fem filamentous microorganisms were present in sludge during the project period, and the two lines did not differ notably and mixed liquor ozonation was not seen to affect the filament index.

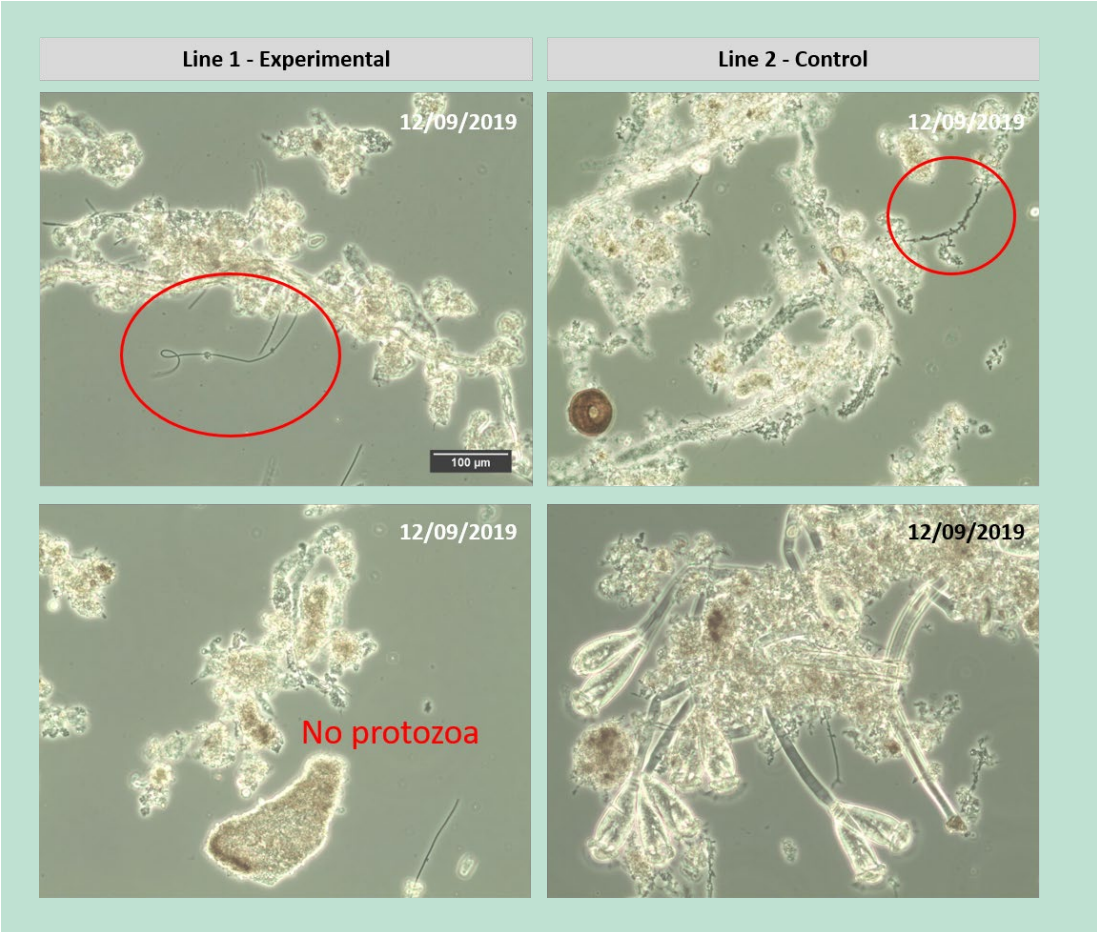

FIGURE 17. Notable effects of second ozone condition. No attached growth on the filaments in line 1 (experimental line). No animals present in line 1 (experimental line), whereas the control line 2 had Vorticella, Arcella and Nematodes (not shown)

For further information see Appendix 4.

4.3.7 Changes in microbial community by DNA sequencing

During the project, weekly sludge samples were analysed through DNA sequencing to classify the composition of the microbial community.

Before the multiple point ozonation conditions, the microbial community were very similar in the experimental and control line.
When mixed liquor ozonation was started, the microbial community in the two lines started to differ. The relative abundances of some genus increased, others deceased, and some disappeared almost completely.

Microbial community changes during the ozone conditions are shown in FIGURE 18, see Appendix 4 for further information regarding the construction of the map. The “timeline” is shown by the colour, where the dark points are from the beginning of the project and the light points are the latest measurements. The experimental line is shown by dots, and the reference line is shown by triangles. If the points (triangle and dot) are positioned close to each other, the microbial community in the two lines are similar. During the “Baseline” period until mixed liquor ozonation starts, the microbial communities in the two lines are similar which is seen as the “dots” and “triangles” are positioned closely and follow the same pattern. When mixed liquor ozonation starts the microbial communities in the two lines starts to differentiate, which is seen by the “dots” and “triangles” move away from each other.

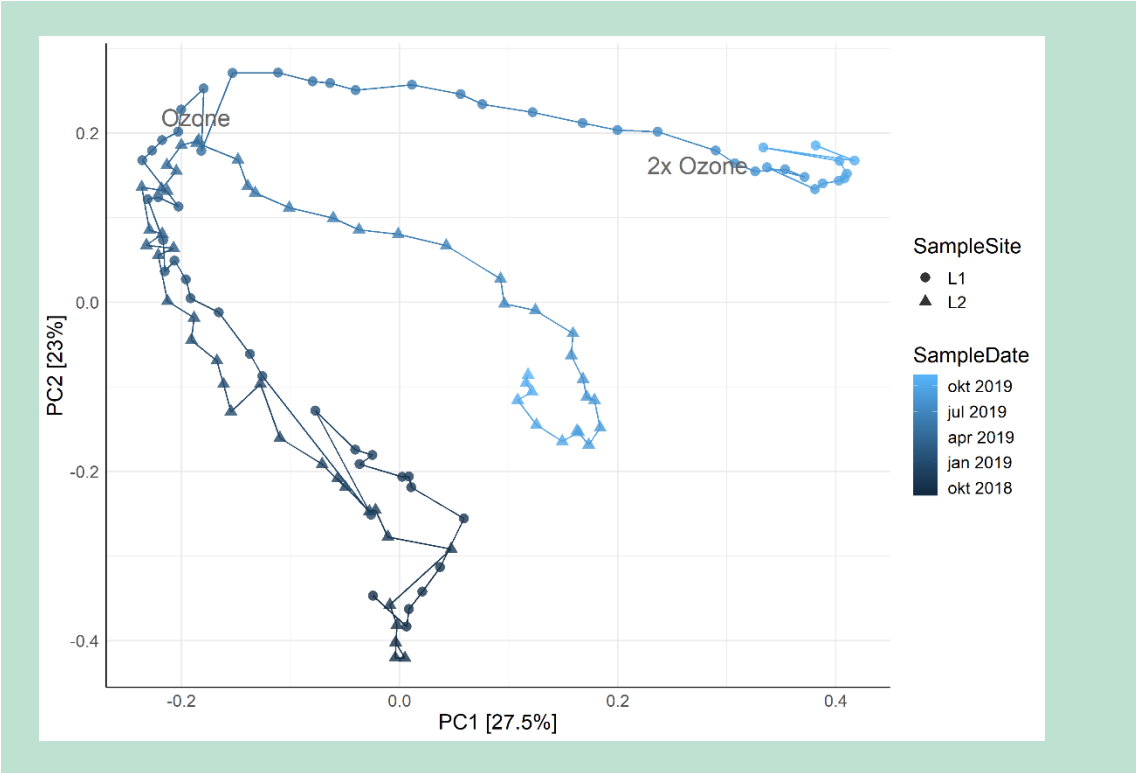

FIGURE 18. Principal Components Analysis (PCA). Line 1 = experimental (•) and line 2 = control (Δ). The distance between the points represents the differences in microbial community composition between the samples. The relative contribution of each axis to the total inertia in the data is indicated in percent at the axis titles.

The abundances changes are probably affected by the microorganism’s position in the sludge floc. The relative abundance of some known microcolony formers rises for example the ammonia-oxidizing bacteria *Nitrosomonas* and the nitrite-oxidizing bacteria *Nitrospira*. These organisms are most often found to be sitting inside the sludge flocs which may protect them from the ozone. The relative abundance of these bacteria is seen in FIGURE 19
The relative abundance of some filamentous bacteria decreases during the mixed liquor ozonation and almost disappears while others are unaffected.

The overall most abundant phosphorous accumulating organism (PAO) in Danish WWTPs (*Tetrasphaera*) don't seem affected in the first ozonation condition, but during the second condition with higher ozone dose (7.2 mgO3/l), the relative abundance drops. The abundance of *Tetrasphaera* was always below 2% in the experimental line. In other Danish WWTP this organism can be present in up to 40% relative abundance. There isn't a dedicated biological phosphorus removal at Brædstrup WWTP, and the low abundance of *Tetrasphaera* is therefore expectable.
The relative abundance of another PAO (*Dechloromonas*) increases in the experimental line during the first ozonation condition but drops at the end of the second condition. This organism is a well-known microcolony former and might be situated inside the flocs and protected from the ozone.
Other highly abundant bacteria with unknown function in activated sludge, shows same tendency as describe above, some were unaffected, some increased and others decreased/disappeared.

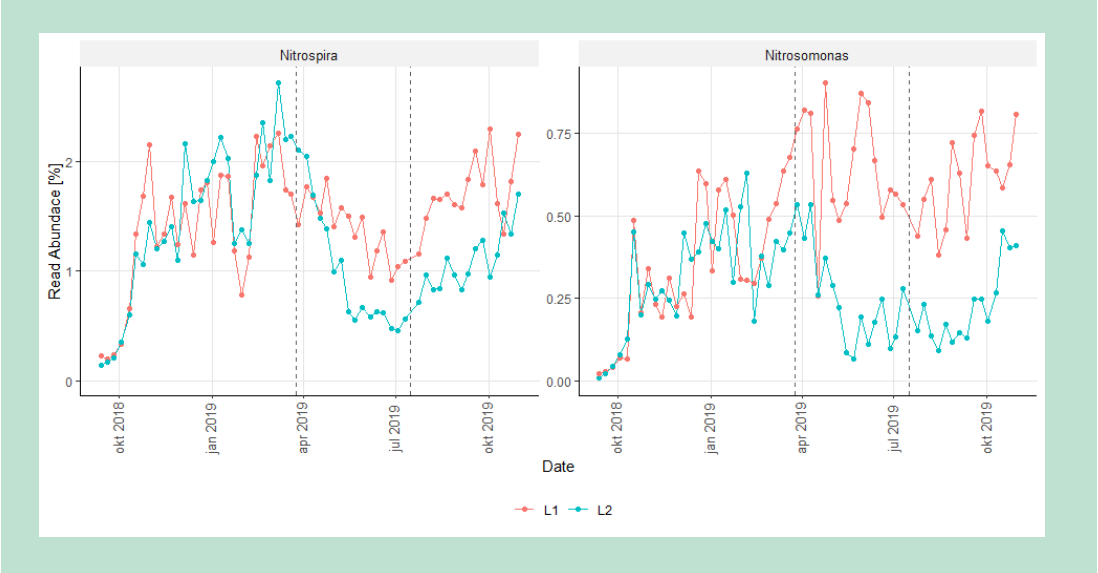

FIGURE 19. Time series of known nitrifiers in line 1 – experimental (red) and line 2 - reference (blue). The dotted vertical lines represent the start of the two ozone conditions. Note the Y-axis varies in the figures.

Bacteria that disappear might be those which are positioned at the outer surface of the flocs and thereby are less protected from the ozone, as shown in FIGURE 17 where the attached growth at the filaments are stripped off.

The microbial community changes from ozone didn't cause a loss of any of the known process-critical genera.

The microbial diversity was characterized through alpha diversity and Shannon diversity index. These showed similar diversity during the "baseline" campaigns but especially during the second ozone condition, the diversity in the experimental line decreased indicating that some species were removed by the mixed liquor ozonation.

For further information see Appendix 4.

4.4 Cost of multiple point ozonation

Different economical parameters for 2 points ozonation are presented in TABLE 9. CAPEX is a measure for the capital expense in relation to upgrade, maintain and improve the existing conventional activated sludge WWTP. OPEX is a measure of the operational expenses. TCO is a measure of total cost of ownership. The following assumptions are done for the economical calculations.

- WWTP capacity – 100,000 PE.
- Wastewater flow – 8,300,000 m³/year
- For CAPEX calculation 15 years lifetime for equipment and 30 years lifetime for building structures is assumed.
- OPEX: chemicals and energy, excluding manpower.
- Ozone dose: 5 – 10 mg O3/l of wastewater.
- The ozone dosage used for the calculations of expenses is optimized compare to ozone dosage used in the project (approximately 11 mg O_3/l). The experimental analysis results showed an optimum ozone dose of approx. 4 mg/l in mixed liquor. The supplementary micropollutant removal by tertiary ozone dose depends on the wastewater treatment plant.

TABLE 7. CAPEX, OPEX and TCO for 2 points ozonation at a 100,000 PE WWTP.

Ozone dose [mg/l Water]	CAPEX [mDKK/year]	OPEX [mDKK/year]	TCO [mDKK/year]	TCO [kr/m³]	TCO [kr/PE/year]
-	1.1	-	-	-	-
5	-	1.6	2.6	0.32	26
10	-	3.1	4.2	0.51	42

The energy consumption for the 2 points ozonation is approximately 0.1 kWh/m³.

For a “normal” household (consisting of 2.4 PE) the yearly cost is approximately 100 DKK/year for removal of micropollutants at a WWTP equipped with multipoint ozonation.

4.5 Conclusion

The two separate treatment lines at Brædstrup WWTP, made it especially suited for direct comparison between conventional activated sludge (CAS) and CAS combined with multiple point ozonation or PAC addition.

The average removal of pharmaceuticals during the first condition of multiple point ozonation (mixed liquor dose of 3.98 mgO_3/l and a tertiary dose of 7.20 mgO_3/l) was 78% after mixed liquor ozonation and by adding tertiary ozonation step, it increased to 93%. In the same period, June 2019, the conventional biology within the reference line removed 63%. Switching the ozone dosages around a total average removal of 86% was seen (79% by mixed liquor ozonation). The small extra removal seen in the process tanks and the lowered overall removal support an optimal division between the dosages as in the first condition, i.e. having a low mixed liquor dose and a high tertiary dose.

For both ozonation conditions the concentration of all pharmaceuticals was reduced to levels below PNEC.

The results show that 3.98 mg/l dose of ozone is not sufficient to oxidize the micropollutants in the sludge. Due to the high competition in the liquid phase, the transferred ozone is rapidly consumed by the micropollutants and dissolved organic matter. As the ozone dose increases to 7.2 mg/l, there is enough transferred ozone to react with both the water phase and solid phase micropollutants.

The ecotoxicological tests showed lower or equal ecotoxicity of the wastewater from the experimental line compared to inlet wastewater and wastewater treated in conventional biology treatment. Thereby no effects of possible toxic intermediate product from the ozonation were observed.

The mixed liquor ozonation lowered the SVI thereby enhancing the sludge's ability to settle. Some microorganisms disappeared during ozonation, but no critical species were lost, and general treatment performance was maintained. Only for a high dose of ozone in sludge in the experimental line (7.20 mg O3/L) showed a reduction in microbial diversity.

During the first ozonation condition both mixed liquor and multipoint ozonation caused an additional removal of antibiotic resistant bacteria. During the second ozonation campaign no supplementary removal was observed by the multiple points ozonation.

Moreover, multiple point ozonation showed no significant removal of genes for antibiotic resistance compared to the reference line.

The effect of mixed liquor ozonation on sludge characteristics were examined in different ways including physical- and chemical characterization, biological performance, microscopy analysis, and DNA sequencing:

- The onset of mixed liquor ozonation significantly reduces Sludge Volume Index (SVI) and Diluted SVI (DSVI) of sludge. This is a reversible process, i.e. after termination of mixed liquor ozonation, the sludge SVI and DSVI increased again. Hence, the settling properties are enhanced by mixed liquor ozonation.
- CST measurements could not conclude any impact of mixed liquor ozonation of dewaterability. This may be because CST values are already low, i.e. dewaterability is high.
- No change in floc properties as a result of the treatments could be concluded.

The total cost of ownership calculation showed, for a “normal” household (consisting of 2.4 PE) the yearly cost is approximately 100 DKK/year for removal of micropollutants at a WWTP equipped with multipoint ozonation.

5. Mixed liquor activated carbon dosing

5.1 Experiments

The effected of dosing powdered activated carbon (PAC) in the process tank where tested. Two experimental conditions were conducted doubling the PAC concentration in the second condition. During the first condition 5 mgPAC/l were added to the mixed liquor in aerobic process tank in the experimental line, and 10 mgPAC/l in the second condition. The tests were conducted in full scale since this is an innovating way of using activated carbon to remove pharmaceuticals and micropollutants.

The biological performance of the wastewater treatment was not affected by the addition of PAC to the experimental line, as the effluent concentrations of COD, TN and TP were all similar between experimental and control lines and below the limit threshold values throughout the baseline and PAC campaigns. This result is in correlation with results from measurements of oxygen uptake rate, denitrification and nitrification rates and BioP activity. In addition, SVI and DSVI were similar during the PAC campaign, i.e. the SVI and DSVI of sludge in the experimental line increased to reach and follow the level of the control line.

Further information is given in Appendix 4.

5.2 Removal of pharmaceuticals

During the PAC conditions, 33 compounds were included in the survey, since the anti-estrogen substance Fulvestrant was added to the analysis program. The removal efficiency was calculated for 18 compounds in the first PAC condition and 22 compounds in the second PAC condition, using the same rules for calculation of removal efficiency used for the multiple point ozonation conditions, see section 4.2.1.

For the two PAC conditions, an average removal efficiency for each pharmaceutical is calculated. Removal efficiencies for the individual pharmaceuticals is used to calculate the average removal efficiencies for all pharmaceuticals. Average removal efficiency for all pharmaceuticals during the first and second PAC conditions are presented in TABLE 10.

TABLE 8. Average removal efficiency of pharmaceuticals in the two PAC conditions.

PAC campaign	Removal efficiency reference line [%]	Removal efficiency experimental line [%]
1.condition 5 mgPAC/l	45	73
2. condition 10 mgPAC/l	54	74

From TABLE 10 it is seen that PAC dosing in the process tank increase the removal of pharmaceuticals compared to the biological treatment. Doubling the PAC dosing caused the average removal efficiency to increase by 1 %.

The removal efficiencies for the individual pharmaceuticals during the PAC conditions are shown in FIGURE 20 and FIGURE 21.

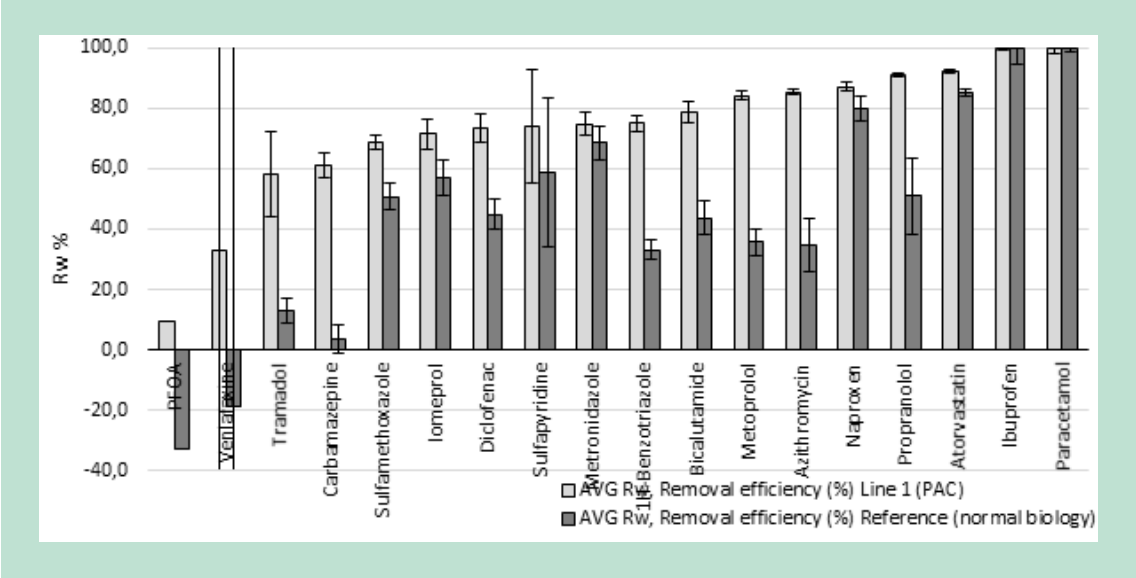

FIGURE 20. Compound specific removal efficiency (Rw %) in percentage with standard deviations for 18/33 compounds that fulfilled the rules for removal percentage calculation during the first condition of PAC addition (based on 3 samples). Results shown against reference line with no interference within the biology.

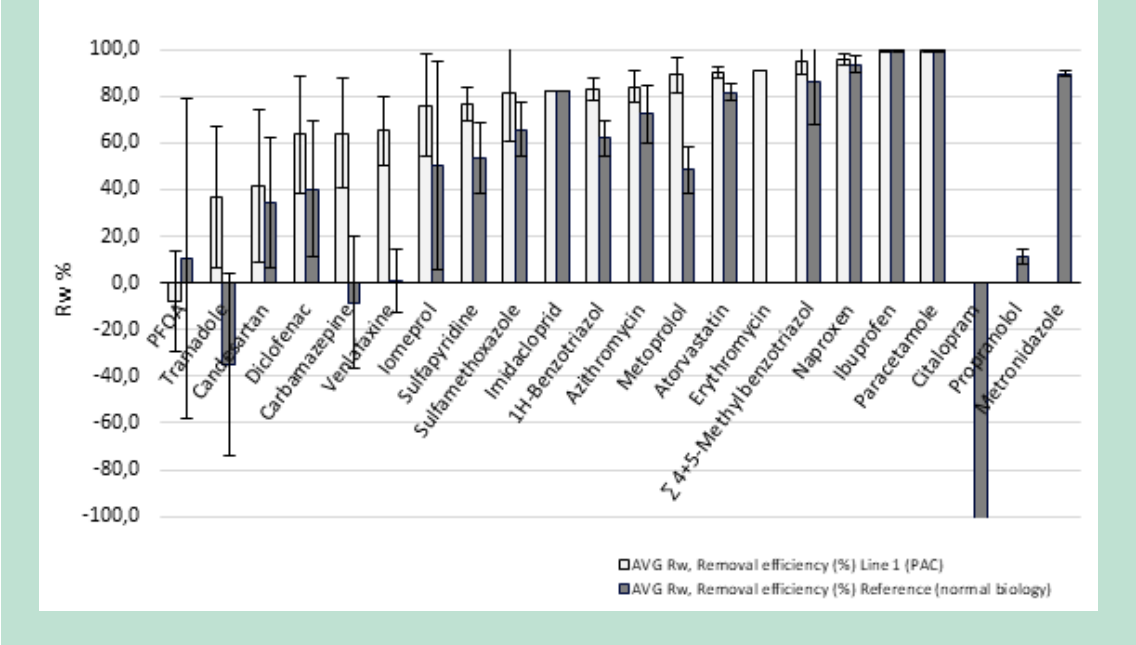

FIGURE 21. Compound specific removal efficiency (Rw %) in percentage with standard deviations for 22/33 compounds that fulfilled the rules for removal percentage calculation during the first condition of PAC addition (based on 3 samples). However, Rw for citalopram, propranolol, and metronidazole not calculable in experimental line. Results shown against reference line with no interference within the biology.

From FIGURE 20 and FIGURE 21 it is seen that PAC dosing affects the removal efficiency of most compounds including hard biodegradable compounds such as carbamazepine. For some

compounds for example metronidazole the PAC dosing had a minor effect on the removal efficiency.

PFOA and PFOS concentrations have been measured during the PAC conditions. The results showed very low (< 10 ng/L) concentration. therefore, it's not possible to conclude a significant effect of PAC on it.
Moreover, it is well known that PFOA and PFOAS are difficult to adsorb because they have an hydrophilic chemical group that reduce their adsorption onto PAC. These 2 molecules are also quite large, so their diffusion into PAC pores is more difficult than for other micropollutants.

During the first PAC condition the concentration of two compounds (diclofenac and venlafaxine) were not reduced below PNEC in the experimental line. During the second PAC condition 4 compounds (Atorvastatin, Azithromycin, Candesartan and Diclofenac) were above PNEC in the outlet from the experimental line. These compounds and others were above PNEC in the inlet and the outlet from the reference line. Comparing of measured concentrations and PNEC are presented in FIGURE 22.

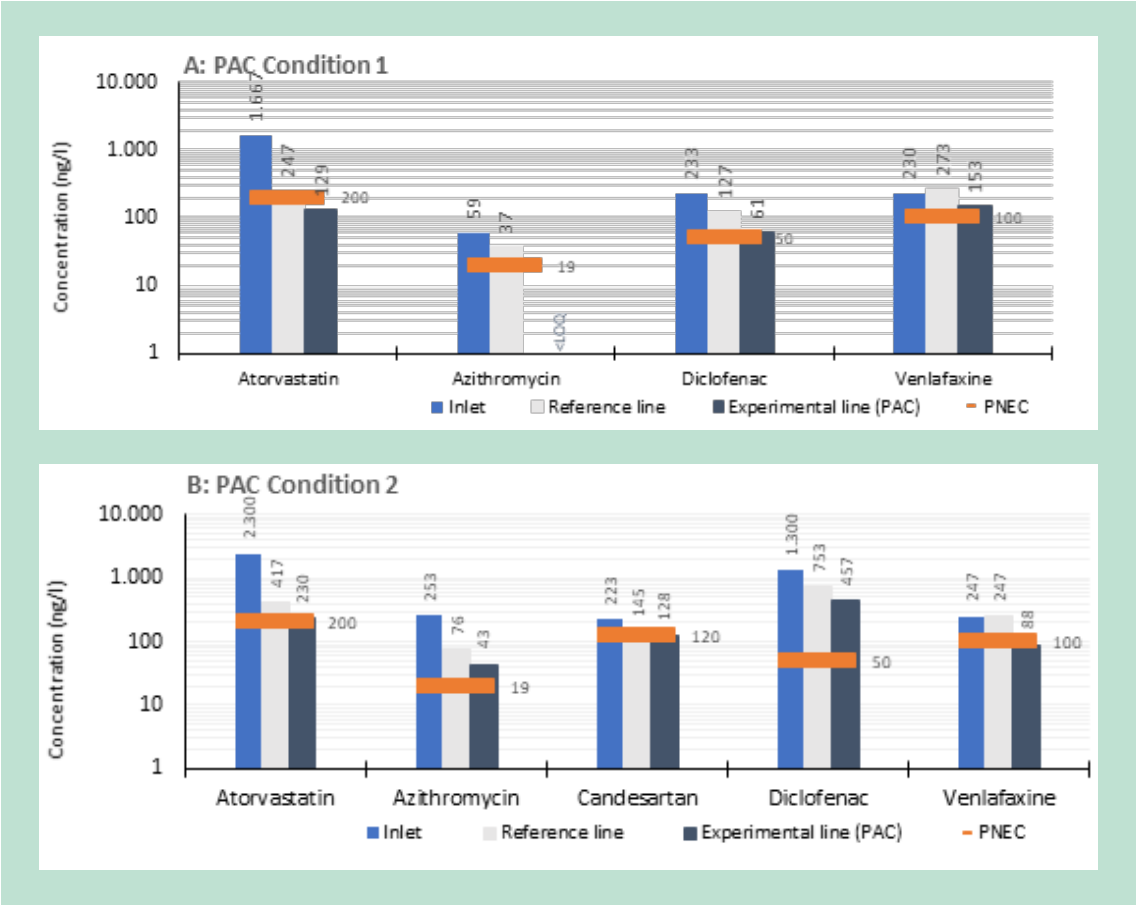

FIGURE 22. PNEC exceedance during the two conditions of Powder Activated Carbon (PAC) addition directly into the process tank of the experimental line (based on 3 samples in both conditions). Condition 1 = 5 mgPAC/l, Condition 2 = 10 mgPAC/l. Note logarithmic scale.

In summary one point PAC additions of 5 mgPAC/l and 10 mgPAC/l is not enough to reduce the concentration of all measured substances to levels below PNEC and mixed liquor PAC dosing can thereby not stand alone based on these findings.

For further information see Appendix 1.

5.3 Cost of PAC dosing

Different economical parameters for PAC dosing are presented in TABLE 11. CAPEX is a measure for the capital expense in relation to buy, maintain and improve the plant. OPEX is a measure of the operational expenses. TOC is a measure of total cost of ownership. The low PAC dosage used for the calculation is the PAC dosage used in the second PAC campaign. The high PAC dosage used in the calculation is higher the tested in this project. The following assumptions are done for the economical calculations.

- WWTP capacity – 100,000 PE.
- Wastewater flow – 8,300,000 m³/year
- For CAPEX calculation 15 years lifetime for equipment and 30 years lifetime for building structures is assumed.
- OPEX: chemicals and energy, excluding manpower and sludge disposal.

TABLE 9. CAPEX, OPEX and TCO for PAC dosing at a 100,000 PE WWTP.

PAC dose [mg/l Water]	CAPEX [mDKK/year]	OPEX [mDKK/year]	TCO [mDKK/year]	TCO [kr/m³]	TCO [kr/PE/year]
-	0.6	-	-	-	-
10	-	1.7	2.3	0.27	23
15	-	2.5	3.1	0.37	31

5.4 Conclusion

The average removal of pharmaceuticals during the first condition of PAC (5 mgPAC/l) was 73% and the conventional biology within the reference line removed 45%. Removal efficiency during the second condition of PAC (10 mg PAC/l) was 74%, and the reference line removed 54% in average by biology.

During both PAC campaigns the outlet concentration of some pharmaceuticals were above PNEC, and complementary treatment is thereby necessary to remove all pharmaceutical to a safe level.

6. Comparison of Mixed Liquor PAC dosing vs, Mixed liquor ozonation

6.1 Removal of pharmaceuticals

The effected of dosing powdered activated carbon (PAC) in the process tank vs. the effect of mixed liquor ozonation is shown in TABLE 12.

TABLE 10. Average removal efficiency of pharmaceuticals in the PAC conditions vs. Mixed liquor ozonation conditions.

	5 mgPAC/l	10 mgPAC/l	3.98 mg O3/l	7.2 mg O3/l
Removal efficiency reference line [%]	45	54	63	65
Removal efficiency experimental line [%]	73	74	78	79

The result in TABLE 12 shows, removal by mixed liquor ozonation (78-79%) is slightly better than removal by adding PAC to mixed liquor (73-74%).

The effects of the season, weather and temperature conditions, biological treatment is not negatable in micropollutant removal. By looking at general performance of the plant, the results show less removal efficiency by biology in cold season, when PAC conditions experimented. The removal efficiency in reference line during PAC conditions is 45-54% while during ozonation conditions in spring and summer time is higher in range of 63-65%, which proof the impacts of other parameters on the results.

All in all, it can be concluded that PAC addition to process tank shows comparable results to mixed liquor ozonation in term of selected micropollutant average removal efficiency.

6.2 Total cost of ownership

The comparison of CAPEX, OPEX and total cost of ownership for dosing powdered activated carbon (PAC) in the process tank vs. the effect of mixed liquor ozonation are shown in TABLE 8.
In all figures, it is assumed:

- WWTP capacity – 100,000 PE.
- Wastewater flow – 8,300,000 m³/year
- For CAPEX calculation 15 years lifetime for equipment and 30 years lifetime for building structures
- OPEX: chemicals and energy, excluding man power and sludge disposal.

TABLE 11. Total Cost of Ownership in PAC conditions vs. Mixed liquor ozonation conditions.

Ozone dose [mg/l Water]	CAPEX [mDKK/year]	OPEX [mDKK/year]	TCO [mDKK/year]	TCO [kr/m³]	TCO [kr/PE/year]
5 mg O3/l	0.9	1.6	2.6	0.32	26
10 mg O3/l	0.9	3.1	4.2	0.51	42
5 mg PAC/l	0.6	0.8	1.4	0.17	14
10 mg PAC/l	0.6	1.7	2.3	0.27	23

The estimated figures in TABLE 13 shows the total cost of ownership in PAC addition to wastewater is considerably less than mixed liquor ozonation.

6.3 Other reflections

Compare to PAC addition, multiple point ozonation proved other positive reflections:

- Micropollutant removal from sludge
- SVI and DSVI improvement
- Disinfection effect

6.3.1 Disinfection effect

In order to evaluate the disinfection effect of multiple point ozonation and PAC addition on disinfection effect, the Ecoli has been measured during both ozonation and PAC conditions in inlet of the plant, outlet of reference line, after mixed liquor ozonation and after multiple point ozonation. The results are presented in Table 7 and Table 8.

TABLE 12 E.coli concentrations (E.coli/100ml) During ozonation sampling conditions. Note: On October 17th (2nd ozonation condition) a contamination of samples had occurred, thus, the day is taken out.

E. Coli/100 mL	1st ozonation condition			2nd ozonation condition	
Date	02.07.2019	08.07.2019	10.07.2019	23.10.2019	30.10.2019
Inlet	>82000000	14000000	22000000	>82000000	>82000000
Reference line	4100	15000	9100	1600000	6500000
Mixed liquor ozonation	3600	1200	200000	1700	16000
multiple point ozonation	<38	<38	<38	78	<38

TABLE 13 E.coli concentrations (E.coli/100ml) During PAC sampling conditions.

E. Coli/100 mL	PAC condition			2nd PAC condition	
Date	24.03.2020	25.03.2020	30.04.2020	05.05.2018	06.05.2015
Inlet	>820000	>82000000	>820000	>820000	>820000
Reference line	2500000	29000	260	2900	2000
PAC addition	25000	20000	720	1600	1600

By looking at the inlet concentrations of E. coli they corresponded well with the inlet levels measured at all times at Brædstrup WWTP. The reference line seems to have higher levels during autumn/wintertime. By comparing the concentrations from October (2019) and March (2020) with the levels of July (2019) and May (2020) it is clear, that the reference line removes less E. coli during the cold/wet times of the year. This could be due to shorter retention time within the plant. Moreover, PAC does reduce the amount of E. coli, however, never to same level as ozonation did. By doubling the dose of activated carbon, no extra percentage removal regarding E. coli has been seen. This corresponds well with the findings of pharmaceutical removal (from 73% removal with a PAC dose of 5 mg/l to 74% average removal by adding 10 mg/l instead).

The results show that multiple point ozonation is quiet effective in reducing E. coli to less than limit of quantification, which proves the disinfection effect of multiple point ozonation.

7. Perspective

7.1 Obtained results and further experiments

The mixed liquor ozonation showed increased removal of micropollutants up to 15% and other positive effects for example micropollutant removal from solid phase of sludge and lower SVI. Depends on micropollutant removal expectation, it is of interest to investigate the optimum mixed liquor ozone dosage to achieve the most positive effects in one side and reduce the operation costs in another side.

Since ozonation in mixed liquor applies considerable amount of pure oxygen in aeration tank (10-20% contribution), it is of interest to evaluate the contribution of mixed liquor ozonation in aeration system capacity. Theoretically, it's expected that by 5 mg/l ozone injection, 45 mg/l oxygen will be applied to aeration tank (90% oxygen vs. 10% Ozone).

In Brædstrup WWTP, after secondary clarifier, sludge is thickened and dewatered by centrifuge and finally is used for agriculture. It could be interesting to investigate the effect of mixed liquor ozonation in stabilization, reduction of total mass of solids, pathogens removal and dewaterability of sludge in a wastewater treatment plant, where sludge is stabilized in digesters before dewatering.

Moreover, in this study, the effect of mixed liquor ozonation on sludge dewaterability has been tested in Aalborg University in laboratory scale by measuring CST values. Although the results didn't show meaningful effect on CST values, but due to different aeration systems in line 1 and line 2 and other parameters interfered the results, it is of interest to investigate the effect of mixed liquor ozonation on sludge dewatering system at full scale before and after ozonation.

In SUEZ, Ozonation integrated to different biological processes like Conventional Activated Sludge (CAS), Biofilters, MBBR and Membrane Bioreactors have been studied in several pilot studies. In Brædstrup WWTP, this has been tested in full scale in CAS process. It would be interesting to test ozonation integrated to other biological processes at full scale as well.

In this study, mixed liquor ozone injected trough a hydro venturi ejector. It is of interest to investigate the optimum ozone gas and sludge flow ratio on micropollutant removal from sludge.

One concern using ozone for pharmaceutical removal is the possible production of intermediate products which might have a higher ecotoxicity than the parent compound. In Multiple point ozonation solution the Ozone/DOC is very low (0.2-0.3) and it is expected not to produce any toxicity due to ozonation. The ecotoxicological tests in this study proved, and even showed lower effects from the ozonated water compared to wastewater treated in the reference line. This can be proved by real time toxicity biomonitoring system like Toxmate at full scale in future.

During the PAC campaigns PFOA was measured. PFOA is a component of the PFAS (polyfluoroalkyl substances) along with PFOS and others. These compounds are carcinogenic, harmful to reproduction, acute toxic, persistent and possible bioaccumulating. During the PAC campaigns low removal of PFOA was observed. Therefore, further investigation of the ability of activated carbon and ozonation to remove PFOA and other PFAS is of interest.

7.2 Economical

The focus on micropollutants is increasing in Denmark and in Europe in general. In Denmark so far, the focus has been on pollution source point of hospital wastewater. In Europe the focus is towards centralised/domestic WWTPs. Some examples are listed below.

- In Switzerland larger WWTP or WWTP discharging into lakes with drinking water interest or recipients with low dilution should be able to remove 80% of selected micropollutants. So far several plants have been upgraded to treat micropollutant from domestic wastewater.
- In Sweden a total of SEK 85 million (2019) was allocated from the Government to support investment in domestic sewage treatment plants to reduce emissions of pharma residues and other environmental pollutants to the sea, lakes and water sources. The first reference for micropollutant treatment is in Lindkøbing with the capacity of 250 000 p.e. using tertiary ozonation system.
- In Luxemburg all WWTO have to plan to add future micropollutant treatment.
- In Germany micropollutants removal is required in two "länder" (Baden-Württemberg and Nordrhein-Westfalen).
- In Denmark:
  - at the new "super" hospital in Aalborg (NAU), SUEZ designs and build a local WWTP for treatment of hospital wastewater. This MUDP project have been one of the main references SUEZ have used to win the tender.
  - in Hjørring municipality in Denmark, it is investigated if the centralised WWTP should be upgraded for micropollutants removal or if hospital wastewater should be treated.
  - Herning vand ensures that the final treatment solution is in operation by the end of 2023. Herning vand is implemented 2 MUDP projects to develop a biological treatment solution, most recently with Mereff pilot project.
  - SK Forsyning is implementing a MUDP project and focuses on ozone and advanced oxidation processes in combination with activated carbon at large scale.
  - Hillerød WWTP joined CWpharma 2 international search project to investigate the performance of tertiary ozonation and GAC filter in micropollutant removal from wastewater.

The focus on micropollutants in these countries and legalisation in some countries (for example Switzerland and some German "länder") and legalisation in other countries will probably follow, will enhance the market for micropollutant removal.

Since the technology tested in this project is based on existing core technologies, used in an innovative way (multipoint ozonation and dosing of PAC in the process tank instead of purely tertiary treatment), the technology is well-known to the customers.

Multiple point ozonation, as well as mixed liquor ozonation and PAC addition are patented solutions in SUEZ.

The expected market values for micropollutant removal in different regions and countries are listed below.

- The Scandinavian market value is expected to be 100 M€ during the next 5-7 for hospital WWTPs and 150 M€ for WWTP during the next 5-10 years.
- The Switzerland market value is expected to be 1.2 billion during the next 20 years for upgrading of WWTP to meet the requirements described earlier. SUEZ target is 15-30 M€/year.
- The French market for construction/retrofits of WWTP to perform micropollutant removal is 30 M€/year.

The total estimated European market value in the above selected markets is:

- Hospitals: Project value for micropollutant solutions 150 M€ over 10 years
- Main WWTP: Project value 600 M€ over 10 years.

The estimated direct market for technology and knowhow supplies for micropollutant removal is thus substantial over the next 5-10 years. If a 15% market share is assumed, it accumulates to more than 100 M€ for selected European countries only.

8. Media awareness and publications during the project

8.1 Articles in technical magazines

During the project several articles have been published in technical magazines and national media. These are presented in TABLE 14.

TABLE 14. Articles published in technical magazines and national media

Magazine/Media	Dato	Article
Spildevand	#5 2017	Miljøfremmede stoffer - Det miljøteknologisk udviklings- og demonstrations program (MUDP) har bevilliget støtte til et nyt test- og demonstrationsprojekt til fjernelse af miljøfremmede stoffer ved flerpunktsdosering af ozon på et centralt renseanlæg. Projektet gennemføres af et samarbejde mellem Suez Water (projektleder), Samn Forsyning, KD-Maskinfabrik, universiteterne i Aalborg og Aarhus samt COWI.
	#1 2019	MUDP-projekt på Brædstrup Renseanlæg – Ny metode til fjernelse af mikroforureninger om medicinrester og kemikalier på eksisterende renseanlæg bliver i denne tid afprøver på renseanlægget i Brædstrup.
	#5 2019	Demonstration af flerpunkts ozonering – fjernelse af mikroforurenede stoffer fra kommunalt spildevand i fuldskala.
DanskVand	#4 2018	Fremtidens renseteknologi med ozon testes på Brædstrup Renseanlæg
	#2 2020	Effektiv fjernelse af mikroforureninger på Brændstrup Renseanlæg
	#6 2020 (not published yet)	Resultater fra Danmarks første fuldskala centrale renseanlæg til fjernelse af lægemidler fra spildevand.
Ingeniøren/Watertech	29/10-2019	Ozon fjerner medicinrester effektivt
	25/9-2020	Første salg af ozon-renseanlæg med ny teknologi i Danmark
	29/10-2020	Ozon fjerner medicinrester effektivt – rester fra medicin i spildevandet er et stigende problem, og både vandmiljø og mennesker er i risikozonen. Et lovede forsøgsprojekt i Midtjylland har fjernet 93 procent af stofferne, relativt billigt.
Energy Supply	17/10-2019	Efter succesfuld test med Ozon: Gar efter de små hospitalsanlæg
Horsens Folkeblad	25/3-2019	Verdenspremiere på ny teknologi: Renseanlæg ska løse stigende dansk problem.
	14/10-2019	Forsøg med ozon er en kæmpe succes: Ny teknik renser spildevand for medicinrester.
	25/10-2019	Banebrydende teknologi er testet i Brædstrup: Ny rensetekniк mod giftstoffer i spildevand er klar til brug
Danmarks Radio	4/3-2020	Vi skider og tisser medicin ud i kloakken

During the project several webinars have been hosted. Webinars are given in TABLE 15.

TABLE 15. Webinars hosted during the project.

Webinar	date
DANVA Webinar: Rensning for miljøfremmede stoffer – fokus på medicinrester.	18/5-2020
SUEZ Water webinar om mikroforurenende stoffer	19/8-2020
SUEZ Water webinar om mikroforurenende stoffer	20/8-2020

During the project results and other information have been shared by theme days, advisory committee meeting and a by a closing event. These are presented in TABLE 16

TABLE 16. Theme days and steering group meetings

Theme day	date
Hvorfor mikroforureninger/medicinrester skal fjernes kommunalt – presentation at Brændstrup WWTP.	9/10-2019
Steering group meeting	15/6-2020
Closing event	24/9-2002

Other activities which promotes the project and the overall topic are presented in TABLE 17

TABLE 17. Other activities to promote the project and overall topic.

Activity	date
Production of website for sharing results (mpozone.com)	August 2019
Contribution to formation of “focus group” which gathers information about micropollutants in wastewaters, discharge to recipient, degradation in WWTP.	
IWA presentations • Micro-pollutant Removal By Multiple Point Ozone Injection In Full-scale Municipal Wastewater Treatment Plant (platform presentation) • Full-scale Technical And Financial Comparison Of Multiple Point Ozonation Vs. Activated Carbon Adsorption (poster pitch presentation)	9-14 May 2021

The Social media have been used to promote the project, see TABLE 18

TABLE 18. Social media activities which have promoted the project.

Activity	date
Del af en artikel fra Spildevand #5 2019	10/12-2019
Del af en artikel fra DanskVand #2 2020	30/04-2020
DANVA webinar invitation til d. 18.05	12/05-2020
Del af Horsens Blad artikel fra 25/10-2020	29/09-2020
Del af Jeppe Lund Nielsen MUDP præsentation fra afslutningsevent (video)	29/10-2020
Del af Jeromine Albertini MUDP præsentation fra afslutningsevent (video)	06/11-2020
Del af Nana Jensen MUDP præsentation fra afslutningsevent (video 1) (Ikke udgivet endnu)	12/11-2020
Del af Nana Jensen MUDP præsentation fra afslutningsevent (video 2) (Ikke udgivet endnu)	20/11-2020
Del af Mads Koustrup Jørgensen MUDP præsentation fra afslutningsevent (video) (Ikke udgivet endnu)	26/11-2020
Del af Dorthe Lüneborg Baggesen MUDP præsentation fra afslutningsevent (video) (Ikke udgivet endnu)	03/12-2020
Del af Thomas Møller MUDP præsentation fra afslutningsevent (video) (Ikke udgivet endnu)	09/12-2020
Del af Anne Mette Granhøj Hansen MUDP præsentation fra afslutningsevent (video) (Ikke udgivet endnu)	17/12-2020
Del af Per Krøyer Kristensen MUDP præsentation fra afslutningsevent (video) (Ikke udgivet endnu)	18/12-2020

Youtube have been used to share presentation of obtained results and information about the overall topic, see TABLE 19.

TABLE 19. Youtube videos used to share results and information about the project.

Activity	Date
Hvad er mikroforurenende stoffer, og hvor er det et problem – Jeppe Lund Nielsen	22/10-2020
Erfaring fra udlandet (på engelsk) – Jeromine Albertini	22/10-2020
Præsentation af resultaterne 1/2 - Nana Jensen	22/10-2020
Præsentation af resultaterne 2/2 – Mads Koustrup Jørgensen	22/10-2020
Fjernelse af mikroforurenende stoffer ved multipoint ozoninjektion og PAC - hvad er det? – Nana Jensen	22/10-2020
SAMN Forsyning erfaring med drift - Dorthe Lüneborg Baggesen	22/10-2020
Kilder til lægemidler i spildevandet, resultater og sammenligning af råvands data – Thomas Møller	22/10-2020
NOVANA-data - miljøfarlige forurenende stoffer målt på renseanlæg - Anne Mette Granhøj Hansen	22/10-2020
Økonomien ved implementering af fjernelse af mikroforurenende stoffer i Danmark – Per Krøyer Kristensen	22/10-2020
Velkommen og åbningstale - Michael Lind Frandsen	23/10-2020

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Removal of micropollutants by application of multiple point ozonation and powder activated carbon

The focus on micropollutants in treated wastewater have increased in the recent years. In Switzerland, most centralised WWTPs are using ozonation and/or activated carbon as a tertiary treatment for micropollutant removal.

In this project ozone and activated carbon are used in an innovative way. Ozonation is applied in the process tank and as a tertiary treatment called multiple point ozonation. Experiments have also been performed with powdered activated carbon (PAC) added to the process tank.

The technologies are tested at Brædstrup WWTP in Samn Forsyning. This WWTPs has two separated biological treatment lines. The two lines separate in the outlet of the sand- and grease trap. Inlet measurements are thereby the same, and the performance of the tested technologies can be directly compared to conventional activated sludge treatment.

By using ozenation an average removal of 93 % of micropollutants were obtained, and concentrations of all measured micropollutants were below PNEC (predicted no effect concentration) after ozonation. The ecotoxicity of the ozonated treated wastewater was equal or lower compared to conventional treated wastewater.

By dosing activated carbon (PAC) in the process tank, an increased removal of micro-pollutants is achieved compared to conventional treatment.

For a 100,000 PE WWTP treating 8,300,000 m³/year multipoint ozonation can be implemented and operated for 26-42 DKK/year per inhabitant depending on the applied ozone dosage.

For a 100,000 PE WWTP treating 8,300,000 m³/year PAC dosing in the process tank can be implemented and operated for 23-31 DKK/year per inhabitant depending on the applied PAC dosage.

I de seneste år har der været et øget fokus på mikroforureninger i renset spildevand. I Schweiz benytter de fleste centraliserede renseanlæg ozonering og/eller aktivt kul som en tertiær behandling til fjernelse af mikroforurening.

I dette projekt anvendes ozon og aktivt kul innovativt. Ozonering tilføres både i procestanken og som en tertiær behandling, bedre kendt som flerpunkts ozonering. Endvidere er der udført forsøg med tilsætning af pulveriseret aktivt kul (PAC) direkte til procestanken.

Teknologierne er testet på Brædstrup Renseanlæg, Samn Forsyning. Renseanlægget består af to adskilte biologiske behandlingslinjer. Der er fælles indløb for de to linjer, men i udløbet fra sand- og fedtfanget deles spildevandsstrømmen til de to proceslinjer. Indløbet for de to linjer er dermed de samme, og ydeevnen af de testede teknologier kan direkte sammenlignes med konventionel aktiv slambehandling.

Ved ozonering i procestanken blev der opnået en gennemsnitlig fjernelse af 93% af mikroforurenende stoffer, og koncentrationer af alle målte mikroforureninger var under PNEC (Predicted No Effect Concentration) efter ozonering. Økotoksiciteten af det ozon-behandlede spildevand var lig med eller lavere sammenlignet med det konventionelt behandlede spildevand.

Ved dosering af aktiv kul (i form af PAC) i procestanken opnås en øget fjernelse af mikroforurenende stoffer sammenlignet med konventionel behandling.

For et 100.000 PE renseanlæg, der behandler 8.300.000 m³/år, kan fler-punkts ozonering implementeres og drives for 26-42 DKK/år pr. indbygger afhængigt af den anvendte ozondosis.

For et 100.000 PE renseanlæg, der behandler 8.300.000 m³/år, kan PAC-dosering i procestanken implementeres og betjenes for 23-31 DKK/år pr. indbygger afhængigt af den anvendte PAC-dosis.

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Ministry of Environment and Food of Denmark
Environmental Protection Agency

Removal of micropollutants by application of multiple point ozonation and powder activated carbon

Appendix 1 – Effect of ozonation and PAC conditions on micropollutant removal

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Contents

1 Context/preface

The initial purpose of the project was to remove organic micropollutants (especially pharmaceuticals), which can have effluent concentrations above their Predicted No Effect Concentration (PNEC), by applying ozone multiple places and/or powdered activated carbon (PAC). Furthermore, to demonstrate that it can be implemented to an already existing wastewater treatment plant without economically expensive technology investments and vast expansions. Previous studies have been finalized in laboratory and on pilot scale in THRIUMPH R&D program in France. Hence, this project is the first multiple point ozonation demonstrated in full scale on an already existing WWTP. Following successful demonstration of pharmaceutical removal, it is expected that the technology not only can be implemented on most municipal Danish WWTPs, but correspondingly at point sources as well, e.g. hospitals. Ozone is known to be a strong oxidant, due to its high redox potential 2,07 V. Hence, it reacts fast with the pharmaceuticals (Margot et al., 2015).

An onsite pre-screening of 365 compounds was conducted prior to tests. By mapping the pharmaceuticals and their concentrations in the inlet and outlet water of the plant it was possible to identify those present in the effluent with potentially toxic concentrations for the receiving environment (conc.>PNEC). In total 36 compounds - 3 corrosion inhibitors, 1 insecticide, 2 x-ray contrast media and 30 pharmaceuticals – were investigated.

Overall, this project consists of two different treatment solutions. Firstly, multiple points ozonation, 1) application of ozone to the aerated tank in activated sludge process (termed: mixed liquor ozonation) and 2) in the treated secondary effluent (termed: tertiary ozonation) before being discharged to a waterbody, and secondly PAC addition to the aerated tank.

The project is structured as follow:

Characterization phase

- Pre-screening semi quantification of 365 compounds to mapping the content of pharmaceuticals in treated wastewater. the output was the selection of 36 compounds.

Baseline test

- 3 campaign in 3 months
  - Quantification of 36 compounds in raw and secondary treated wastewater and sludge- solid and liquid phases- and to identify those present in treated water (effluent) which potentially exceeds toxic limits for the receiving environment.
  - Sludge analysis and evaluation of plant performance.

Phase 1:

- Two periods of a 110 days
  - First condition: an ozone mixed liquor (also referred to as mixed liquor) dose of 3.98 mg O3/L and a tertiary of 7.20 mg/LO3.
  - Second condition: an ozone mixed liquor (also referred to as mixed liquor) dose of 7.20 mg/LO3 and a tertiary treatment of 3.98 mg/LO3.

Phase 2

- Powered Activated Carbon (PAC) added into the aerated tanks
- Two periods of 40 days
  - First condition: 5 mg/l PAC
  - Second condition:10 mg/l PAC

2 Introduction

A wide range of chemicals used daily in homes, workplaces or in the urban environment ends up in sewers due to “down the drain” products, such as detergents and their additives, or personal care products, but also pharmaceuticals and their metabolites that are in urine and faeces, and several household chemicals such as food or plastic additives, or flame retardants contained in textiles ends up at the wastewater treatment plants.

The consumption of chemical products has led to increasing chemical pollution of surface and groundwaters, with still largely unknown effects on human health and aquatic life. Once in the environment, these compounds are referred to as “micropollutants”. Micropollutants are defined as chemical compounds present at low concentrations (e.g. nano- to micrograms per litre) in the environment, and which, despite their low concentrations, can generate adverse effects for living organisms (Margot et al., 2015). This include hydrophobic compounds such as, heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), etc., but also the hydrophilic (polar) compounds designed to be biologically active such as pesticides and pharmaceuticals. Pharmaceuticals, personal care products or biocides, are mainly discharged into municipal sewer systems and, because of only partial removal in conventional wastewater treatment plants (WWTPs) (Verlicchi et al., 2012), their residuals reach the aquatic ecosystems. Thus, municipal WWTP effluent is considered as the main vector of these compounds into the environment.

In recent years, an increasing focus on the presence of pharmaceuticals in the surrounding environment originating from wastewater discharge have become a topic in Denmark and other European countries. At present, Switzerland is the only country regulating pharmaceuticals and micropollutants in their wastewater. Their goal is to remove up to 50% of micropollutants by upgrading 1/7 of all wastewater plants using ozonation and activated carbon. In Denmark, particularly, the wastewater companies connected to larger hospitals is concerned about the risk of discharging residual pharmaceuticals to the environment. Hospitals have been/is considered as “hot-spots” for pharmaceutical release due to the high load of pharmaceuticals used and emitted through hospital wastewater into municipal sewers. However, the fraction of the total pharmaceuticals distributed from hospitals compared to private homes (the community) is relatively low (around 4%) (Sundhedsdatastyrelsen). However, certain pharmaceuticals (X-ray contrast media, cytostatics and some antibiotics) are used in much higher quantities at hospitals than in private homes.

Since, conventional WWTPs have been designed to remove bulk contaminations such as solids, organic matter, nitrogen and phosphorus nutrients. Even though, partly elimination of some of the simplest pharmaceuticals (e.g. paracetamol) can be observed in conventional WWTPs. Some of the main mechanisms are adsorption into the primary sludge, mixed liquor or the biodegradation. Nevertheless, a large quantity of refractory micropollutants are not at all or only partially degraded and remain in the secondary treated wastewater. Complementary treatments are necessary to reduce the micropollutant concentrations and reach values compatibles with a good state of the environment.

3 Micropollutants removal

The fate of micropollutants during plant operation is controlled by their physical and chemical properties (hydrophobicity, biodegradability, volatility) and the type of treatment. Some micropollutants entering the WWTP are removed partially or completely during the primary and secondary treatments.

Removal efficiency may be enhanced by optimizing conventional treatments through increasing levels of solids and nutrients removals (Ternes, Joss and Siegrist 2004). Even so, incomplete elimination from water is still observed for a wide range of substances that are less biodegradable, poorly absorbable, hydrophobic or for those that enter the WWTP in high concentrations. Consequently, implementing specific additional treatments is needed to reduce effectively the micropollutant discharge by WWTPs. Hence, it is necessary to understand what occurs in traditional WWTPs to minimize the release of micropollutants to the aquatic environment.

3.1 Micropollutant compounds

Back in 2000 the Water Framework Directive (WFD) 2000/60/EC was adopted. The goal of the WFD is to ensure the full integration of the economic and ecological objectives in water quality and quantity management. The overall key objective is to get good status for the over than 111 000 surface waters (coastal waters, rivers, lakes and so on) and the over 13 000 groundwaters in EU territory. But what does “good status” mean? For groundwaters it means securing good quantitative and chemical status and for surface waters it is achieving good ecological and chemical status. Here we will focus on surface water.

Surface water:
Good ecological status is defined in Annex V of the WFD, in terms of the quality of the biological community, the hydrological characteristics and the chemical characteristics. As no absolute standards for biological quality can be set which apply across the community, because of ecological variability, the controls are specified as allowing only a slight departure from the biological community which would be expected in conditions of minimal anthropogenic impact. Good chemical status is defined in terms of compliance with all the quality standards established for chemical substances at European level. The Directive also provides a mechanism for renewing these standards and establishing new ones by means of a prioritization mechanism for hazardous chemicals. This will ensure at least a minimum chemical quality, particularly in relation to very toxic substances, everywhere in the Union.

Generally, WFD is an EU directive which commits European union member states to meet environmental objectives. The European Commission’s science and knowledge service, the Joint Research Centre (JRC), have made a Watch List (WL). The surface Watch List under the WFD is a mechanism for obtaining high-quality Union-wide monitoring data on potential water pollutants for the purpose of determining the risk they pose and thus whether Environmental Quality Standards (EQS) should be set for them at EU level (**Error! Reference source not found.**).

A Danish guideline was in 2013 proposed to regulate hospital wastewater to sewer, as point sources and therefore, discharge of pharmaceuticals will have to be regulated as industries (*Local Government Denmark, 2013*). The list proposed guiding limit values for a list of pharmaceuticals (some was updated in *AMK 2015*). They were selected based upon their usage i.e. predicted effluent concentration from hospitals, the stability score, and protentional hazardous impact, i.e., predicted environmental no effect concentrations (PNEC).

3.2 Selection of compounds

In this study a combination of compounds from various different lists - AMK-list, Swiss-list and MERMISS-list - have been chosen. These can be seen in ***Error! Reference source not found.***. Switzerland have been the first country to implement legislation on pharmaceutical removal and the Swiss-list is their guideline/legislation list. The Swiss approach is to base the action on micropollutants around a set of indicator compounds. These represent all the compounds Switzerland would like to remove – compounds of concern in terms of ecotoxicity in the receiving waters.

Table 1 List of investigated compounds in Brædstrup.

	ATC Family	Compound	ATC code	PNEC (ng/l)	PNEC Reference*
Alimentary tract and metabolism	Steroid medicament	Prednisolone	A07EA01	2.000	AMK 2013
Cardiovascular system	Lipid modifying agents	Atorvastatin	C10AA05	200	AMK 2013
	Cardiac therapy	Ibuprofen	C01EB16	4.000	AMK 2015
	Agents acting on the renin-angiotensin system	Candesartan	C09CA06	120	AMK 2015
	Agents acting on the renin-angiotensin system	Losartan	C09CA01	1.000.000	FASS
	Beta-blocking agent	Metoprolol	C07AB02	7.300	FASS
	Beta-blocking agent	Propranolol	C07AA05	100	AMK 2015
Antiinfectives for systemic use	*Antibiotics*	Azithromycin	J01FA10	19	Recommendations for 2nd watch list, EU
		Cefalexin	J01DB01	-	-
		Ciprofloxacin	J01MA02	89	AMK 2015
		Clarithromycin	J01FA09	120	Recommendations for 2nd watch list, EU
		Erythromycin	J01FA01	200	Recommendations for 2nd watch list, EU
		Ofloxacin	J01MA01	100	AMK 2015
		Sulfamethoxazole	J01EC01	120	AMK 2015
		Sulfapyridine	J01EB04	-	-
		Trimethoprim	J01EA01	-	-
Antineoplastic and immunomodulating agents	Antiandrogens	Bicalutamide	L02BB03	100	AMK 2015
	Antimetabolite	Capecitabine	L01BC06	200	AMK 2015
	Anti-estrogens	Fulvestrant	L02BA03		-
	Immunosuppresant	Mycophenolic acid	L04AA06	100	AMK 2015
Musculo-skeletal system	Analgesic	Diclofenac	M01AB05	50	Recommendations for 2nd watch list, EU
	Analgesic	Naproxen	M01AE02	6.400	AMK 2015
Nervous system	Antiepileptic	Carbamazepine	N03AF01	500	AMK 2015
	Antiepileptic	Gabapentin	N03AX12	-	-
	Analgesic	Tramadol	N02AX02	2.300	AMK 2013
	Analgesic	Paracetamol	N02BE01	9.200	AMK 2015
	Antidepressant	Sertraline	N06AB06	0,52	AMK 2015
	Antidepressant	Venlafaxine	N06AX16	100	AMK 2015
	Antidepressant	Citalopram	N06AB04	8.000	AMK 2015
	Psycholeptic	Zopiclone	N05CF01	-	-
Antiparasitic products, insecticides and repellents	Antiprotozoals	Metronidazole	P01AB01	12.500	FASS
Antiparasitic products, insecticides and repellents	Neonicotinoid insecticide	Imidacloprid	QP53AX17	8,3	Recommendations for 2nd watch list, EU
Contrast media (x-ray)	Contrast media (X-ray)	Iomeprol	V08AB10	1.000.000	FASS

	Contrast media (X-ray)	Iohexol	V08AB02	1.000.000	FASS
Corrosion inhibitors		(methyl) 1H-Benzotriazole		-	-
		∑ 4+5-Methylbenzotriazole		-	-
		Dimethylbenzotriazole		-	-
Perfluorinated Substances		PFOA PFOS			

** Local Government Denmark (KL): Proposal for administrative basis for pharmaceuticals in hospital wastewater, recommended maximum concentration for connection to sewer, Input for KL working group concerning hospital wastewater. Prepared by DHI, June 2013, (in Danish); AMK (2015). BIOFOS' Arbejdsgruppe omkring hospitalsspildevand Opdaterede Anbefalede Maksimale Koncentrationer (AMK) for lægemiddelstoffer. 2015; the Swedish Farmaceutiska Specialiteter - www.fass.se; European Union updated watch list (Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List, EUR 29173).*

3.3 Removal capabilities by conventional wastewater treatments

The classical configuration used for wastewater treatment consists of multiple steps, (i) pre-treatments, (ii) primary treatment, (iii) secondary treatment, and (iv) tertiary treatments.

Pre-treatments removes the coarse wastes, sand and fat and grease, respectively by bar screen, decantation channel and in tanks where skimmers collect the floating fat.

Primary treatment is composed of a primary clarifier (or sedimentation tank) that removes the suspended solids that enter the WWTPs through sedimentation. Micropollutants are removed mainly by sorption onto primary sludge, as distribution of a compound into organic (lipophilic) layer is predominant way of sorption (Ternes et al., 2004). For pharmaceuticals and hormones, removal efficiency in primary treatment ranged up to only 28 % (diclofenac and estriol). No considerable reduction was reported for ibuprofen, naproxen, sulfamethoxazole and estrone in primary treatment (Carballa et al., 2004).

During secondary treatment, micropollutants are biologically degraded to various degrees, resulting in mineralization or incomplete degradation (transformation products). The micropollutants are subjected to a range of mechanisms, including dispersion, dilution, partition, biodegradation, and abiotic transformation. Biodegradation/biotransformation and sorption are the two major removal mechanisms during biological treatment, while volatilization occurs to a minor degree (Verlicchi et al., 2012).

Lastly, tertiary treatment, removes nutrients such as ammonium (by biological nitrification), nitrate (by biological denitrification) and phosphate (generally by chemical precipitation) or in some cases to eliminate residual suspended solids (by sand filtration) or pathogens (by disinfection).

General speaking, the micropollutants with high biodegradable and adsorption constant could be well retained after a primary and secondary biological treatment. Other parameters related to the biologicals which can influence the removal of micropollutants from wastewater are:

The process parameters: hydraulic retention time (HRT), the sludge retention time (SRT) but also the sludge concentration

The particular retention level to remove the micropollutants attached to the particulate matter: conventional vs physiochemical treatments.

The organic sanitation level: carbonaceous vs nitrification treatments

The influence of HRT on the removal of pharmaceuticals was reported for compounds with low sludge affinity (low K_d) and medium good to poor biodegradability (half-lives in the range of the HRT). Here, a higher removal efficiency was observed at higher HRT, because when the HRT increases the biological diversity also increases which increases the number of micropollutant biodegradation pathway. Substances that are highly biodegradable (half-lives much shorter than HRT, and thus always removed) or hardly degradable (half-lives much longer than HRT), as well as compounds mainly removed by sorption, are unlikely to be influenced by HRT (Verlicchi et al., 2012). Temperature do also play an important role in the efficiency of the WWTPs. Temperature stimulates microbial activity and thus higher removal of those pharmaceuticals that are biodegradable. Therefore, higher removal efficiencies are often observed during summertime compared to winter. The pH of the wastewater can also affect the removal efficiency of some ionisable substances if the charge in pH can affect their charges (switch to another form: cationic, neutral, anionic or zwitterionic), which mainly concern substances with a pKa value close to the pH of the wastewater (e.g. quinolone antibiotics) (Verlicchi et al., 2012).

The main mechanisms involved in degradation of micropollutants in WWTPs are (i) sorption, (ii) biological transformation, (iii) volatilization or (iv) abiotic degradation. Biodegradation/biotransformation and sorption are the two major removal mechanisms during biological treatment, while volatilization occurs to a minor degree (Verlicchi et al., 2012).

Sorption:
For hydrophobic or positively charged micropollutants sorption play a major role in removal, especially if they are poorly biodegradable. Adsorption mainly occurs by two processes:

Hydrophobic interactions, in which the absorption occur between the aliphatic and aromatic groups of a compound and the lipophilic cell membrane of microorganisms as well as the fat fractions of sludge, and

Electrostatic interactions between positively charged groups of the micropollutant and the negatively charged surfaces of microorganisms and sludge (e.g. amino groups) (Ternes et al., 2004)

Adsorption is therefore a complex process dependent on the physio-chemical properties of the substance (charge, hydrophobicity) and the properties of the sludge (i.e. surface charge, specific surface area and/or mineral content). The compounds that tend to be sorbed onto solids are expected to be better eliminated by activated sludge treatment than other low-cost secondary treatments (trickling filter beds, anaerobic lagoon and constructed wetlands) (Camacho-Muñoz et al., 2012). This can be due to the promoted biodegradation under forced aeration during the conventional treatments, together with the enhanced sorption by large amounts of sludge generated in conventional treatment systems.

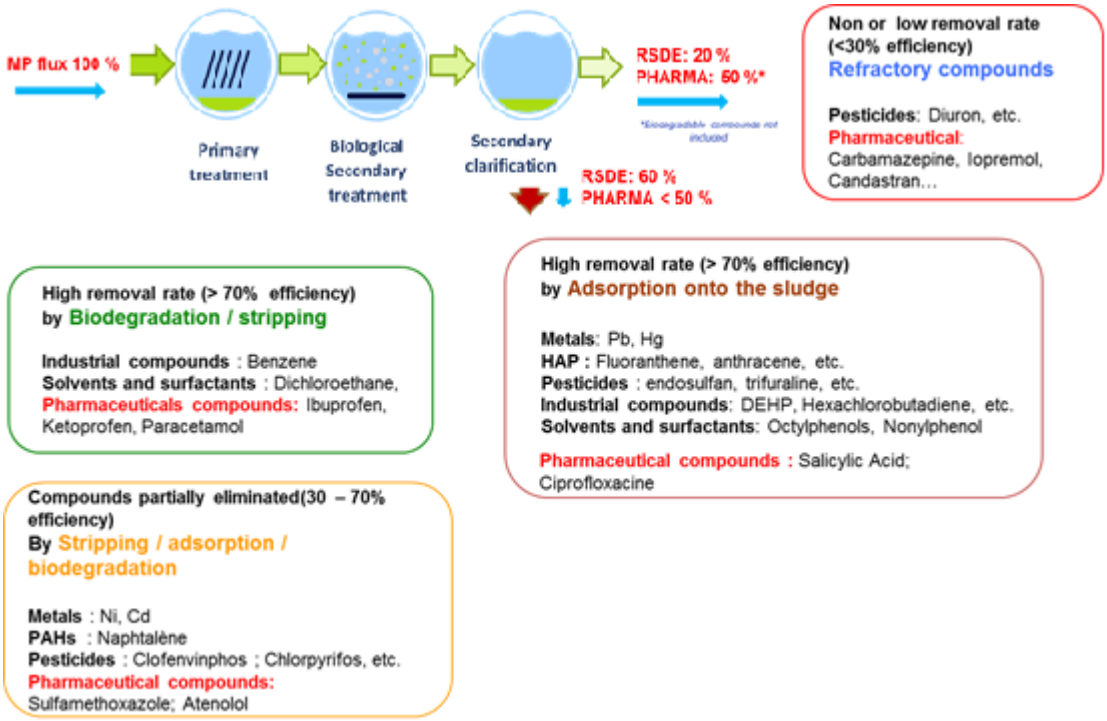
Biodegradation mechanism:
For many hydrophilic organic micropollutants, biological transformation is the main removal mechanism during wastewater treatment. Micropollutant concentrations in wastewater are usually too low (ng/l to µg/l) to support the growth of microorganisms or to induce the corresponding enzymes and/or cofactors for their biodegradation (probably no acclimatization / adaptation occurs at these concentrations).
Biodegradation of micropollutants can occur via different mechanisms:

1) Single substrate growth of a small subset of specialist oligotrophic organisms, which is less common in WWTPs and more likely to occur in receiving water or sediment (Daughton and Ternes, 1999);

2) Co-metabolism, in which micropollutants are decomposed by enzymes generated for other primary substation degradation (e.g. ammonia monooxygenase (AMO)) and are not used as carbon and energy source for microbial growth;

3) Mixed substrate growth, in which micropollutants are used as carbon and energy source and become mineralized (Vader et al., 2000). For pharmaceuticals, even if the compounds fall into the same therapeutical group, their biodegradability can show great variability. For example, Salgado et al. 2012 reported, among NSAIDs, diclofenac exhibited low (75%) biodegradation, whereas ibuprofen and ketoprofen were biodegraded to a much higher extent (>75%).

Therefore, biological transformation of micropollutants generally requires the presence of other growth substrates (carbon and energy sources) (Tran et al., 2013). The schema hereunder gives some examples of levels of efficiencies expected according with the physicochemical characteristics of the compounds.



Negative elimination/no elimination

In WWTPs, there are circumstances where the effluent concentrations of some micropollutants exceed their influent concentrations. This can be explained by the presence of some substances, e.g. human metabolites and/or transformation products in the influent, which can subsequently be transformed back to parent compounds during biological treatment (e.g. diclofenac, carbamazepine, erythromycin, and sulfamethoxazole) (Göbel et al., 2007; Kasprzyk-Hordern et al., 2009). In addition, some pharmaceuticals excreted with feces are probably partly enclosed in feces particles and released during biological treatment. The negative removal has also been ascribed to the daily concentration fluctuations during the sampling period, the analytical uncertainty, or desorption of molecules from sludge and suspended particulate matter (Clara et al., 2004; Köck-Schulmeyer et al., 2013).

Low apparent removal of certain compounds may be due to the reformation of these substances during the treatment, by the biological cleavage of the conjugated molecules. Indeed, many human drugs metabolites are the hydroxylated (-OH), amine (-NH_2), carboxylated (-COOH) or conjugated (with glucoronic or sulphuric acids) forms of the parent molecules. Deconjugation of the metabolite during the treatment can thus reform the parent drugs. This is for instance reported for N_4-acetyl sulfamethoxazole, reconverted to the antibiotic sulfamethoxazole (Göbel et al., 2007) and for several other pharmaceutical metabolites (ibuprofen, diclofenac, carbamazepine, or oestrogens) (Verlicchi et al., 2012). Some chemicals can also be converted to other. For instance, the biological oxidation of the hormone estradiol leads to the formation of the hormone estrone (Shi et al., 2013).

Low apparent removal of the dissolved fraction can also be due to the release in the dissolved phase during the treatment of pharmaceuticals trapped into faeces particles. This is suspected for drugs excreted with bile and faeces, such as macrolide and fluoroquinone antibiotics (erythromycin, azithromycin, ciprofloxacin, norfloxacin) (Göbel et al., 2007; Verlicchi et al., 2012).

4 Brædstrup WWTP characterization phase

4.1 Brædstrup WWTP

Brædstrup WWTP was built in 1992. It receives urban and industrial wastewater from Brædstrup and some minor surrounding communities. In 1992 the capacity of the plant was 8000 PE (Person Equivalent) and Brædstrup WWTP was just one of six minor WWTPs in that area. In the following years four of these was closed and the wastewater redirected to Brædstrup WWTP. Then in 2007, due to the Danish municipal reconstruction (Kommune sammenlægningen), a new strategy plan was presented. Small WWTPs was closed and Brædstrup WWTP was expanded from the originally 8000 PE to 16.000 PE and completed in 2012.

Brædstrup WWTP key figures are indicated in Table 2.

Table 2 Brædstrup WWTP key figures

Parameter	Unit	Value
Capacity	PE	16.000
Annual flow average	m^3/year	1.300.000
Daily flow average	m^3/d	3.500
Q max dry weather	m^3/h	360
Q max rain weather	m^3/h	1.100
Sludge production	ton/year	1.500

The plant is composed of several different steps. Principally there is two lines – line 1 and line 2 (the oldest) having two independent process tanks. After removal of coarse particles, the wastewater goes through a sand- and grease filtration step. Normally, with a waterflow up to 800 m^3/h, the water is then divided equally between the two lines. However, if the waterflow exceeds 800 m^3/h, line 1 can receive up to 700 m^3/h, while line 2 still gets 400 m^3/h and thereby reaching the plants limit of 1100 m^3/h. In Line 1, when the wastewater reaches the process tanks, in an OOO tank, bio phosphorous removal, nitrification and denitrification occur in anaerobic zone, anoxic zone, and an oxygen rich aeration tank, using the bottom air diffusers.

In line 2, there are 2 CSTR system in parallel, where phosphorous removal, nitrification and denitrification occur, by using the surface aerators. From the process tanks, the water inters one dedicated clarification tank, in which the sludge settles on the bottom and the treated wastewater is discharged to a small creek which is connected to Gudenåen. In dry weather the hydraulic retention time (HRT) in both line is 2-3 days. The wastewater treatment must comply with requirements stated in the water environmental plan and other stricter requirements. The treatment requirements for the discharge treated effluent are indicated in Table 3

Table 3 Treatment requirements for the discharged treated effluent.

Parameter	Unit	Value
COD	mg/L	50
BOD_5	mg/L	10
Total P	mg/L	0,25
Total N	mg/L	8,0
$N\text{-}NH_4$	mgN/L	2,0
Suspended solids	mg/L	20

Table 4 Quality of the wastewater - average from a weekly sample in the period October 2018 – January 2019. Written in green are the effluent concentration of the two lines (L1 and L2) fulfilling treatment requirements (see Table 3 Treatment requirements for the discharged treated effluent.). The measured minimum and maximum concentrations are shown in parenthesis.

Baseline	Date 2/10-18 - 28/1-19	avg.	(minimum – maximum)
COD (mg/L)	Inlet	**514,8**	(225,0 - 1024)
	L1 out	**15,3**	(8,0 - 22,0)

	L2 out	**16,9**	(9,0 - 24,0)
BOD_5	Inlet		
	L1 out		
	L2 out		
Total P (mg/L)	Inlet	**6,2**	(2,5 - 12,2)
	L1 out	**0,1**	(0,1 - 0,3)
	L2 out	**0,1**	(0,1 - 0,2)
Total N (mg/L)	Inlet	**58,9**	(30,3 – 99,9)
	L1 out	**4,7**	(2,2 – 8,1)
	L2 out	**3,0**	(1,2 – 6,7)
$N\text{-}NH_4$	Inlet	**33,0**	(12,8 – 58,0)
	L1 out	**1,4**	(0,6 -2,2)
	L2 out	**0,7**	(0,2 – 2,2)
TSS (mg/L)	Inlet	**392,5**	(164,0 - 828,0)
	L1 out	**5,4**	(2,0 – 10,2)
	L2 out	**5,1**	(1,6 – 9,2)
VSS (%)	L1 sludge return spot	**64,5**	(59,6 – 67,8)
	L2 sludge return spot	**63,3**	(58,9 – 67,1)

5 Methodology for sampling campaigns

Samples were taken at three (four) locations:

- Inlet of wastewater treatment plant: municipal wastewater including hospital effluent
- Outlet of wastewater treatment plant: outlet of both secondary clarifier
- After tertiary ozonation (only during ozonation campaigns)

Sampling dates/project outline:
24-hour flow proportional samples have been taken during a 21 months period (Table 5). Periods with no extreme rainfall were avoided as far as possible.

Table 5 Sampling dates for experimental phase

Baseline test									Ozone 1. Campaign						Ozone 2. Campaign			PAC 1. campaign			PAC 2- Campaign		
									Midway Campaign			Endway campaign											
29-11-2018	13-11-2018	09-11-2018	16-10-2018	12-10-2018	10-10-2018	18-09-2018	14-09-2018	12-09-2018	07-05-2019	13-05-2019	15-05-2019	12-07-2019	10-07-2019	09-07-2019	31-10-2019	24-10-2019	17-10-2019	27-03-2020	26-03-2020	25-03-2020	06-05-2020	04-05-2020	30-04-2020

Prior the experimental phase of this project a so-called pre-screening were conducted. Here, the detectable compounds were found and a selection of substances in focus were defined. Afterwards, a baseline test period in the fall of 2018 were done. Here the biological performance of the two lines at Brædstrup WWTP were found and the substances measured. The first campaign of ozonation started in the early spring of 2019 and ran until mid-July. Followed by the second campaign of ozonation, which ended in late October 2019. The first of the two Powder Activated (PAC) experiments did not start continuously until start January and ran until March

2020, followed by the second PAC campaign from March to May 2020. The overall project structure can be seen pictured below (Figure 1).

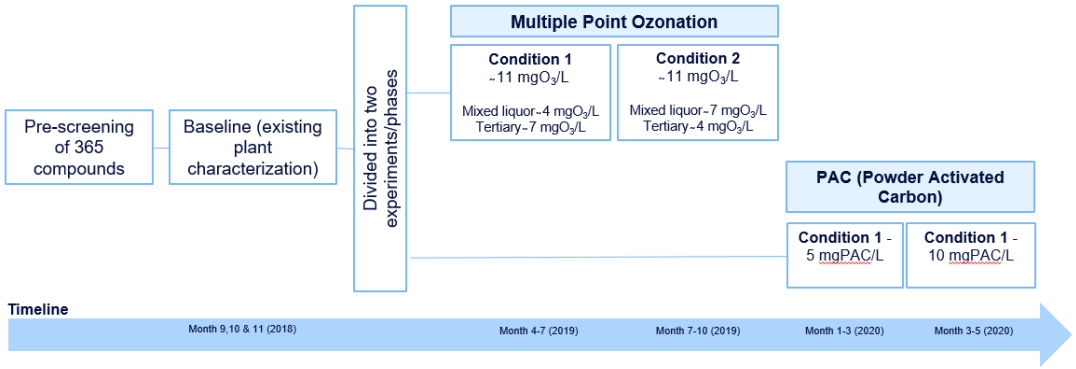


Figure 1 Schematic representation of the experimental programme. Also shown are the specific dosages of both ozone and Powder Activated Carbon (PAC). Please note that ozone and PAC never is dosed simultaneously.

Sampling method: Sampling actions for micropollutant analysis were based on the following standards and guides:

- The standard NF EN ISO 5667-3 "Water quality - Sampling - Part 3: Guidelines for the storage and handling of water samples"
- The guide FD T 90-523-2 "Water Quality - Sampling Guide for Environmental Quality Monitoring of Water in the Environment - Wastewater Collection"
- The operational technical guide AUQAREF. Sampling and conditioning practices for the research of priority and emerging micropollutants in collective and industrial sewer systems. Action I-B-02 - Support to contractors, monitoring of discharge.

In short - The sampling was carried out continuously over a 24-hour period, at controlled temperature and flow proportional. The samples were refrigerated at a temperature no greater than 4°C. Thus, the sampler was equipped with glass or High-Density Polyethylene (HDPE) containers. The containers were filled through a Teflon pipe with an internal diameter greater than 9 mm.

Cleaning procedure: A preliminary cleaning step was conducted before the start of each measurement campaign for the sampler and the collecting bottle.

- Coarse cleaning with water
- Alkaline detergent
- Acetic acid (80%) diluted ¼
- Ultra-pure acetone
- Rinsing with water free from micropollutants

Shipping of samples: All bottles / samples are labelled with sampling location (i.e. Brædstrup Wastewater Treatment Plant) and date. The samples were packed in flamingo/cooling boxes along with cooling elements to maintain a temperature of 4°C during transport. The samples were then dispatched to the laboratories by rapid transport the same day, with express delivery within 24 hours maximum.

5.1 Analysis of compounds

Baseline: All in all, 9 daily samples of inlet-outlet of Brædstrup WWTP were shipped to IUTA laboratory in Germany. The IUTA is a research institute in the field of energy and environmental technology located in Duisburg, Deutschland. Among the 36 target compounds, the analysis of 1H-Benzotriazole, Carbamazepine, Ciprofloxacin, Clarithromycin, Diclofenac, Ibuprofen, Metoprolol, Paracetamol, Sulfamethoxazole and Tramadol were carried out according to SAA 9.6.1. (Determination of pharmaceuticals, pesticides, and industrial chemicals in water samples by LC-MS / MS, November 2017). The analysis of the other compounds was done by LC-MS/MS. Only measured in the 3 sampling campaigns of November are Gabapentin, Iohexol, Losartan, Mycophenolic acid, Sertraline and Trimethoprim.

Multiple point ozonation condition 1: 2 sampling campaigns, Midway and Endway campaign have been conducted during ozonation condition 1. For each campaign, Three 24H-flowproportionel samples were done and shipped to IUTA laboratory in Germany. Among the target compounds, the analysis of Iomeprol, 1H-Benzotriazole, ∑ 4+5-Methylbenzotriazol, Carbamazepine, Ciprofloxacin, Clarithromycin, Diclofenac, Ibuprofen, Metoprolol, Paracetamol, Sulfamethoxazole and Tramadol were accredited according to DIN EN ISO/IEC 17025:2005, DAkkS (Deutsche Akkreditierungsstelle GmbH), accreditation certificate no. D-PL-19759-01-00., accreditation. The analysis of the other compounds was done by LC-MS/MS.

Multiple point ozonation condition 2: Three 24H-flowproportionel samples were done and shipped to IUTA laboratory in Germany. Among the get compounds, the analysis of Iomeprol, 1H-Benzotriazole, ∑ 4+5-Methylbenzotriazol, Carbamazepine, Ciprofloxacin, Clarithromycin, Diclofenac, Ibuprofen, Metoprolol, Paracetamol, Sulfamethoxazole,Tramadol, PFOA and PFOS were accredited according to DIN EN ISO/IEC 17025:2005, DAkkS (Deutsche Akkreditierungsstelle GmbH), accreditation. The analysis of the other compounds was done by LC-MS/MS.
PAC conditions 1 and 2: Three 24H-flowproportionel samples were done and shipped to IUTA laboratory in Germany. Among the target compounds, the analysis of Iomeprol, 1H-Benzotriazole, ∑ 4+5-Methylbenzotriazol, Carbamazepine, Ciprofloxacin, Clarithromycin, Diclofenac, Ibuprofen, Metoprolol, Paracetamol, Sulfamethoxazole, Tramadol, PFOA and PFOS were accredited according to DIN EN ISO/IEC 17025:2005, DAkkS (Deutsche Akkreditierungsstelle GmbH), accreditation. The analysis of the other compounds was done by LC-MS/MS. Fulvestrant is only measured during PAC campaigns.

6 General performance of the plant

General performance of Brædstrup WWTP in term of conventional biological parameters like Flow in Line 1, line 2 and the mixed liquor temperature in process tanks in both Line 1 and Line 2, COD, Total Nitrogen (TN) and Total Phosphorous (TP) in the inlet, and outlet of each line have been monitored in all duration of the project and presented in this chapter.

6.1 Daily operation of Brædstrup WWTP

The overall performance of Brædstrup WWTP was evaluated according to conventional wastewater parameters.

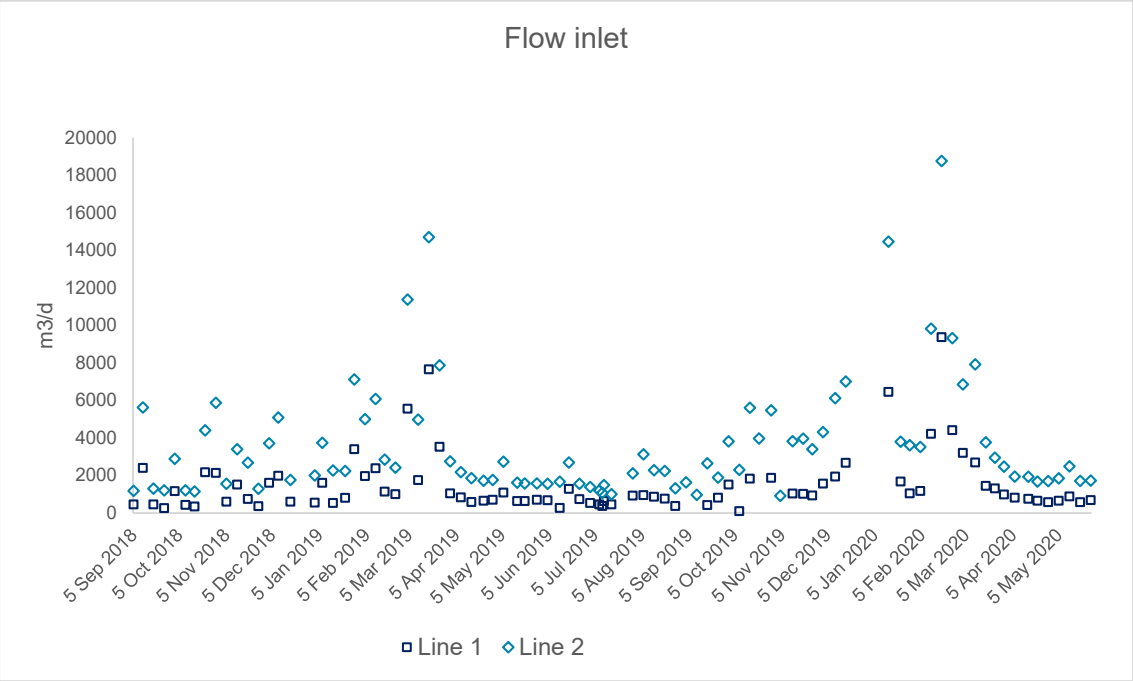


Figure 2 The distribution of Inlet flow in Line 1 and Line 2

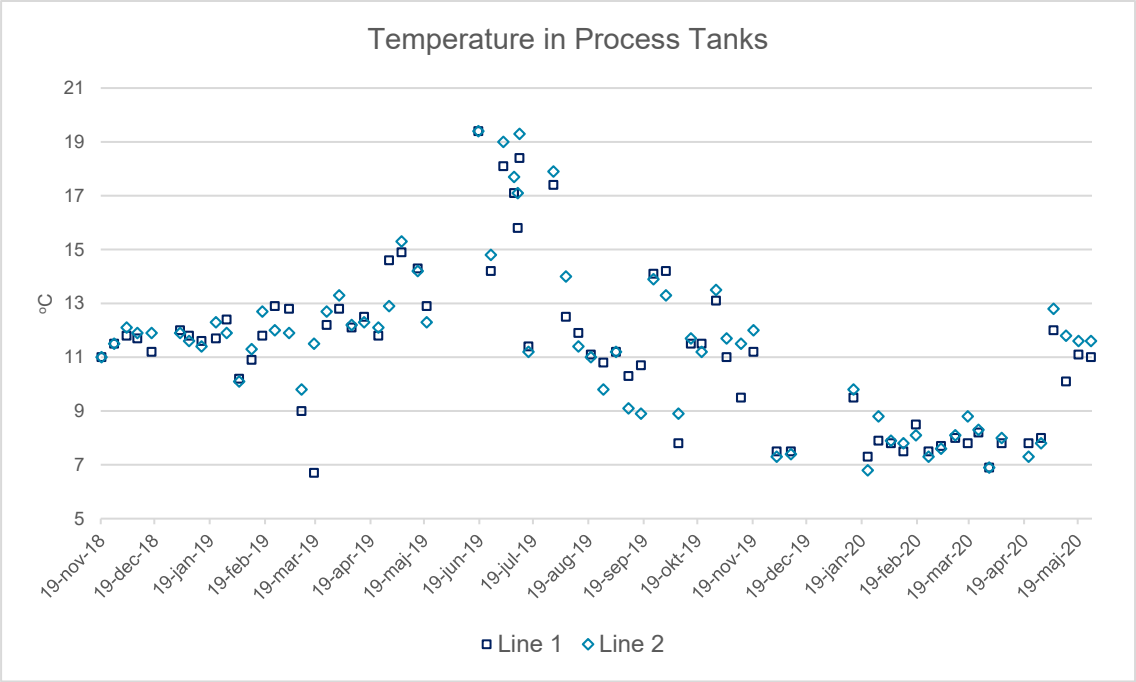


Figure 3 The content of Temperature in process tanks Line 1 and Line 2

The concentration of chemical oxygen demand, COD, in the period of operation in inlet and out let line 1 and outlet line 2 are shown in *Figure 4*

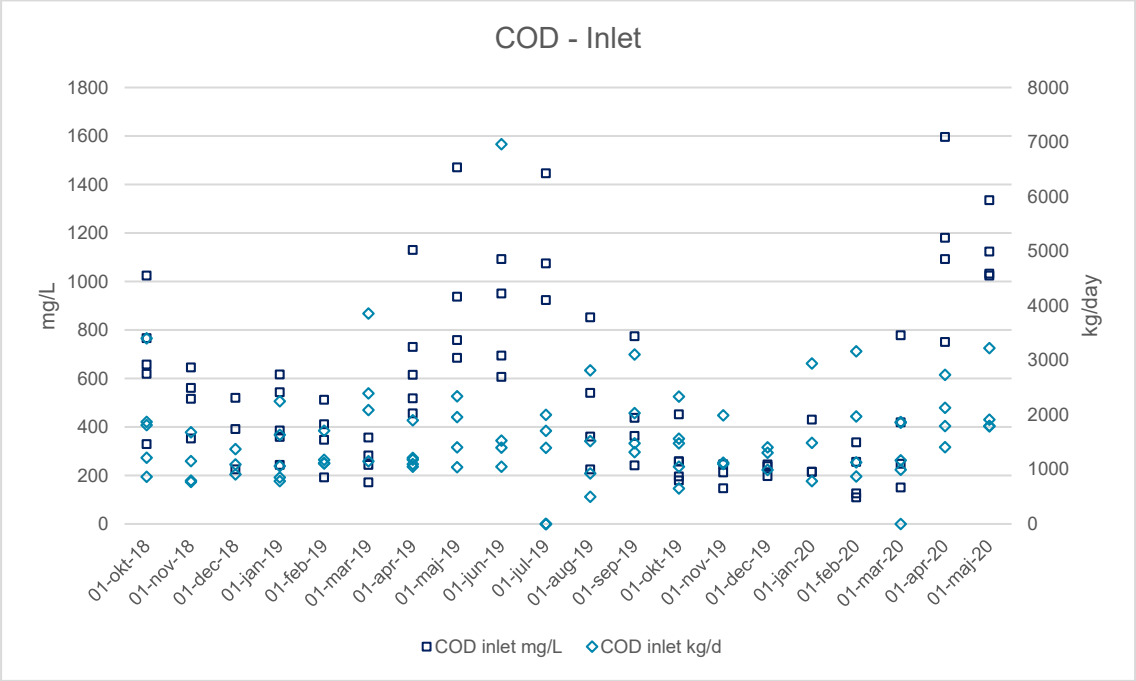


Figure 4 The content of COD Inlet concentrations and Inlet load

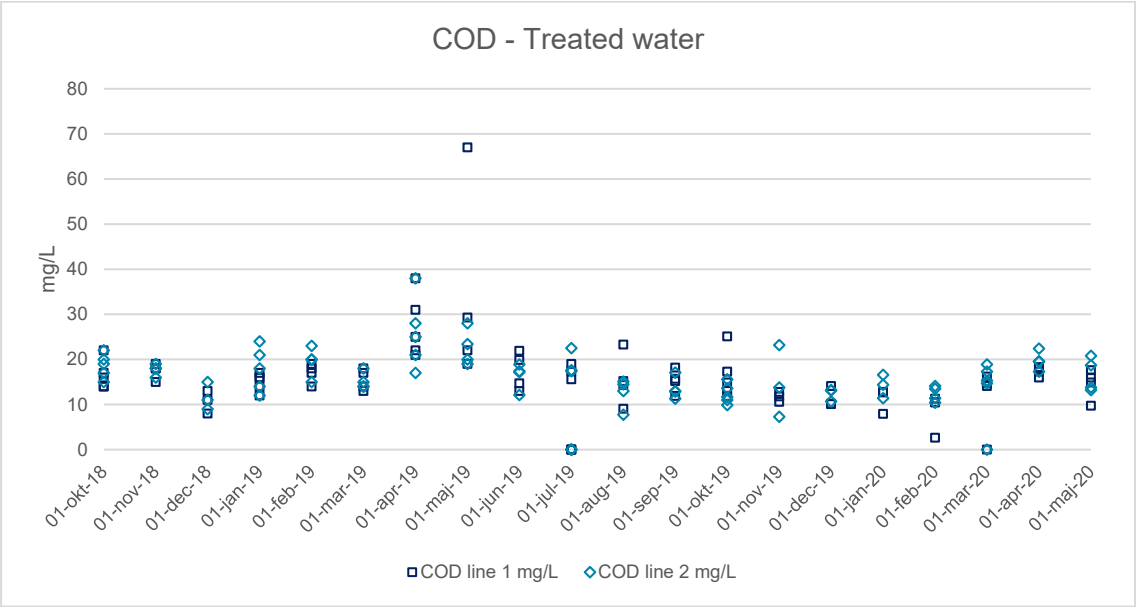


Figure 5 The content of COD Line 1 and Line 2 outlet concentrations

The unfiltered COD (COD total) was on average 560 mg/l in inlet and 17 mg/l in effluent of Line 1 and Line 2, with variations in periods with rainwater mixing into the sewer. Average inlet values for dissolved fraction of COD of 60 mg COD/l and effluent values of 13 mg COD/l in each line have been observed. The results show mixed liquor ozonation or PAC addition in the biological process tank in Line 1 do not make significant effect in performance of the plant in term of COD removal.

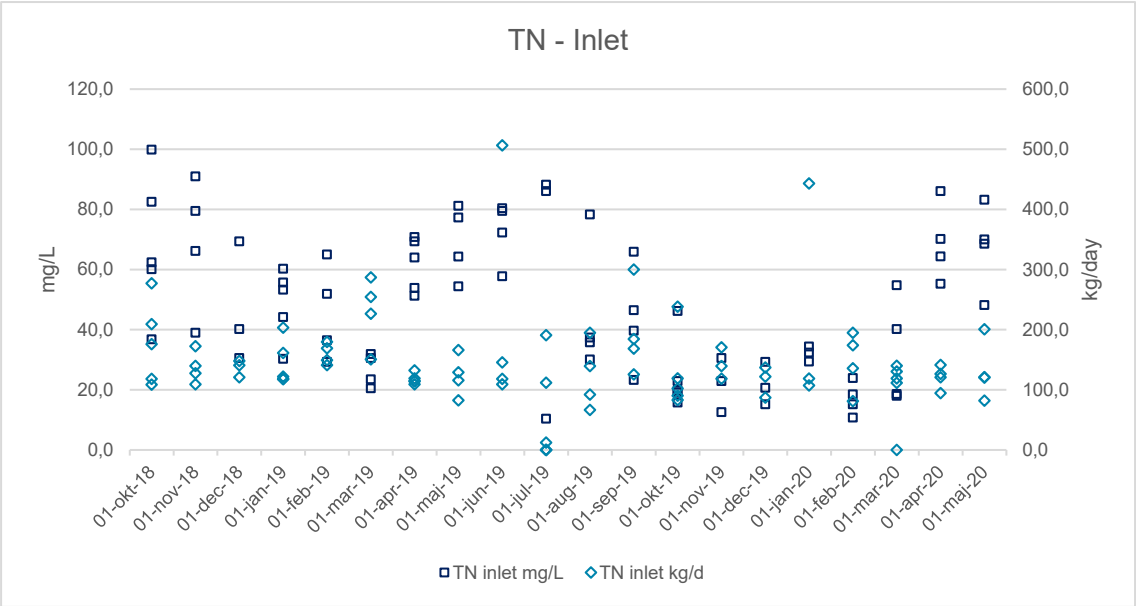


Figure 6 The content of TN Inlet concentrations and Inlet load

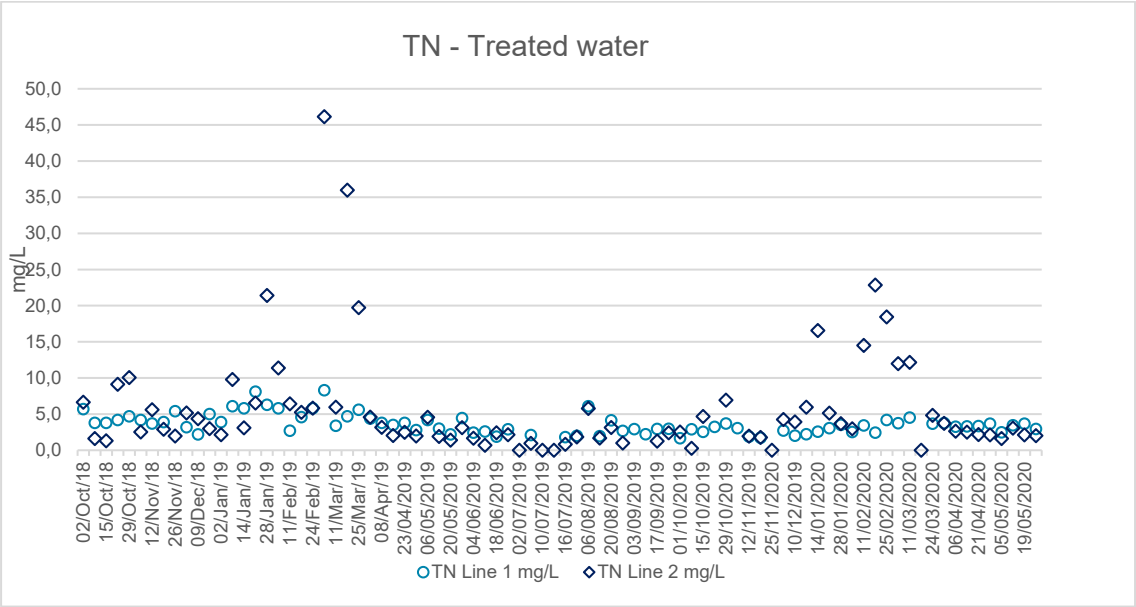


Figure 7 The content of TN Line 1 and Line 2 outlet concentrations

As it shows in Figure 6, the inlet concentration of TN varies, the average TN concentration is 48 mg/l. Nevertheless, the TN concentration in outlets of both Line 1 and Line 2 are more stable, with average of 3,8 mg/l in Line 1 and 2,9 mg/l in Line 2. The results show mixed liquor ozonation or PAC injection in the biological process tank in Line 1 do not make significant effect in performance of the plant in term of TN removal.

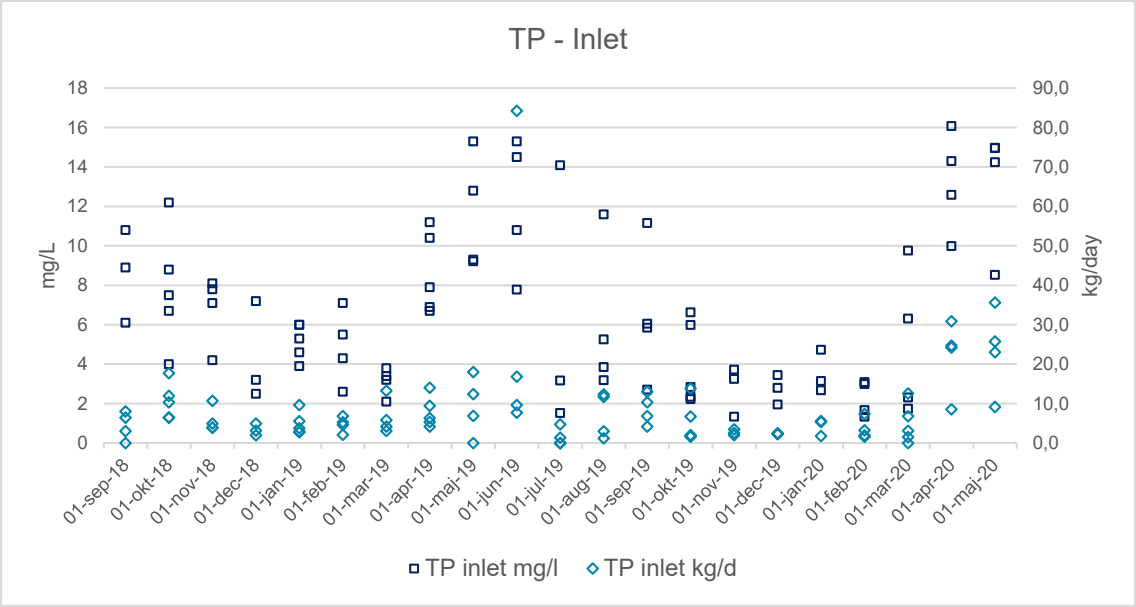


Figure 8 The content of TP Inlet concentrations and Inlet load

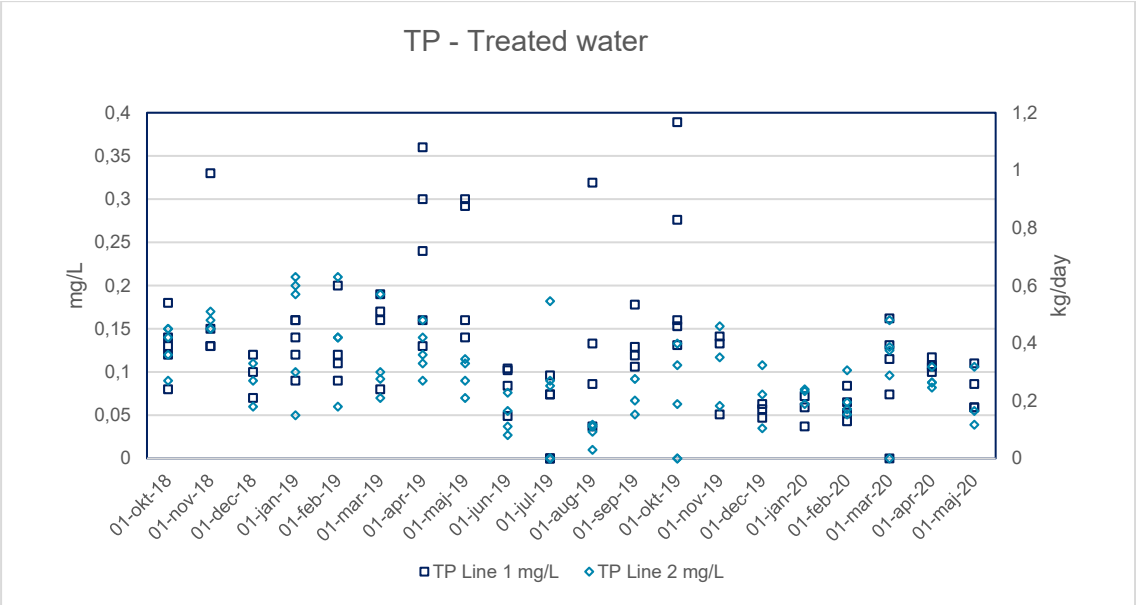


Figure 9 The content of TP Line 1 and Line 2 outlet concentrations

The TP analysis results in inlet and outlet of the plant is shown in Figure 8 and Figure 9. The average inlet TP concentration is 6,9 mg/l. TP is eliminated partly biological and partly chemical by adding aluminum based industrial waste product. The average of TP outlet concentrations in Line 1 is 0,14 mg/l and in Line 2 is 0,13. The results show mixed liquor ozonation or PAC injection in the biological process tank in Line 1 do not make significant effect in performance of the plant in term of TP removal.

7 Baseline test:

7.1 Occurrence of pharmaceuticals in raw wastewater

Three sampling campaigns composed of three days were carried out in the fall of 2018 (3 in September 3 in October and 3 in November) (known as the baseline).

Five pharmaceuticals were never above their Limit of Quantification (LQ) in the inlet of the plant. These being - the two antibiotics cefalexin and ciprofloxacin, the steroid medicament prednisolone and finally the antidepressant sertraline and psycholeptic zopiclone. Others were not above LQ in some days, but other days above, e.g. capecitabine and metronidazole. The top three most abundant pharmaceuticals (with concentration range) in the influent of the WWTP was paracetamol (7.100-160.000 ng/l), gabapentin (18.000-50.000 ng/l) and ibuprofen (9.000-39.000 ng/l). Even though, Brædstrup does not receive wastewater from hospitals, the two x-ray contrast media – iohexol and iomeprol - you would expect to find mainly in hospital effluents are found in the range 6.400-24.000 ng/l and 250-25.000 ng/l, respectively.

The concentration in inlet wastewater varied from 11 ng/l for citalopram to 160.000 ng/l for paracetamol. The particularly high for the analgesic paracetamol could be explained by the high consumption and easy accessibility of the compound. The minimum, quartile 25, median, quartile 75 and maximum concentration of the 31 substances quantified in the inlet above their limit of quantifications are shown in (*Figure 10)*.

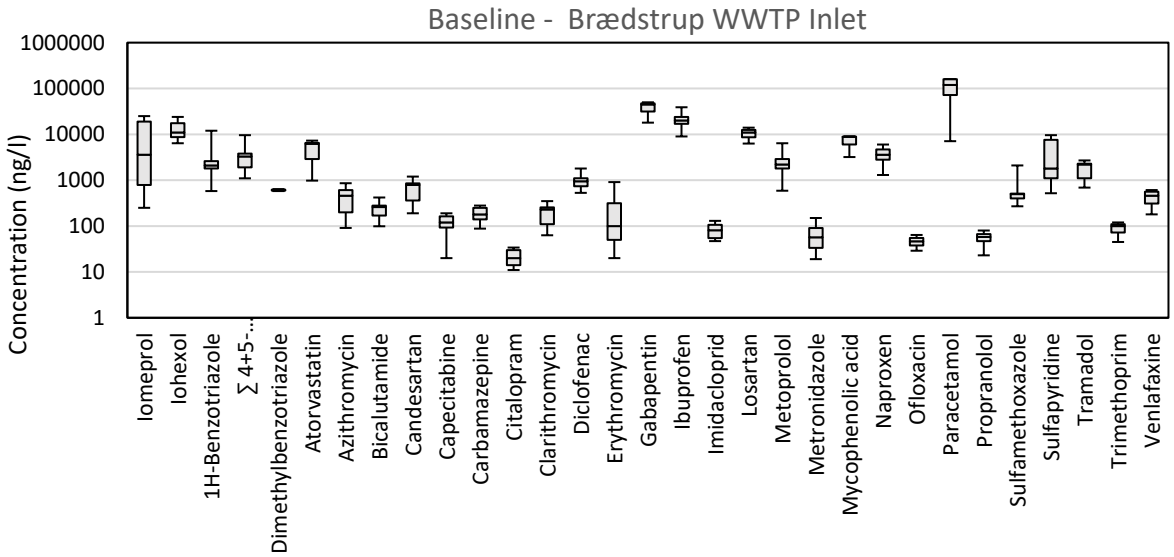


Figure 10 Average (n=9) inlet concentration of the 31 pharmaceuticals quantified above their limit of quantification (LQ) in liquid phase for the baseline samples. It shows the minimum, quartile 25, median, quartile 75 and maximum concentration. Notice logarithmic scale.

As can be observed the consumption and thereby the excretion/release into the sewage system varies a lot, with the painkiller paracetamol being most profound at average concentrations of 111,304 ng/l (±93,048 ng/l) at the inlet at Brædstrup WWTP. Contrary, some of the lowest detectable compounds are the antibiotic ofloxacin and the beta-blocking agent propranolol both occurring with average concentrations >60 ng/l. Also notable are the high standard deviations seen for each substance. Hence, the concentration fluctuations from day to day is high. The average concentration across all detected compounds in the pumping station were 7,526 ng/l.

This high range of distribution is also found in numerous studies from WWTPs (Verlicchi et al. 2012). Concentrations of micropollutants in WWTP influent and effluent reveal significant spatial and temporal variations, which are essentially due to several factors: Size of WWTPs; Water consumption per person and per day; Local common diseases can induce a higher consumption of specific pharmaceuticals in certain periods; Metabolism (excretion rate): products containing potential contaminants (e.g. pharmaceuticals) are metabolized in the human body and are subsequently excreted via urine and faeces, excretion rate plays a role in determining the introduction of pharmaceuticals into raw wastewater; Rainfall as it affects the flow pattern of wastewater influent when a combined sewer system is employed; Elimination efficacy of wastewater treatment processes; Other weather conditions, such as temperature and level of sunlight also can affect the discharge of micropollutants from WWTPs.

Figure 11 gives an overview of the different ATC family of substances are the most profound in the inlet and outlet of Brædstrup WWTP. Especially, the analgesics stand for a high percentage (46%) of the total load of substances. This can be explained by the very high inlet loads of paracetamol (an average inlet concentration of 111,304 ng/l, n=21). Contrary, the antalgics only stand for 10% in the effluent of Brædstrup WWTP which again can be explained by the high biodegradation of paracetamol (an average effluent concentration of 435 ng/l, n=21). The high inlet load of the contrast media (17%) is mainly due to iopromide and the increased effluent load

of contrast media (37%) could suggest the removal during bio-treatment is not as successful as is seen for the easy biodegradable paracetamol, i.e. the percentage of contrast media increases compared to the total load of micropollutants.

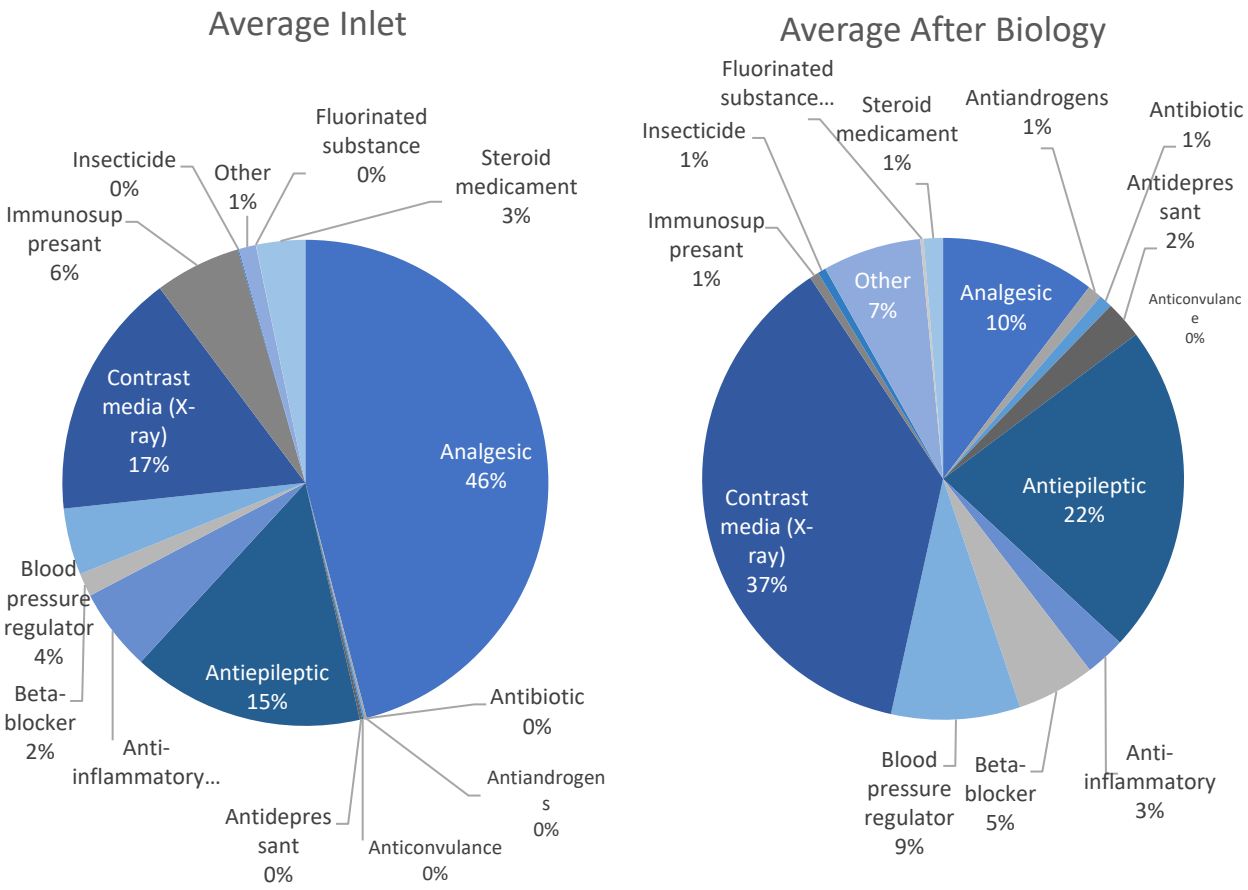


Figure 11 Percentage distribution of the different ATC groups/substance families of the overall concentration at Brædstrup WWTP inlet and outlet after conventional activated sludge (CAS).

7.2 Occurrence of pharmaceuticals in treated water

Line 1:

10 pharmaceuticals were never above their Limit of Quantification (LQ) or just above 1 time (and therefore considered to be below all the time) in the outlet of Line 1. These being the five which also did not quantify in the inlet (Cefalexin, Ciprofloxacin, Prednisolone, Sertraline and Zopiclone) and the rest being removed by the conventional activated sludge (CAS); Ibuprofen Metronidazole, Mycophenolic acid, Dimethyl benzotriazole and Capecitabine. Ibuprofen which in inlet was found at very high concentrations is removed <LQ and Paracetamol is reduced far below the initial inlet concentration.

The concentration of the 26 substances quantified in the influents above their limit of quantifications are shown in *(Figure 12)*.

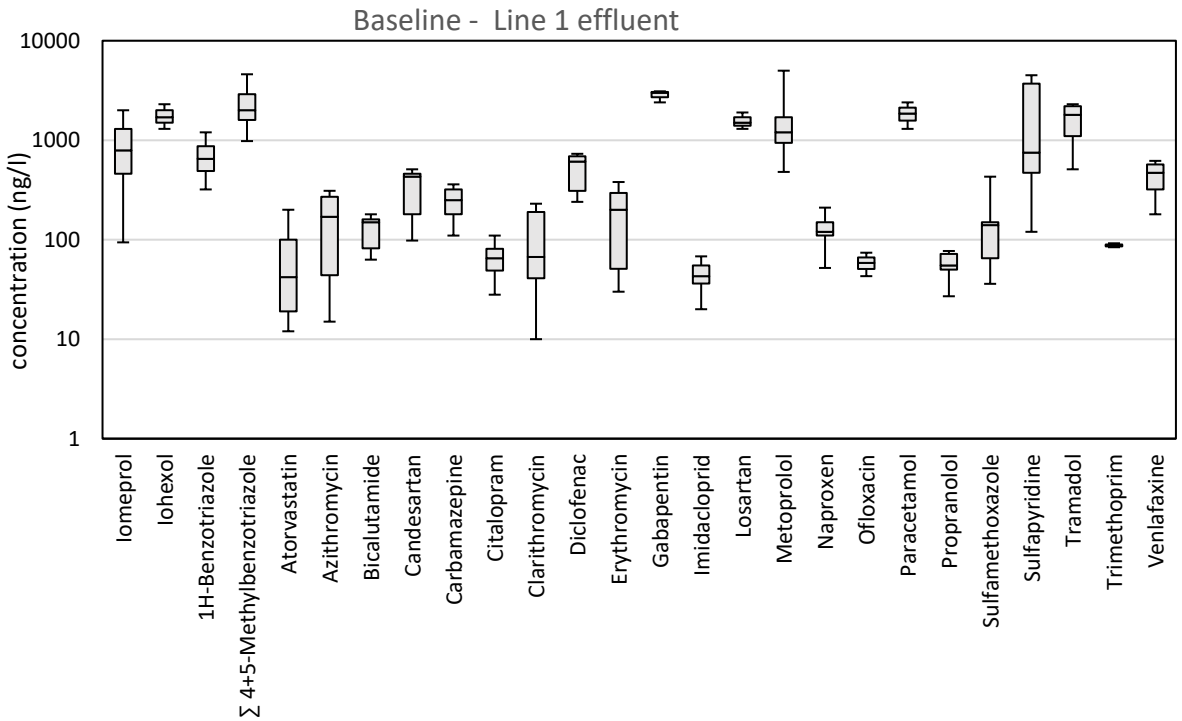


Figure 12 Effluent of Line 1. Average (n=9) concentration of the 26 pharmaceuticals quantified above their limit of quantification (LQ) in liquid phase for the baseline samples. It shows the minimum, quartile 25, median, quartile 75 and maximum concentration. Notice logarithmic y-axis.

A vast variation in ratio between the highest and the lowest concentration is also observed in the effluent of Line 1, e.g. Sulfapyridine shows a factor 37,5 in difference, while Losartan only varies with a factor 1.5.

Line 2:

The concentration of the 27 substances quantified in the influents above their limit of quantifications are shown in *(Figure 13)*.

9 pharmaceuticals were never above their Limit of Quantification (LQ) or just above 1 time (and therefore considered to be below all the time) in the outlet of Line 2. These being the five which also did not quantify in the inlet (Cefalexin, Ciprofloxacin, Prednisolone, Sertraline and Zopiclone) and the rest being removed by the CAS treatment; Ibuprofen Ofloxacin, Dimethyl benzotriazole and Capecitabine.

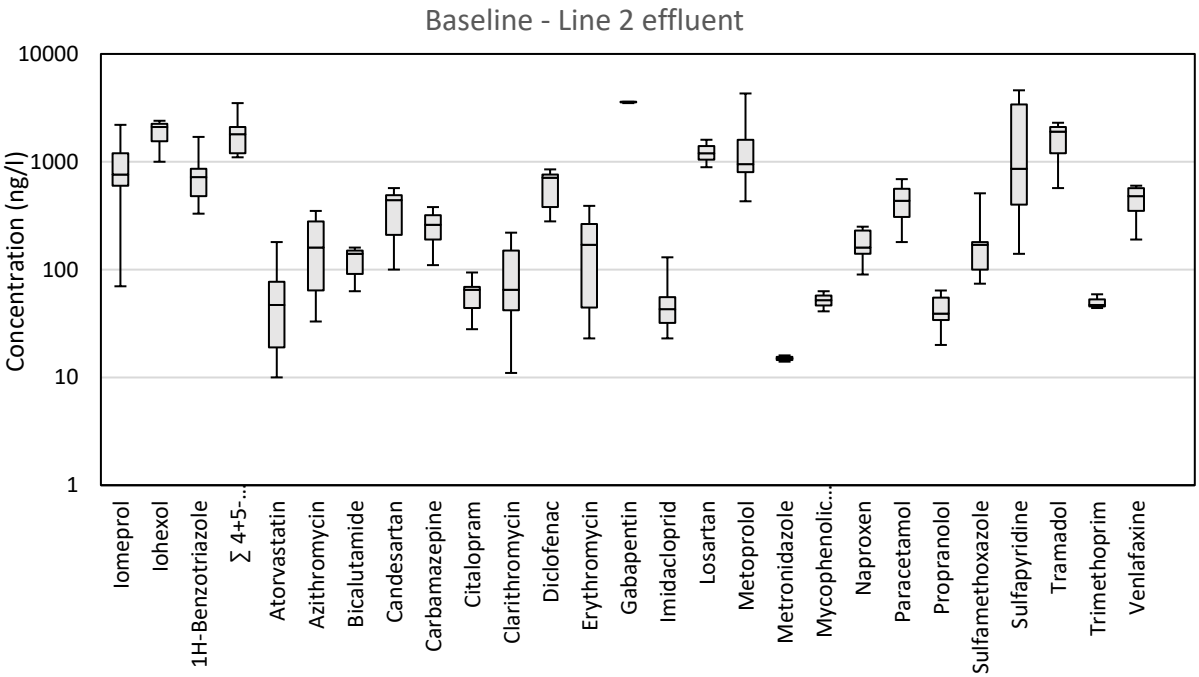


Figure 13 Effluent of Line 2. Average (n=9) concentration of the 27 pharmaceuticals quantified above their limit of quantification (LQ) in liquid phase for the baseline samples. It shows the minimum, quartile 25, median, quartile 75 and maximum concentration. Notice logarithmic y-axis.

7.3 **Removal Rate**

The removal rate (R_w) is calculated as following equation: $Rw\ (\%) = \frac{C_{in} - C_{out}}{C_{in}}$, For a detailed description of removal calculation please see *Appendix A*.

Pharmaceuticals have low volatility (KAW < 10-5) (Hörsing et al., 2011) and thus not expected to be stripped during the WWTP treatment. Removal by sorption can be significant (10-80%) for hydrophobic drugs such as mefenamic acid (log KOW = 5.1) or gemfibrozil (log KOW = 4.8) or for positively charged pharmaceuticals such as several quinolone and macrolide antibiotics like ciprofloxacin and azithromycin. Most other pharmaceuticals have high solubility, low hydrophobicity, and often negative charge at neutral pH (acidic compounds), which means low sorption affinity on biological sludge (negatively charged). They are thus mostly found in the "dissolved" phase and their removal by sorption is often negligible (< 5%) (Verlicchi et al., 2012). Their removal in the primary treatment is usually very poor. Biodegradation or biotransformation is therefore the main removal mechanism for most pharmaceuticals. Biotransformation processes are strongly dependent on the properties of the compound (biodegradability). A few pharmaceuticals (e.g., some Antalgics /anti-inflammatory drugs) are well removed during the biological treatment, but most are only partially or not removed at all.

Removal efficiency during baseline tests were calculated for 29/36 investigated compounds, or about 80% of the investigated substances. Contrary, it was not possible to calculate Rw for 7/36 substances, or about 20%. These 7 compounds were either never measured above detection limit or not fulfilling the removal calculation rules (see Appendix A for a detailed description). A total of 9 samplings days spread from September to November was taken. The specific dates can be found in table 5.

As seen with the concentrations, we also observe a high removal difference pattern for each substance. Removal efficiencies from -325% to almost 100% were observed, depending on the compound and the sampling day. Candesartan, a blood pressure regulator, was only removed

in average 41% in Line 1 and 38% in Line 2 (-60% to 67% on different sampling days). Contrary, the easy biodegradable ibuprofen, an anti-inflammatory, was removed 99.97% (Line 1) and 99.96% Line 2 in average (Figure 14). Indeed, paracetamol and ibuprofen are known to be biodegradable and well removed by biological processes operated at low load (Verlicchi et al., 2012).

The average biological removal for the plant during 1.5 year of surveillance were 55% in total.

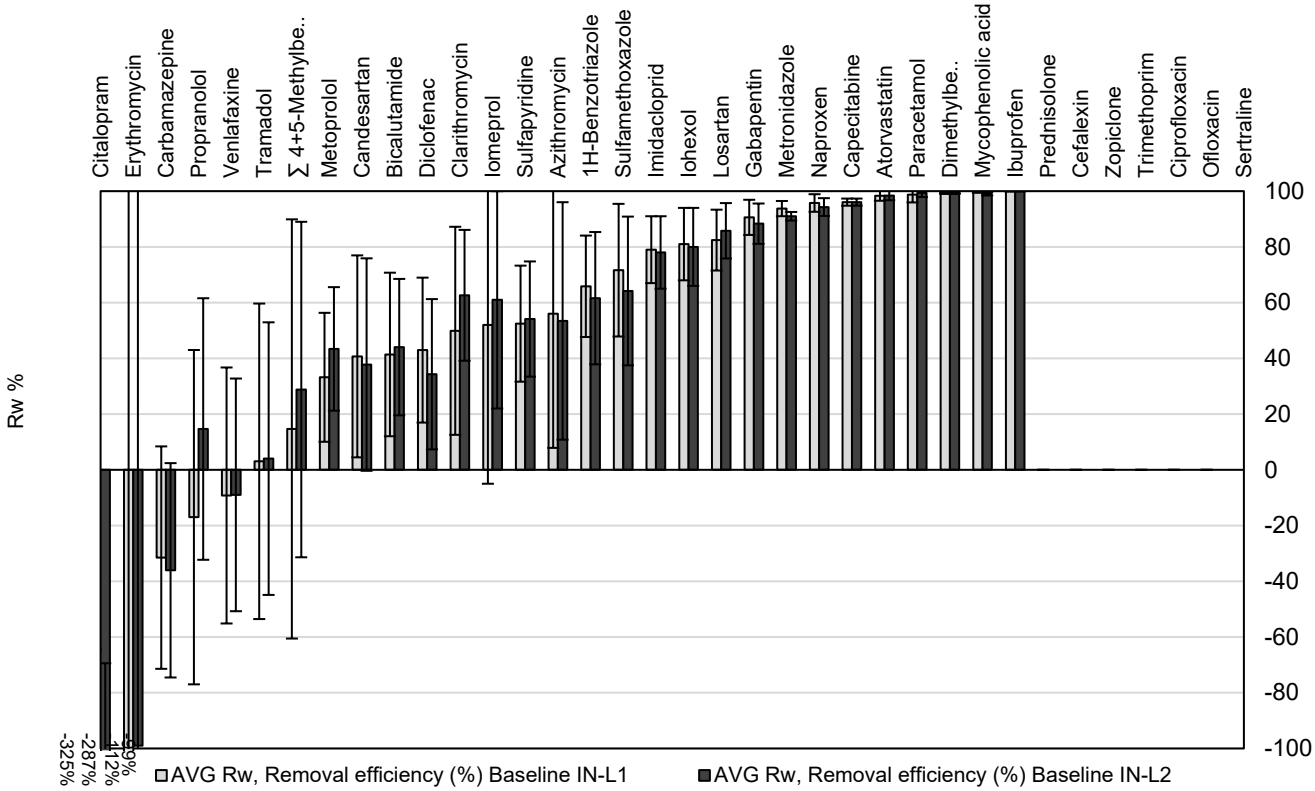


Figure 14 Graphic illustration of removal efficiency for the 36 pharmaceuticals investigated in the Baseline test. Prednisolone, Cefalexin, Zopiclone, Trimethoprim, Ciprofloxacin and Ofloxacin did not fulfil the rules of calculation or below Limit of Quantification at all time. The five compounds with a negative removal are from left to right: citalopram, erythromycin carbamazepine, propranolol and venlafaxine.

While some substances can be efficiently and consistently removed by conventional WWTP, like paracetamol and ibuprofen, the removal of micropollutants is basically under no control. There are some compounds who show negative elimination - carbamazepine, citalopram, propranolol, erythromycin, and venlafaxine. Compounds even in the same usage, family or class were removed at different degrees.

For individual compounds, specific large elimination disparities were also displayed. For example, Bicalutamide was in Line 1 relatively removed (46%) one day, while it showed negative reduction (-23%) the next day. While Line 2 showed 43% removal and -8% on the same correspondingly days. Largely, the removal difference among different compounds in WWTPs could be ascribed to numerous factors such as retention time, inlet concentrations, micropollutant properties and operational conditions.

The important removal variability as well as negative eliminations can result from of the transformation of human metabolites into the parent compounds during the biological treatment (Lishman et *al*., 2006) as well as from important concentration variations in the raw wastewater during sampling. The transformation of the metabolites carbamazepine-glucuronide into its parent compound during secondary biological treatment were made evident in several studies (Göbel et al.

2005; Plosz, Leknes, and Thomas 2010; Zhang, Geißen, and Gal 2008) and may explain the higher concentrations (negative elimination) of carbamazepine measured in the biologically treated effluents.

7.4 Investigation whether the two separate lines are comparable

The two separate treatment line at Brædstrup WWTP have made it especially suited for direct comparison between conventional activated sludge (CAS) and CAS combined with multiple point ozonation or PAC addition. Thus, to say with certainty it is possible to compare the two lines, since the lines do not have the same configuration, several comparisons were made. One of those being the outlet concentrations and thereby also removal efficiency with pure biology. In Figure 15 the ratio Line 1 (L1) versus Line 2 (L2) for each compound can be seen. The average ratio across all compounds is 1.1, hence no significant difference in outlet concentration level is observed.

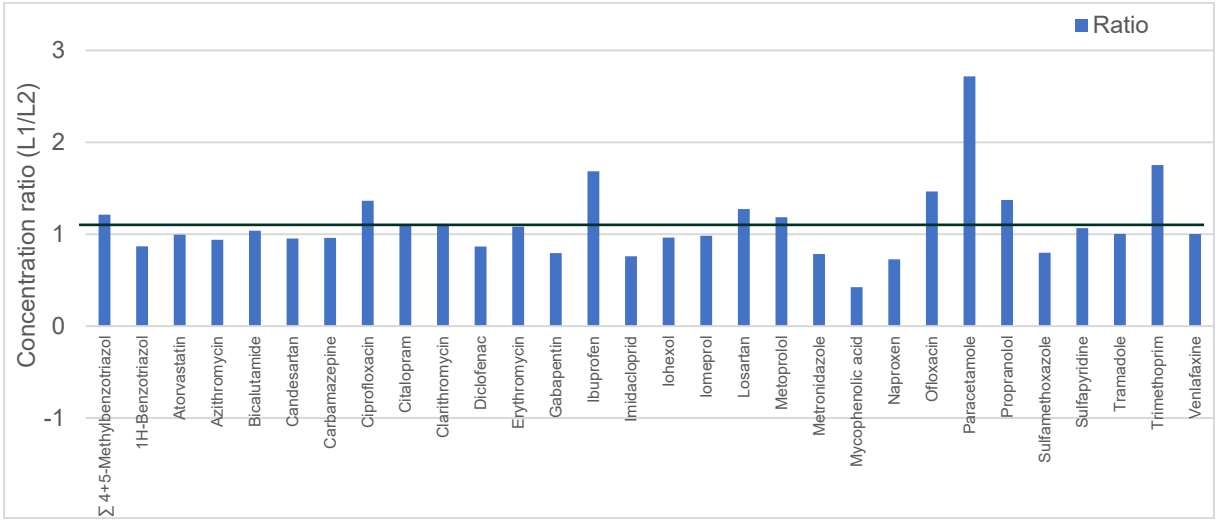


Figure 15 The ratio between Brædstrup WWTP Line 1 and Line 2 outlet concentrations for the detectable 30/36 substances. The average ratio is 1.1 illustrated by the orange line.

If the ratio is above 1, Line 2 would have been lower in outlet concentration. Thus, paracetamol seems to have been in a higher amount in Line 1 (ratio of 2.7). Contrary, the ratio of 0.4 for the immunosuppressant mycophenolic acid shows the effluent of Line 1 to be lower than Line 2. Consequently, if it is assumed that the inlet concentration is divided equally between the two lines a difference in removal efficiency could be the reason. Hence, to determine if that was the case, the removal efficiency ratio between the two lines was assessed.

Comparing the two lines removal capabilities with no interference, we see an average ratio of 0.9, thus supporting similarity across both lines. The negative ratio for propranolol is because it was negatively removed in line 1 (-17%) but positively removed in line 2 (15%) (see Figure 16).

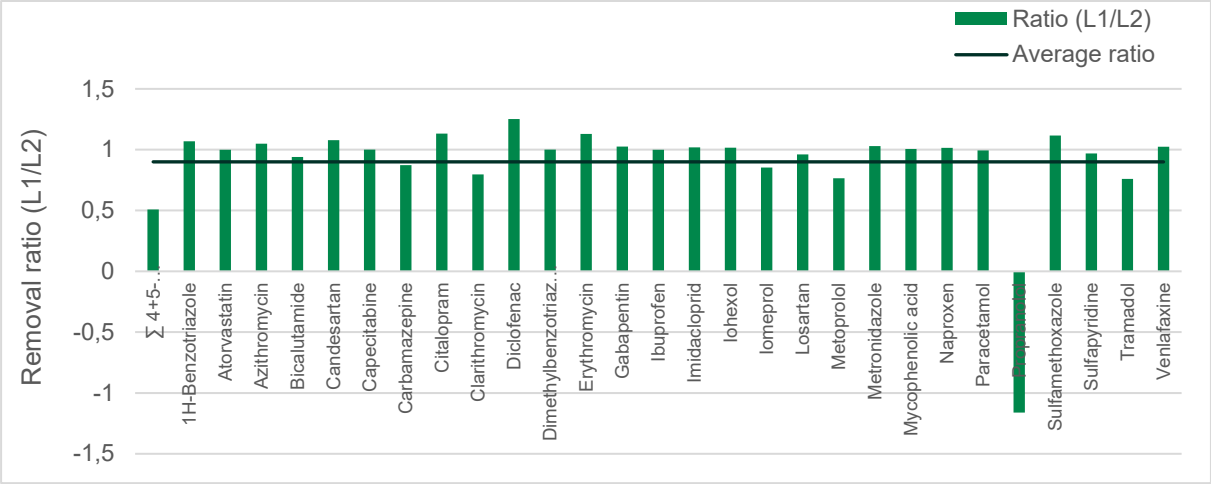


Figure 16 The removal efficiency ratio between Brædstrup WWTP Line 1 and Line 2 for the 29 calculable removal efficiencies. The average ratio is 0.9 illustrated by the orange line.

7.5 Result evaluation based on PNEC value

Predicted No Effect Concentration ($PNEC_{freshwater}$) is the concentration of a substance in any environment below which adverse effects will most likely not occur during long term or short-term exposure. In environmental risk assessment, PNECs will be compared to actual or predicted environmental concentration (PEC) to determine if the risk of a substance is acceptable or not. If PEC/PNECs <1, the risk is acceptable. PNECs need to be derived for various environmental compartments (water, sediment, soil, air, etc.). PNECs are typically derived from long-term laboratory-based ecotoxicity tests using well-defined protocols on a limited number of species. Such information is usually retrieved from relevant literature and/or internationally recognized databases. The quality of the extracted data may vary considerably among individual source documents.

In this report, PNEC values are defined for 31 compounds by combining information from ECHA, Fass.se, new EU recommendations 2018 (JRC rapport - watch list), AMK 2015, AMK 2013, Bkg. 1022 (miljøkvalitetskrav).

It is important to note that in the inlet of Brædstrup WWTP 11 substances were above their $PNEC_{freshwater}$ and subsequently could have a potential negative effect on the receiving waterbody. One of these substances is paracetamol, which as we have stated previously is well removed (almost 100%) during the normal treatment at Brædstrup WWTP. While 6 substances, is above $PNEC_{freshwater}$ in the effluent of the plant and thereby having a potential negative environmental effect. These consist of two antibiotics, one anti-inflammatory, one antidepressant, one antiandrogen and a blood-pressure regulator (Azithromycin, Sulfamethoxazole, Diclofenac, Venlafaxine, Bicalutamide and Candesartan)(*Figure 17*). No dilution has been used in this report. Thus, the outlet concentration is directly compared to $PNEC_{freshwater}$.

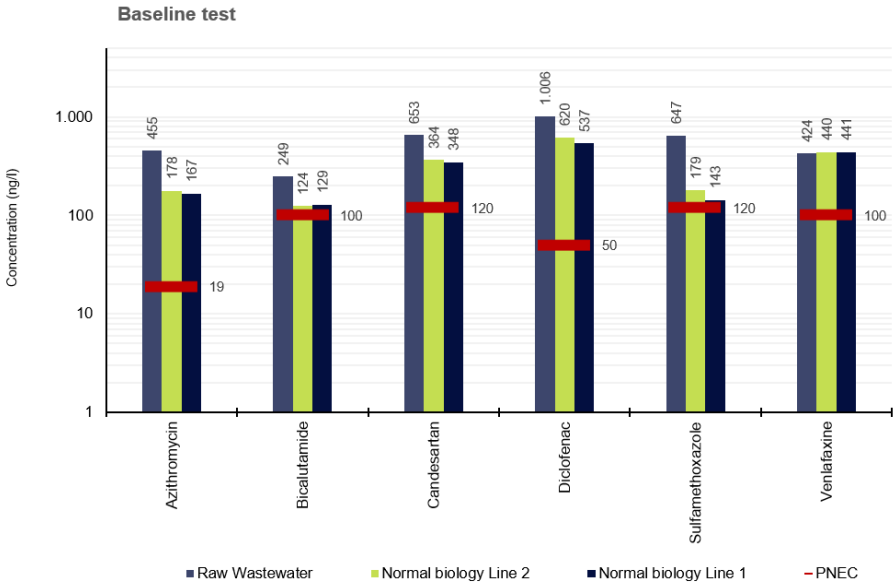


Figure 17 Average inlet concentration and outlet concentration from Brædstrup WWTP (n=9) of those 6 micropollutants above PNEC (red line) in the fall of 2018 (Baseline). Note logarithmic scale.

7.6 Conclusion on the baseline tests

This phase tries to give an understanding on how Brædstrup WWTP operates without interference of the conventional activated sludge line. In total 36 micropollutants were analysed over 3 sampling campaigns each of 3 days length, 24 h flow proportional. The campaign was done in the fall of 2018 (more precisely in September (d.12., 13., 18.), October (d. 10., 12., 16.), and November (d. 9., 13., 29.)). This work helped answer, at least somewhat, several fundamental questions of the performance of Brædstrup WWTP and to some degree which micropollutants are present in the incoming wastewater.

Inlet concentration varied from one day to another day. Among 36 compounds, 31 substances quantified in the influents at least once during the sampling campaign. The inlet concentration also varied for individual compounds during the week. The most abundant pharmaceuticals in wastewater are Paracetamol, Gabapentin, and Ibuprofen, which mainly originates from domestic wastewater. Although, Brædstrup WWTP does not receive hospital effluent, x-ray contrast media was still found in high concentrations compared with the other micropollutants. An explanation could be the fact, that people gets contrast media in the hospital, however, it simply does not fully pass through the body before going back home.

The variation in total concentration of selected compound and for individual compounds during the 9 days of sampling, could be due to different factors as discussed in this report. According to literature review, the same results have been reported in many other studies in other municipal wastewater treatment plants worldwide.

The fate of micropollutants a WWTP depends on their physio-chemical characteristics. Given their diverse properties (e.g., hydrophobicity and biodegradability) and low concentrations, micropollutant removal is commonly incomplete and variable, ranging from negative elimination to 100% removal (as found in Brædstrup). According to literature, biological treatment is commonly unable to remove all micropollutants. However, its efficiency can be improved under favourable conditions (e.g., extended SRT and HRT, warm temperature and fine-tuning redox conditions). The average percentage removal of all compounds, in inlet and outlet, was 57% for Line 1 and 59% in Line 2, during the sampling campaign.

Effluent is discharged to a small recipient nearby, which then is connected to a larger stream, Gudenåen. PNEC for 27 out of 36 micropollutant were available and 11 had inlet concentrations above their respective PNECs. However normal biology could remove 5 of those below their PNEC in both lines, but 6 were still above in the effluent of the lines and thereby posing a threat to the recipient waterbody. These compounds were Azithromycin (antibiotic), diclofenac (anti-inflammatory), Sulfamethoxazole (antibiotic), Venlafaxine (antidepressant), Candesartan (blood pressure medicament) and the antiandrogen Bicalutamide.

8 Multiple point ozonation in full-scale test

8.1 Occurrence of pharmaceuticals in treated water after multipoint ozonation

The first full-scale trial with multiple ozonation with a mixed liquor dose of 3.98 mgO_3/l and a tertiary dose of 7.20 mgO_3/l lasted from March-July 2019. During this period, 2 sampling campaigns have been conducted, called as Ozone Condition 1 midway campaign in May 2019 and and Ozone Condition 1 Endway campaign in July 2019. Analysis results after multiple point ozonation show only 4/30 compounds were above detection limit. These being bicalutamide, iomeprol, ∑4+5-Methylbenzotriazol and 1H-Benzotriazol, none of which were above their subsequent $PNEC_{freshwater}$.
In ozonation condition 2 the mixed liquor dose increased to 7.20 mgO_3/l while the tertiary dose decreased to 3.98 mgO_3/l. Analysis results after multiple point ozonation showed more compounds detected (10/32). Both full-scale multiple ozonation condition 1 and condition 2, removed to a large extent all the organic micro pollution below detection limits.

8.2 Removal efficiency after multipoint ozonation

Removal rate (Rw%) during the ozonation condition 1 calculated for 21/30 compounds, corresponding to 70% of the investigated substances. Contrary, it was not possible to calculate Rw% for the rest, or about 30%. These compounds were either never measured above detection limit or not fulfilling the removal calculation rules (see Appendix A for a detailed description).

A total of 6 sampling days in May and July 2019 was done. The specific dates can be found in table 5. As seen with the concentrations during baseline testing, we do observe a high removal different pattern for each substance. Removal efficiencies from negative to almost 100% were observed, depending on the compound and the sampling day.

The removal efficiency has been calculated after just mixed liquor ozonation and after tertiary ozonation as complementary treatment step. The mixed liquor ozonation was performed on full wastewater volume while the tertiary ozonation was conducted in a 10 m^3/h ozone contact tower with a contact time of 10 minutes.

Some of the hard-biodegradable compounds, such as venlafaxine, tramadol, and carbamazepine, showed a much better removal after mixed liquor ozone dosage. E.g. Venlafaxine were negatively removed (-9%) in the reference line at the same days, while mixed liquor dose of 3,98 mgO_3/l in the mixed liquor got it removed up to 45%. Adding the tertiary ozonation with a dose of 7.20 mgO_3/l remarkable 99% was removed. (*Figure 18* and *Figure **19***).

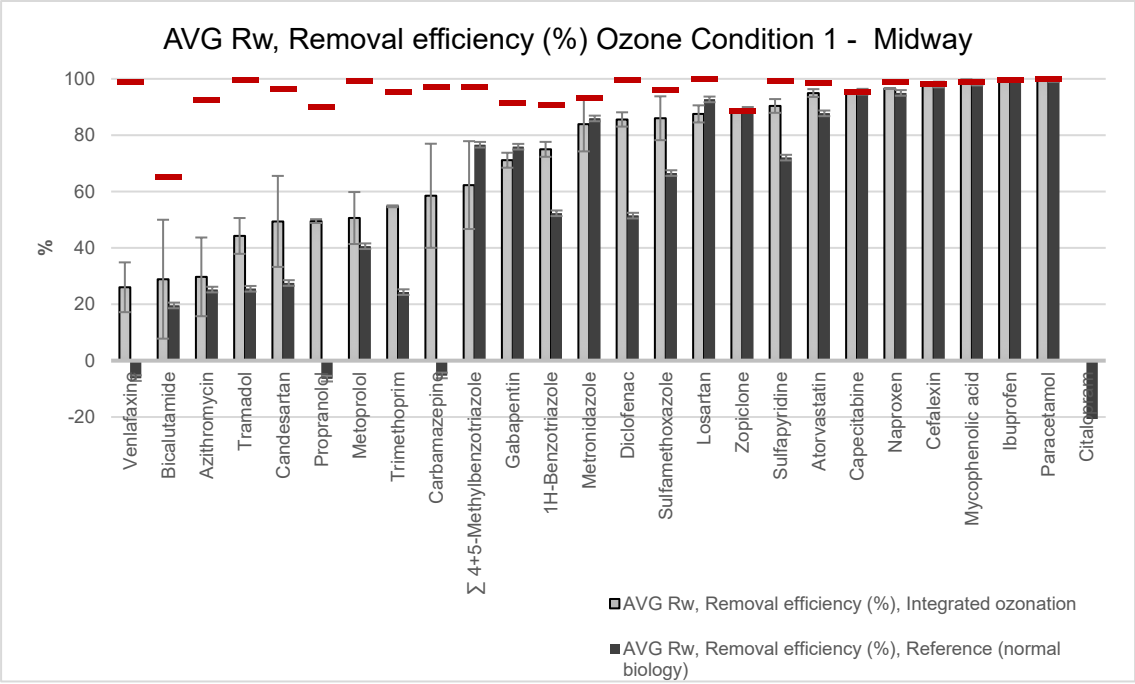


Figure 18 Compound specific removal efficiency during Ozonation condition Midway campaign in percentage with standard deviations for 25/30 compounds that fulfilled the rules for removal percentage calculation during the first campaign of multiple point ozonation. (n=3). Results shown against reference line with no interference within the biology.

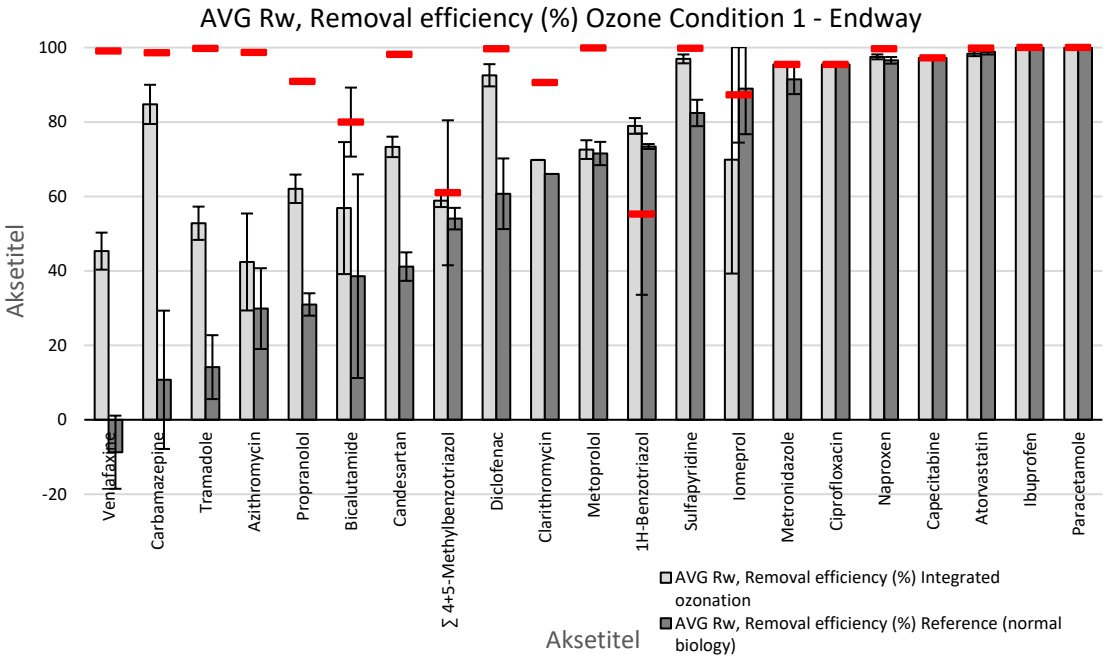


Figure 19 Compound specific removal efficiency during Ozonation condition Endway campaign in percentage with standard deviations for 21/30 compounds that fulfilled the rules for removal percentage calculation during the first campaign of multiple point ozonation. (n=3). Results shown against reference line with no interference within the biology.

The average removal of pharmaceuticals after mixed liquor ozonation during the ozone condition 1 Midway campaign was 72% and during Endway campaign was 78% and when having the tertiary ozonation step 95% and 93% removal were observed, respectively. In the same periods, in May 2019 and July 2019, the conventional biology within the reference line removed by 59% and 63% respectively.

The removal efficiencies in Midway in both Line 1 and Line 2 are relatively lower than the Endway campaign. The reason can be the difference in Mixed liquor temperature in process tanks, which in average increased from 14 °C in May to 18 °C in July.

Compare to the results in Baseline tests, during ozonation condition 2, the hard-biodegradable compounds, such as venlafaxine, tramadol, and carbamazepine, showed much better removal after mixed liquor ozone dosage. Recall, that the ozonation dosages were switched around in the ozonation condition 2 – mixed liquor dose of 7.20 mgO_3/l and a tertiary dose of 3,98 mgO_3/l. Higher mixed liquor ozone dose does not show a significant impact venlafaxine removal rate, while with a mixed liquor dose of 3.98 mgO_3/l, Rw% is 61%, and by increasing the mixed liquor ozone dosage to 7.20 mgO_3/l it gave a Rw% of 69%. The antibiotic azithromycin however shows varied removal rate - 92 % in ozone condition 1, midway campaign, 42% in endway campaign and 83% in the second ozonation condition.

All in all, the same trend of removal can be seen, however, lowering the tertiary ozonation dosage lowers the total average removal from 93% to 86% (*Figure **20***). Suggesting, the optimal division between the dosages are as tested in the ozonation condition 1, i.e. having a low mixed liquor dose and a high tertiary dose.

The average removal of pharmaceuticals during ozonation condition 2 shows after mixed liquor ozonation 79% and when having the tertiary ozonation step an 86% removal was observed. In the same period, October 2019, the conventional biology within the reference line removed 65%.

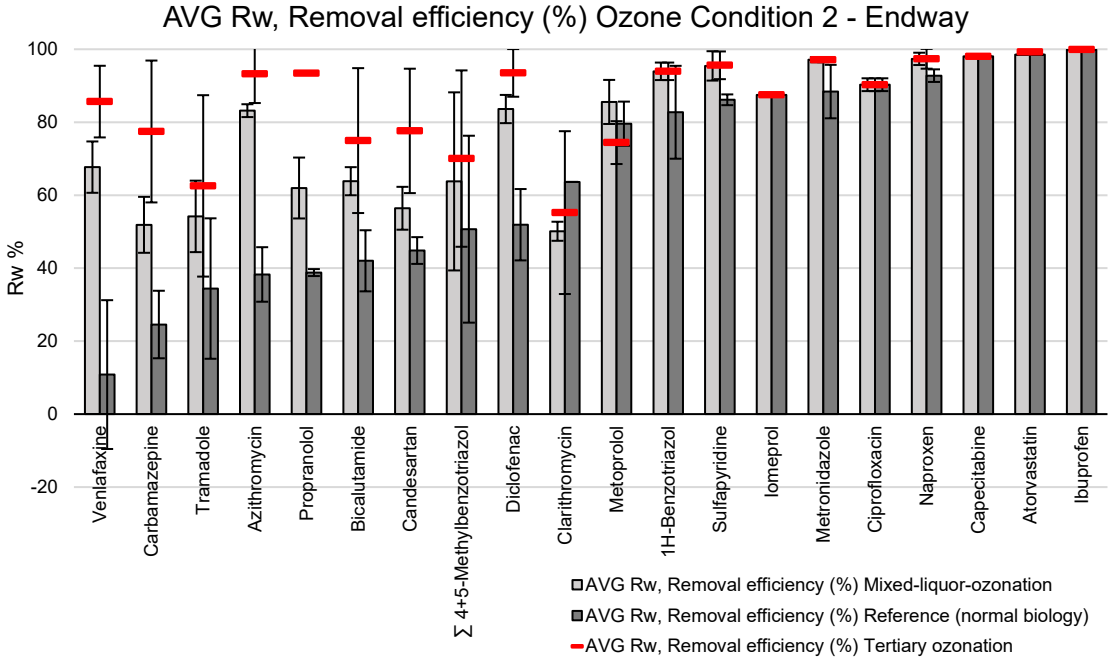


Figure 20 Compound specific removal efficiency in percentage with standard deviations for 20/32 compounds that fulfilled the rules for removal percentage calculation during the second campaign of multiple point ozonation. (n=3). Results shown against reference line with no interference within the biology.

8.3 Result evaluation based on PNEC value after ozone treatment

An average removal after multiple ozonation of 93% during the first ozone set-up (mixed liquor dose of 3.98 mgO_3/l and a tertiary dose of 7.20 mgO_3/l- endway campaign) was observed. If $PNEC_{freshwater}$ were to be used as a guiding limit it is important to study if a percentage removal is enough to reach the subsequent $PNEC_{freshwater}$. After the first condition of ozonation 9/30 substances were above $PNEC_{freshwater}$ in inlet. However, as seen during the baseline study, some compounds might be above $PNEC_{freshwater}$ (*Figure 21*) in the raw wastewater, but nearly or completely removed during the ozonation conditions. In July 2019, 4/9 were above $PNEC_{freshwater}$ in the raw wastewater but completely removed during normal treatment. Opposite the baseline study, bicalutamide was not above $PNEC_{freshwater}$ after conventional biological treatment (Line 2 - reference line) in July. The three others not above $PNEC_{freshwater}$ after conventional biological treatment were atorvastatin, ibuprofen, and paracetamol. However, the insecticide imidacloprid posed a potential negative effect on the receiving waterbody because conventional biology could not remove it below $PNEC_{freshwater}$ (*Figure 21*). Nevertheless, multiple point ozonation removed all harmful measured substances below $PNEC_{freshwater}$ and the full-scale multiple ozonation line did not, according to analytical program, discharge any expected harmful organic micropollution.

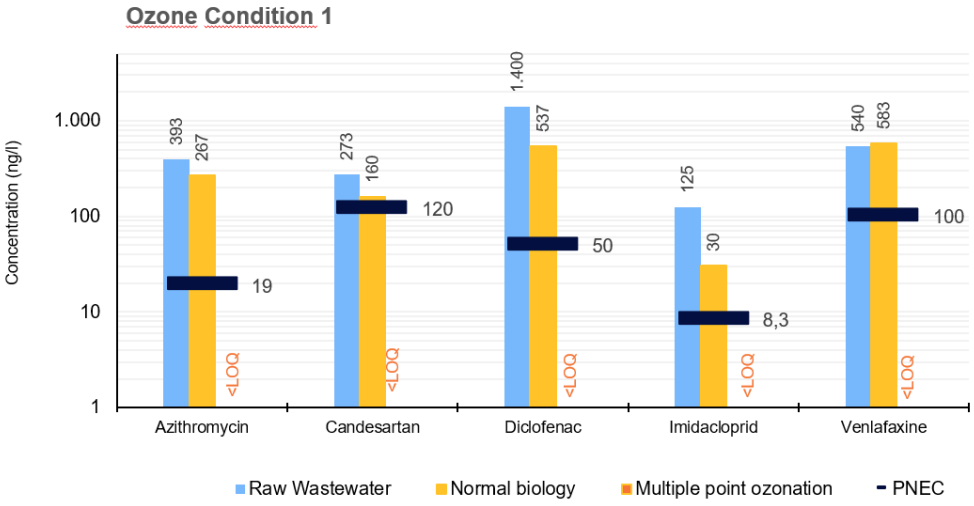


Figure 21 Average inlet concentration and outlet concentration from Brædstrup WWTP (n=3) of those 5 micropollutants above PNEC (red line) in July 2019 (first ozonation condition – endway campaign). Note logarithmic scale.

An average removal after multiple ozonation of 86% during the second ozone set-up (mixed liquor dose of 7.20 mgO_3/l and a tertiary dose of 3.98 mgO_3/l) was observed. During the second campaign of ozonation 5 compounds were observed above their PNEC in raw wastewater (inlet of the plant). However, only three are above in the effluent because the easy biodegradable ibuprofen is degraded along with the beta-blocking agent atorvastatin resulting in three compounds of concern (*Figure 22*).

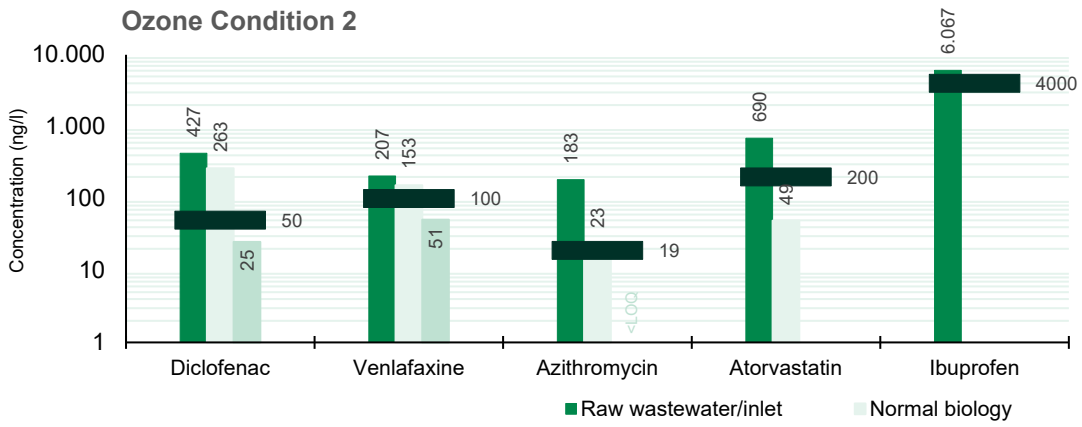


Figure 22 PNEC : The 5 substances with inlet concentrations exceeding their subsequent PNECfreshwater, along with the reference line and the effect of multiple point ozonation. Sampling in October 2019 – second condition of ozonation. (n=3). Note logarithmic scale.

Both ozonation experiments have shown promissing removal down to safe environmental concentrations making sure no ecological negative effects is discharged to the recipient. Further, it can be observed, that biological removal seem to battle with certain compounds namely the anti-depressant venlafaxine, the anti-inflammatory diclofenac and the antibiotic azithromycin.

8.4 Ecotoxicological tests

Among the advanced treatments available for micropollutant mitigation, tertiary ozonation has demonstrated high performances in removing a large range of contaminants while being economically feasible (Ikehata and Gamal El-Din 2005a, 2005b; Ikehata et al., 2006; Margot et al. 2013; Mousel et al., 2017). During the initial phase of ozone application, when the initial ozone demand (IOD) is not yet fulfilled, micropollutants degradation takes place both through direct – molecular ozone- and indirect – hydroxyl radical – oxidation pathways. Direct reactions during IOD occurs mainly with fast-reacting compounds, e.g., with a second-order rate constant above 104 $M^{-1} \cdot s^{-1}$, which reflects the high selectivity of ozone reactions (Buffle et al. 2006b; Wert, Rosario- Ortiz, and Snyder 2009). Moreover, during IOD the removal of these substances is controlled by the ozone mass transfer and thus can be achieved with very low contact time. Thus, due to the high selectivity of molecular ozone and the fast reaction with molecules ozone is often used as a tertiary treatment step (Domenjoud et al., 2017). In the field of wastewater treatment, ozone is commonly used for the removal and oxidation of refractory organic matter (COD) in industrial wastewater treatment as well as for the final disinfection of urban effluent (Lazarova et al., 2013; Paraskeva and Graham, 2002).

Objective of the ecotoxicity study was to qualify and quantify the impact of ozonation compared to the reference line effluent. Based on these findings an evaluation of the effect of ozonation – if negative effects could be seen after treatment with ozone. The ecotoxicological studies were only done at the end of the first campaign of ozonation. While in-vivo bioassays provide a response to the presence of all pollutants in the wastewater sample as well as their bioavailability and physical transfer into the test organism, a major advantage of specific in-vitro responses is that these may indicate which classes of chemicals and which types of effects may cause the main problem for aquatic organisms.

		Impact	Laboratory
In-vivo tests	Chronic reproductive test - Vibrio fischeri	Population growth	Eurofins Denmark

	Growth inhibition of unicellular green algae - Scenedesmus	Photosynthesis and growth	Eurofins Denmark
	Fish embryo/egg test - Danio rerio	Fish early life stages	Eurofins Denmark
In-vitro tests	ER Calux	Estrogen activity of human receptors	CIRSEE / France
	PXR-Calux	Xenobiotic effect	CIRSEE / France
	Ames test	Mutagenic activity	CIRSEE / France
	SOS chromotest	genotoxicity E coli	CIRSEE / France
	A-YES	Human estrogen receptor	IUTA / Germany
	A-YAS	Human androgen receptor	IUTA / Germany

In an extensive interlaboratory study by (Escher et al., 2014) a representative set of water samples was analyzed for a broad range of toxicological effects by using 103 unique in-vitro bioassays. The most relevant modes of toxic action identified in that study were related to nonspecific toxicity, xenobiotic metabolism (activation of aryl hydrocarbon receptor [AhR] and pregnane X receptor [PXR]), hormone-mediated mechanism of action (estrogenic, antiandrogenic, and glucocorticoid activities), and reactive mechanism of action (genotoxicity and oxidative stress). Consequently, we selected four bioassays among these most relevant in-vitro tests covering a variety of modes of action.

CALUX:

Chemical Triggered LUciferase gene eXpression (CALUX) is a bioassay based on the ligand-dependent nuclear receptor that is used to detect particular chemicals or chemical groups in samples. It consists of a modified cell line that has been stably transfected with a luciferase reporter gene with a DNA build under the control of receptor-specific DNA response components that can stimulate the inserted luciferase gene transcription and generate a light-generating enzyme that can be easily measured. In this study, CALUX bio-assays targeting the hormone disrupting Estrogen and PXR (pregnane X receptor) activities were selected due to the existing standard method (ERa CALUX, ISO 19040-3) and the presence of pharmaceuticals in the effluent. These assays were performed at Biodetection Systems (BDS), The Netherlands. The ERa analysis is accredited by ISO17025. Although no clear difference between the line 1 and 2 outlets can be observed (considering the measurement uncertainty for CALUX method is typically below 30%), the tertiary ozonation has a clear positive impact in decreasing the effect on biological activities for both assays.

Table 6 Results of estrogenic and xenobiotic CALUX -bioassays in inlet, one point of ozonation (mixed liquor ozonation), tertiary ozonation (multiple point ozonation), and no ozonation (outlet of reference line).

	ERa CALUX Estrogens ng 17b Estradiol eq./L	**PXR CALUX Xenobiotic** µg Nicardipine eq./L
Inlet	32	51
Mixed liquor O_3	0.46	43
Tertiary O_3	0.018	15
Reference line	0.25	63

For comparing the biological activity levels obtained in the line 1 or 2 outlets, we can also refer to a recently published study that overviewed the quality of effluent wastewater from twelve WWTPs of various size and using different treatment technologies from nine countries (Alygizakis et al., 2019). Excepted one sample that ERα was not detected, ERα responses ranged from

0.54 to 6.6 ng Estradiol eq./L. All samples were proved to be positive for xenobiotic metabolism (PXR), from 20 to 240 µg Nicardipine eq./L

To facilitate water quality assessment, effect-based trigger values (EBTs) have also been proposed. Bioassay responses above EBTs indicate potential risk of adverse effects to the ecosystem. The signal from each of the bioassays can then be compared with the proposed EBTs and allowed for ranking of the toxicity of wastewater effluents. EBTs for the used CALUX® bioassays were retrieved from the literature and are presented in *Table 7*. To date no standard consensus regarding these proposed values has yet been established. Nevertheless, it can be considered that in line 1 or 2 outlets and in larger extend after tertiary ozonation, ER and PXR activities are close or below the proposed EBT values.

Table 7 Effect-based trigger values (EBTs) currently proposed.

Assay	Unit	EBT	Reference
ERa CALUX	ng 17b Estradiol eq./l*	**0.1**	(Escher et al., 2018)
	ng 17b Estradiol eq./l	**0.3**	(Alygizakis et al., 2019)
PXR Calux	µg Nicardipine eq./l	**43**	(Escher et al., 2018)
	µg Nicardipine eq./l	**54**	(Alygizakis et al., 2019)
	(µg DEHP eq./l)	(272)	

**17b-estradiol is also designed under E2 natural hormone.*

SOS chromotest:

The SOS chromotest is a biological assay to assess the genotoxic potential of chemical compounds. The test is a colorimetric assay that by means of a fusion with the structural gene for β-galactosidase, tests the expression of genes induced by genotoxic agents in Escherichia coli. The SOS Chromotest bioassay was conducted at several concentration factors: 1x, 5x and 50x after SPE extraction of wastewater samples. These different concentration factors allow to determine the genotoxicity of the sample under several conditions. The test performed at concentration factor 1x makes it possible to estimate the genotoxicity of the sample under environmental conditions (without concentration factor) while the tests performed at concentration factors of 5x and 50x aim to look for the presence of genotoxic compounds that are less concentrated, but that may potentially lead to carcinogenesis processes in the event of bioaccumulation and/or chronic exposure, but in a more insidious way. Direct quantitative measurements of the genotoxic activity were also obtained from a total of three different dilutions (10, 25 and 50% v/v.) in triplicate for the four wastewater samples.

The SOS induction potency (SIP) is the quantitative parameter to compare the genotoxicity effect, calculated from ratio of the mean number of positive wells for the dose concentration divided by the baseline. The baseline is obtained by adding one standard deviation to the mean number of positive wells of the solvent control. A statistical test (Student test) was performed to determine the significant differences between the SIP obtained for each extract or sample concentration tested and the negative controls (DMSO with or without S9 - S9 is a crude liver enzyme extract that can, under certain conditions, convert materials without any genotoxic activity to active genotoxic entities). Values significantly different at risk 0.05 are marked with an asterisk.

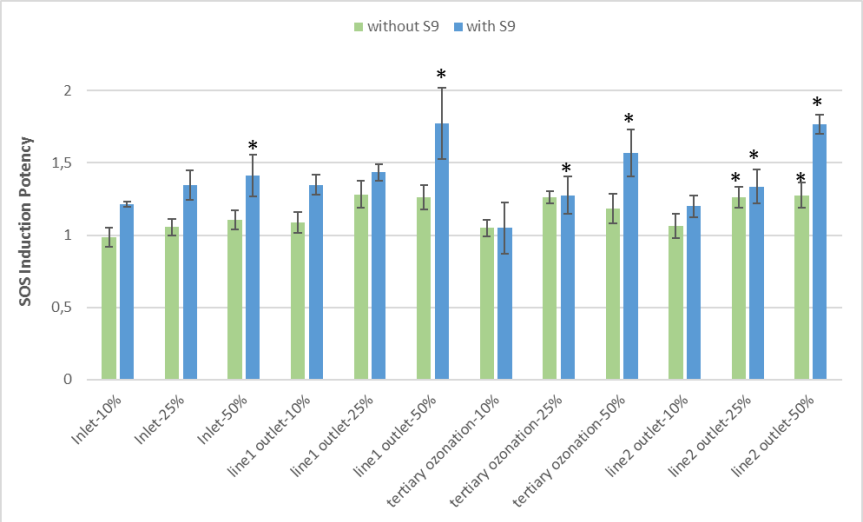


Figure 23 SIP (SOS induction Potency) obtained from several dilutions (10, 25 & 50% vol/vol) of the wastewater samples. Values significantly different at risk 0.05 are marked with an asterisk.

Table 8 SIP values with S9 activation as measured in samples at the dilution of 50% vol/vol. shown with standard deviation.

	SIP mean values (standard deviation)	Equivalent Benzo(a)Pyrene µM (standard deviation)
Inlet	**1.413** (±0.147)	0.9 (±0.4)
Mixed liquor O_3	**1.775** (±0.45)	1.90 (±0.67)
Tertiary O_3	**1.569** (±0.16)	1.33 (±0.44)
Reference line	**1.769** (±0.067)	1.88 (±0.18)

As shown on Figure 23 and Table *8*, the maximum genotoxicity potential is only observed in SOS chromotest conditions with S9 metabolic activation. Regarding SIP values, the sample exhibiting the highest genotoxicity potential is the outline 2 sample (reference) for which genotoxic activity is observed at the dilution of 25%. Otherwise, genotoxic activities are observed for the inlet, line1 outlet and tertiary ozonation at the dilution of 50%. The reference sample (line 2 outlet) is also the only one exhibiting a significant SIP without the S9 activation at the 50% dilution (SIP value 1.277) (*Figure 23*).

Although there is currently no standard consensus regarding a threshold value corresponding to the SOS-inducing potency (SIP) determined by SOS chromotest, Kocak (2015) summarized the different thresholds valued for SIP proposed in the literature. Regarding those thresholds, it can be concluded that all treated samples present a potential genotoxic activity with no significant decrease among the different types of treatment. Summary can be seen below in *Table **9***.

Table 9 Summary of genotoxic activity levels for the 4 samples (genotoxic activity for SIP >1.5, moderate activity for SIP between 1.2 and 1.5)

	SOS chomotest without S9	SOS chomotest with S9
Inlet	No genotoxic activity	**Moderate genotoxic activity**
Mixed liquor O_3	No genotoxic activity	**Genotoxic activity**
Tertiary O_3	No genotoxic activity	**Genotoxic activity**
Reference line	**Moderate genotoxic activity**	**Genotoxic activity**

Ames test:

SOS chromotest and Ames test are complementary and have been used together to broaden the detection capacity and to evaluate the overall genotoxicity in several studies.

Similarly, to the SOS Chromotest, Ames bioassay was conducted at several concentration factors: 1x, 4x, 12x, 40x, 125x and 400x after SPE extraction of wastewater samples. These different concentration factors allow to determine the genotoxicity of the sample under several conditions. The test performed at concentration factor 1x makes it possible to estimate the genotoxicity of the sample under environmental conditions (without concentration factor) while the tests performed at highest concentration factors aim to look for the presence of genotoxic compounds that are less concentrated, but that may potentially lead to carcinogenesis processes in the event of bioaccumulation and/or chronic exposure, but in a more insidious way. The range of concentration factors allows also to establish a dose-response curve. The mutagenic potential of substances is assessed directly and in the presence of metabolic activation. A compound that shows a clear dose response and/or yields multiple fold inductions greater than 2.0, is classified as a mutagen. The mutagen activity of the different samples are summarized in *Table **10***.

Table 10 Summary of mutagenic activity for the 4 samples – Inlet, Mixed liquor O3, Tertiary O3, and Reference line. (smallest concentration factor with fold induction >2.0- ozonation condition 1- endway campaign)

	TA 98 strain-without S9	**TA 98 strain-with S9**	**TA 100 strain-without S9**	**TA 100 strain-with S9**
Inlet	No mutagenic activity	**Mutagenic activity (12.68X)**	No mutagenic activity	No mutagenic activity
Mixed liquor O_3	**Mutagenic activity (40.06X)**	**Mutagenic activity (40.06X)**	No mutagenic activity	No mutagenic activity
Tertiary O_3	Probable mutagenic activity (400X)	Probable mutagenic activity (400X)	No mutagenic activity	No mutagenic activity
Reference line	**Mutagenic activity (40.06X)**	**Mutagenic activity (40.06X)**	No mutagenic activity	No mutagenic activity

A-YES and A-YAS

Yeast Estrogen Screen = YES, Yeast Androgen Screen = YAS

In order to detect estrogenic (YES) and androgenic (YAS) activities of natural and synthetic compounds, mixtures and environmental samples, these in vitro screens were created. The test is based on genetically modified yeast cells (Saccharomyces cerevisiae), which contain the gene for the human estrogen or androgen receptor coupled to a reporter gene (e.g. lacZ). YES assays were performed to evaluate the E2 equivalent (EEQ YES).

The levels of estrogenic activity seemed to be lower after multiple point ozonation compared to the reference line (EEQ YES = 20 ng/l in raw wastewater, 0.25 ng/l in reference line and 0.17 ng/l after multiple point ozonation). Contrary, the androgenic levels of the two lines seemed rather similar (see below).

Summary of ecotoxicity studies:

Highlighted in bold are increased levels.

Ecotoxic effects	**In-vivo tests**		
	Green algae inhabitation	**Vibrio Fischeri**	**Fish embryo**
Inlet	1	>800	1
Outlet Reference	**1**	**>800**	**1**
Multiple point ozonation	1	>800	1

Ecotoxic effects	In-Vitro tests					
	ER Calux (Estrogens ng 17b Estradiol)	**PXR Calux (Xenobiotic µg Nicardipine)**	**SOS chromotest**	**Ames test (mutagenic activity)**	**A-YES (Estrogenic activity)**	**A-YAS (Androgenic activity)**
Inlet	**Very High Risk**	**High risk**	Low/medium genotoxicity	Medium mutagenic activity	20	140
Outlet Reference	**High Risk**	**High risk**	**Medium/high genotoxicity**	**Medium/high mutagenic activity**	0.25	0.37
Multiple point ozonation	No risk	No Risk	Medium genotoxicity	Very low mutagenic activity	0.17	0.57

Understanding the role of specific and sublethal modes of action, such as endocrine disruption, is increasingly important in regulatory assessments of wastewater effluents. There is considerable interest in this approach because effect-based methods can measure the biological activity of environmental samples in a cost-efficient way at very low concentrations. Although such effect-based tools could provide much insight into specific toxic activities of effluents, the interpretation of these data with respect to ecological outcomes remains a challenge due to the lack of Effect-based trigger values (EBT).
First, the results showed that for all the 4 in-vitro bioassays, the lowest biological activity was observed for the tertiary ozonation sample. Comparing outlets of line 1 (with ozonation) and line 2 (without ozonation), similar and never worse biological activities were observed after ozonation.

Based on some figures recently proposed by scientific consortia (i.e. effect-based trigger value 0.4 ng/L EEQ (17b-estradiol equivalents) applicable for both Estrogen CALUX and YES based assays), all the treated samples were below or close to proposed EBT, at least for CALUX Estrogen assay.

8.5 Antibiotic resistance – genes and bacteria

The occurrence and spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) is a serious health protection problem worldwide. WWTPs are an important reservoir in the development of drug resistance phenomenon and they provide a potential route of antibiotic resistance gene (ARGs) dissemination in the environment (Leonard et al., 2015). According to the World Health Organization, the occurrence of antibiotic resistance among bacterial populations is regarded as one of the major hazards and challenges to public health in the 21st century (WHO, 2014), since it has serious economic consequences as well as risks to the health and lives of both humans and animals. One of the main reservoirs of ARB and ARG pollutants and the source of their spread in the natural environment (Kotlarska et al., 2015) are wastewater treatment plants (WWTPs) and treated wastewater discharged to surface water bodies (Osinka et al., 2019). WWTPs receiving high concentrations of microbial contaminants with wastewater from hospitals, agriculture, and industry stimulate the transfer of genetic information between pathogenic and environmental microorganisms. In addition, the conditions prevailing in wastewater treatment plants, such as a high content of microorganism populations, the relative abundance of nutrients, and the presence of sub-threshold levels of antibiotic substances in wastewater (Rizzo et al., 2013), provide an environment favourable for the survival of ARB and the transfer of ARGs.

WWTPs are not specifically designed to remove the antibiotics ARB and ARGs (Osinka et al., 2019). The main requirement imposed on WWTPs is to ensure the optimum values of organic matter, nitrogen, and phosphorus, since the discharge of wastewater containing high levels of these elements may contribute to oxygen depletion and an increase in the trophic state of the receiving waters. However, the penetration of microbial contaminants with the treated wastewater is not usually subject to regulations or monitoring. It should be mentioned that, despite the reduction in the total number of bacteria and ARB in the wastewater treatment process, large numbers of bacteria exhibiting multi-drug-resistance characterized by higher virulence could still penetrate into the environment with the wastewater (Osinka et al., 2017). Objective of the antibiotic resistance study was to qualify and quantify the impact of ozonation compared to the reference line effluent. Based on these findings an evaluation of the effect of ozonation to reduce antibiotic resistance compared with conventional activated sludge. The effect of ozonation was also investigated at a microbiological and genomic level, by studying the efficiency of the process with respect to the inactivation of antibiotic-resistant heterotrophic bacteria, as well as to the reduction of the abundance of selected antibiotic resistance genes.

Antibiotic resistant bacteria (ARB):

Five antibiotic targets were selected based on their coverage of different antibiotic classes: sulfamethoxazole (sulfamides), trimethoprim (diaminopyrimidines), ciprofloxacin (fluoroquinolones), ofloxacin (fluoroquinolone) and the Extended Spectra Beta Lactamase resistance, ESBL (β-lactam family - cephalosporine resistance).

Figure 24 gives an overview of the measured concentrations (coli forming units per millilitre - cfu/ml) at Brædstrup WWTP Inlet, outlet reference line, and outlet after multiple point ozonation for heterotrophic plate count (HPC), ciprofloxacin (CIP)-resistant HPC, sulfamethoxazole (SMX)-resistant HPC, Trimethoprim (TMP)-resistant HPC and ofloxacin (OFL)-resistant HPC, and the ESBL producing E. coli on two specific dates. The sampling was performed in the end of each ozonation campaign.

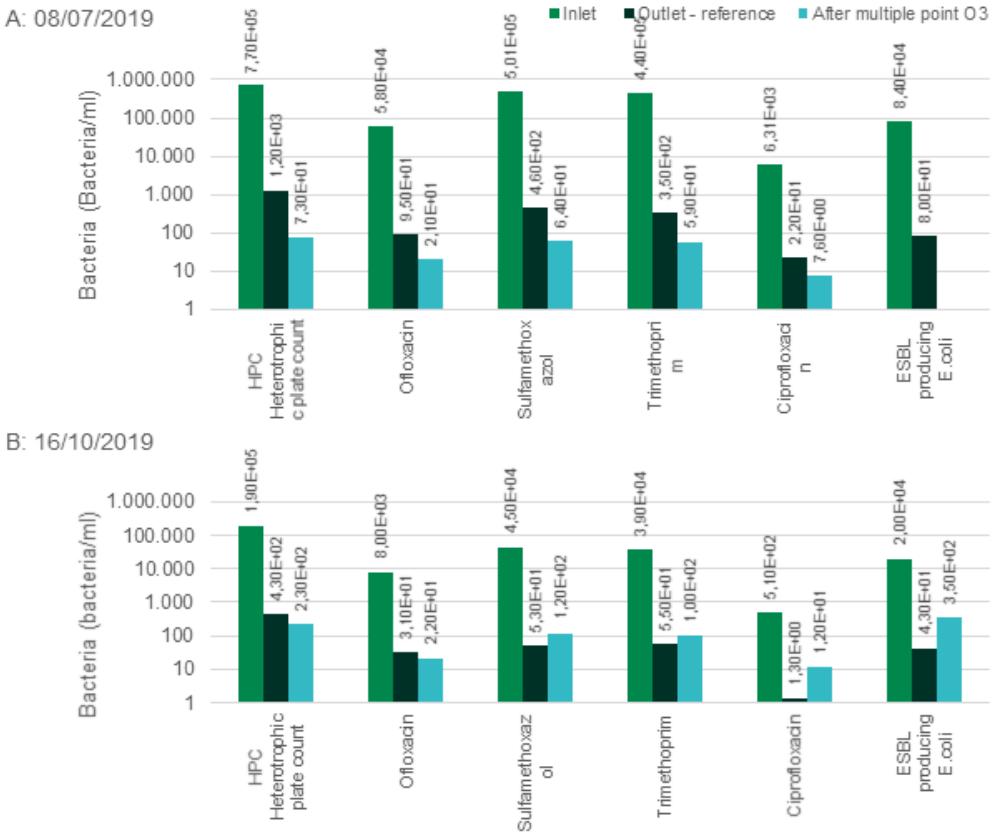


Figure 24 Concentration (cfu.ml-1) at Brædstrup WWTP Inlet, outlet reference line, and outlet after multiple point ozonation for heterotrophic plate count (HPC), ciprofloxacin (CIP)-resistant HPC, sulfamethoxazole (SMX)-resistant HPC, Trimethoprim (TMP)-resistant HPC and ofloxacin (OFL)-resistant HPC, and the ESBL producing E. coli (note unit cfu/100ml). A : the 8th of July 2019 and B : the 16th of October 2019. Note: logarithmic scale.

In inlet samples, sulfamethoxazole and trimethoprim-resistant bacteria are the two most prevalent ARB. In July, their respective concentrations and abundances were higher (65% and 58%) than in October (24 and 20% respectively). For all the targeted antibiotics, the proportion of ARB in October were about two-fold lower than in July, suggesting the possible variations in ARB abundance, depending on the collection flows and multiple sources of antibiotic resistance.

Similarly, multidrug-resistant ESBL E. coli strains are present in untreated wastewater, in ratio of ESBL-Ecoli to total number of E. coli (0.76 and 0.71% in both sampling campaigns), in similar values of previous published study (0.56 to 0.75%) based on Swedish wastewater samples (Jørgensen et al., 2017).

Results from the 8th of July (Figure 24A) from quantification of antibiotic resistant bacteria clearly showed that multiple point ozonation reduced the concentration of antibiotic resistant bacteria compared directly to the reference line in which only regular biology existed. At this point the doses of ozone were split as follows – a low dose of 3,98 mgO_3/l in the mixed liquor, and a high tertiary dose of 7,20 mgO_3/l. This division of dosages seemed to be efficient as a disinfectant, especially E. coli had an extremely positive removal after being subjected to ozone. However, during the second condition of ozonation, in which the dosages were switched, the effect of ozonation did not seem to be as effective, suggesting a higher tertiary dose of ozone to have the highest effect on ARB and e. coli.

The removal of ARBs and E. coli can be seen in Table 11. In outlets of secondary clarifiers of the reference line (line 2), similar log-removals in reference to inlet concentrations were observed from July and October conditions, with an overall efficiency comprised between 2.5 - 3 logs, whatever the resistant or non-resistant bacteria population. Log reduction is a measurement of how thoroughly the concentration of a contaminant is reduced by a decontamination process. The ratio of the levels of contamination before and after the process is known as the common logarithm, so an increment of 1 corresponds to a reduction in concentration by a factor of 10.In a recent review (Hiller, Hübner, Fajnorova, Schwartz, & Drewes, 2019), it was showed that conventional treatment performance could be somewhat similar (from 1 to 3 log removal), according the antibiotic target and the type of biological treatment. When comparing the log removals after mixed liquor ozonation (line 1) with the reference line (line 2), an incremental removal was observed in July (+0.3 to +0.8 extra log removal).

However, while the applied ozone dose in the mixed liquor in October was higher than that applied in July, no supplementary removal was observed, in comparison with the reference line. This statement was observed whatever the targeted quantification (heterotrophic plate count or E. coli, associated to antibiotic resistance or not). This can be explained that even if the disinfection efficiency of ozone depends on the ozone exposure, in wastewater matrices, the concentrations of DOC, suspended solids (SS), residual nitrite concentrations, and particulate matter can result in a rapid depletion of ozone and conduct to a limited efficiency for disinfection and antibiotic resistant bacteria.

The impact of the tertiary ozonation also depended on the applied ozone dose, according the July (highest ozone dose) and October (lowest ozone dose). In July, the multipoint ozonation achieved an additional log-removal from +0.4 to +1.2 in comparison with the reference line. A similar trend was observed for E. coli and ESBL-producing E. coli for which the removal efficiency after tertiary ozonation was up to 1.9 log higher than for the reference line.

By contrast, in October, no higher removal of ARB was observed in the line equipped with ozonation whatever the treatment step (mixed liquor only or combined with tertiary ozonation). However, another noticeable difference between July and October results is the pattern observed after tertiary ozonation for E. coli and ESBL-producing E. coli with high concentration observed in October (above 200 MPN/ml and 350 CFU/100ml respectively) while concentrations below the limit of quantification were observed three months before for both.

Table 11 Log-Removal after treatment stages for heterotrophic plate count (HPC), ciprofloxacin (CIP)-resistant HPC, sulfamethoxazole (SMX)-resistant HPC, Trimethoprim (TMP)-resistant HPC and ofloxacin (OFL)-resistant HPC, Total E.coli, and the ESBL producing E. coli.

Antibiotic resistant bacteria	**Total HPC**	**CIP-HPC**	**TMP-HPC**	**OFL-HPC**	**SMX-HPC**	**Total E. coli**	**ESBL-E.coli**
July 8th, 2019							
Inlet / Outlet line 1 - after mixed liquor O3	3.6	2.8	3.8	3.3	3.8	4.2	3.4
Inlet / Tertiary Ozonation	4.0	2.9	3.9	3.4	3.9	>5.3	4.9
Inlet / Outlet line 2	2.8	2.5	3.1	2.8	3.0	4.0	3.0
October 16th, 2019							
Inlet / Outlet line 1 - after mixed liquor O3	2.6	2.3	3.7	2.4	2.6	3.1	2.5
Inlet / Tertiary Ozonation	2.9	1.6	2.6	2.6	2.6	2.0	1.8
Inlet / Outlet line 2	2.6	2.6	2.9	2.4	2.9	2.5	2.7

Antibiotic Resistant Genes (ARG):

Antibiotic resistance genes (ARGs) encode the ability for bacterial cells to grow in the presence of antibiotics. The main important trait of ARG is their ability of being shared among bacteria through horizontal gene transfer mechanisms and this trait is likely the greatest challenge to combating antibiotic resistance. ARG are quantified by molecular methods, which target DNA (here DNA sequences encoding for antibiotic resistance genes) and their molecular quantification present the main advantage to avoid culture bias since it is estimated that 90-99% or more of environmental bacteria are not readily cultured using standard methods.

For each sample, 100 ml are concentrated by membrane filtration and subsequent to extraction of DNA from respective filters, ARGs are typically quantified by quantitative Polymerase Chain reaction (qPCR) using a wide range of primers that target specific ARGs as available in the literature (Gorecki et al., 2019).

In this study, different genes conferring resistance to β-Lactam Antibiotics (blaKPC), quinolones (qnrS), sulfonamides (sul1), as well as a class 1 Integrase (intI1) were screened, quantified and normalized against the total bacteria population using 16SrRNA gene quantification.

As observed in the ARB-based study, sulfonamide resistance gene (sul1) and integrase coding gene (intI1) are the most prevalent ARG (*Figure 25*). Although the use of sulfonamide antibiotics in humans is not so extensive anymore (10% of the total antibiotic consumption), their high prevalence may be the result of the combination of prolonged use of these antibiotics in both humans and animal husbandry, their association with Mobile Genetic Elements such class1 integron and the presence of residues from these antibiotic families in wastewater.

Except for the IntI1 concentration that was significantly higher in October sample, similar ranges of ARG levels were found in the two sampling conditions. To compare the levels of ARG concentration found in the influents and effluents, one can refer to the cross-sectional study performed on influents and effluents of 62 conventional Dutch WWTPs. While the absolute concentrations are higher in the Dutch cross study, the ratios sul2/16SrRNA or qnrS/16SrRNA are in the same range of values (from 0.5 to 0.7).

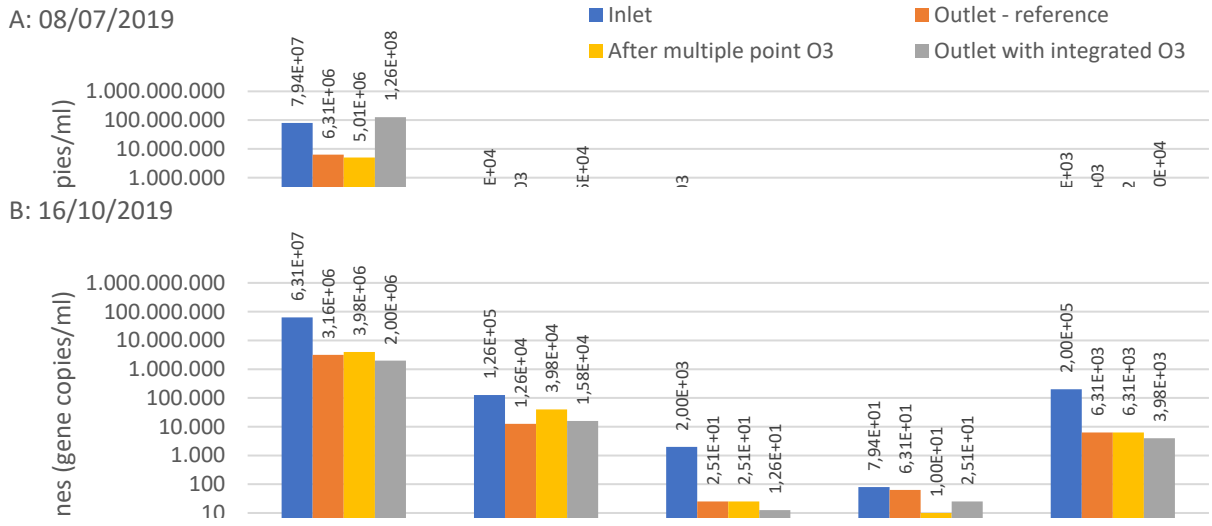


Figure 25 Concentration (gene copies.ml-1) at Brædstrup WWTP Inlet, outlet reference line, and outlet after multiple point ozonation for β-Lactam Antibiotics (blaKPC), quinolones (qnrS), sulfonamides (sul1), class 1 Integrase (intI1), the total bacteria population 16SrRNA gene quantification. A : the 8th of July 2019 and B : the 16th of October 2019. Note: logarithmic scale.

Table 12 Log-removal at the treatment stages for 16SrRNA gene copies and antibiotic-resistant genes (sul2, qnrS, Intl1 and blaKPC)

Antibiotic resistant genes	16SrRNA	Sul2	qnrS	Intl1	blaKPC
July 8th, 2019					
Inlet / Outlet line 1 – only mixed liquor O3	-0.2	-0.1	1.4	-0.5	-0.7
Inlet / Tertiary Ozonation (multiple point O3)	1.2	1.4	2.7	1.0	-0.6
Inlet / Outlet line 2 (reference)	1.1	0.9	1.2	0.4	-0.6
October 16th, 2019					
Inlet / Outlet line 1 – only mixed liquor O3	1.5	0.9	2.2	1.7	0.5
Inlet / Tertiary Ozonation (multiple point O3)	1.2	0.5	1.9	1.5	0.9
Inlet / Outlet line 2 (reference)	1.3	1.0	1.9	1.5	0.1

The log-removals of ARG are reported in *Table 12*. They ranged from zero (especially at the outlet of line 1, July samples) to around 2-log and with an average of 1.2-log (outlet of secondary clarifiers). These performances are in agreement with the Dutch published study in which ARGs and the class I-integrase gene were removed by an average 1.76 log reduction factor, in a similar extent than the total bacteria genes (measured as 16S rRNA gene), although a little bit higher than this study.

Fort both conditions, no significant effect of mixed liquor ozonation compared to the reference line (line 2) was observed; the high organic content of the mixed liquor may react with the majority of the produced oxidizing species, resulting in a small active fraction able to compromise DNA integrity into a condition in which it could not act as PCR template. Although in July, the tertiary ozonation had a higher removal effect (especially on qnrS and Intl1 genes) compared with the reference treatment line. However, no significant difference between multiple point ozonation and reference line was observed in the samples treated and collected in October. This could be explained by the decrease of the applied ozone dose between the two dates.

In a pilot case study (Iakovides et al., 2019), continuous ozonation in three experimental conditions (HRT and ozone dose, figure 5) was evaluated regarding its efficiency to remove selected genes and ARGs. One important observation made in this study was that the resistant E. coli decreased throughout ozonation, whereas the reduction of the abundance of the selected genes observed after treatment was apparently transient, the examined genes were detected after 72 h at almost the same levels as the initial values (1-log cycle below the initial), under dark and ambient temperature (22 to 25°C). Although samples were stored at around 4°C after samples, the 24-hour time used for composite sampling and well the time for shipment from Denmark to France could have replaced this transient incubation time.

Summary of the Antibiotic resistance study:

	ARB Abundance	**ARG** Abundance
Inlet	**High**	**High**
Outlet Reference	Low	Medium/low
Multiple point ozonation	Medium	**High/medium**

The capacity of ozonation operated in multipoint dosing, was also investigated in terms of its efficiency to inactivate antibiotic- resistant bacteria and to remove antibiotic resistance genes. In outlets of secondary clarifiers of the reference line (line 2), similar log-removals in reference

to inlet concentrations were observed from July and October conditions, with an overall efficiency comprised between 2.5 and 3 logs, whatever the resistant or non-resistant bacteria population.

Compared to the reference line, the additional log-removals induced by the multipoint ozonation dose towards total culturable bacteria resistant to CIP, OFL, TMP and SMX were respectively of about +0.3 to +0.8 after mixed liquor ozonation (whatever the applied dose) and +0.4 to +1.2 after tertiary ozonation with the highest dose. When decreasing the dose applied for the tertiary ozonation (October conditions), no benefits of the tertiary disinfection was observed towards the removal of ARB. Unlike the inactivation of culturable bacteria, the efficiency of ozonation on the removal of ARG was less noticeable (from 0 to 2.7 log removal in gene copies), suggesting the inability of ozonation to completely compromise the DNA integrity, either due to a less active oxidizing fraction (especially in the mixed liquor) or due to the inability of the quantitative PCR approach (based on the amplification of small DNA sequence within antibiotic resistance genes) to distinguish between damaged and undamaged DNA (Stange, Sidhu, Toze, & Tiehm, 2019). Nevertheless, the qPCR approach remains to date the most well-adopted method for quantifying abundance and treatment efficiency towards ARG.

9 Powder Activated Carbon addition in full-scale test

9.1 Occurrence of pharmaceuticals in treated water after PAC addition

20/33 compounds in raw wastewater was detected more than once at the three samples taken during PAC condition 1. During the PAC condition 2, 27/33 compounds was more than once detected above their limit of quantification. The total average detected concentration during condition 1 was 7,744 ng/l and 8,464 ng/l in condition 2. Compared with the average effluent concentrations of 347 ng/l for PAC condition 1 and 520 ng/l for PAC condition 2. At the same time in the reference line, the average effluent concentration in March 2020 was 615 ng/l and in May it was 1,032 ng/l.

9.2 Removal efficiency after PAC addition

Removal efficiency during the first condition of PAC addition is calculated for 18/33 investigated compounds, or about 55% of the investigated substances. Contrary, it was not possible to calculate Rw for the rest, or about 45%. These compounds were either never measured above detection limit or not fulfilling the removal calculation rules (see Appendix A for a detailed description). A total of 3 samplings campaigns in March was done. The specific dates can be found in table 5.

A high removal difference pattern for each substance concentration has been observed during PAC conditions. Removal efficiencies from negative to almost 100% were observed, depending on the compound and the sampling day.

PAC had a positive removal effect on the antiepileptic carbamazepine from 4% by biology to 61.2% when adding 5 mgPAC/l. Some did also seem not to be affected by the PAC, e.g. metronidazole (69% by conventional biology to 74.9% after sorption to PAC (Figure 26).

The average removal of pharmaceuticals during the first condition of PAC was 73% and the conventional biology within the reference line removed 45% in March 2020.

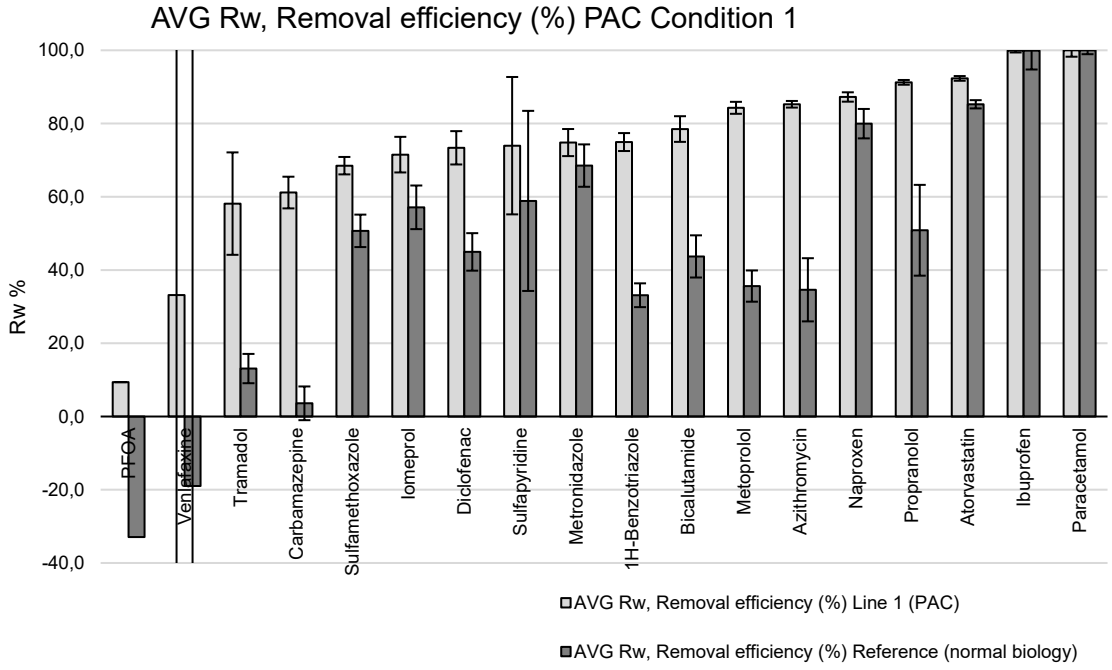


Figure 26 Compound specific removal efficiency in percentage with standard deviations for 18/33 compounds that fulfilled the rules for removal percentage calculation during the first condition of PAC addition. (n=3). Results shown against reference line with no interference within the biology.

Removal efficiency during the Second condition of PAC addition is calculated for 22/33 investigated compounds, or about 66% of the investigated substances. Contrary, it was not possible to calculate Rw for the rest, or about 34%. These compounds were either never measured above detection limit or not fulfilling the removal calculation rules (see Appendix A for a detailed description). During the second condition of PAC addition it was not possible to calculate removal efficiency (Rw%) for citalopram, propranolol, and metronidazole in the experimental line. A total of 3 samplings days in March was done. The specific dates can be found in table 5. Focusing on the average removal for the calculable data from the experimental line we reach a total removal efficiency of 74%. The reference line managed in May 2020 to reach 54% total average removal by biology (Figure 27).

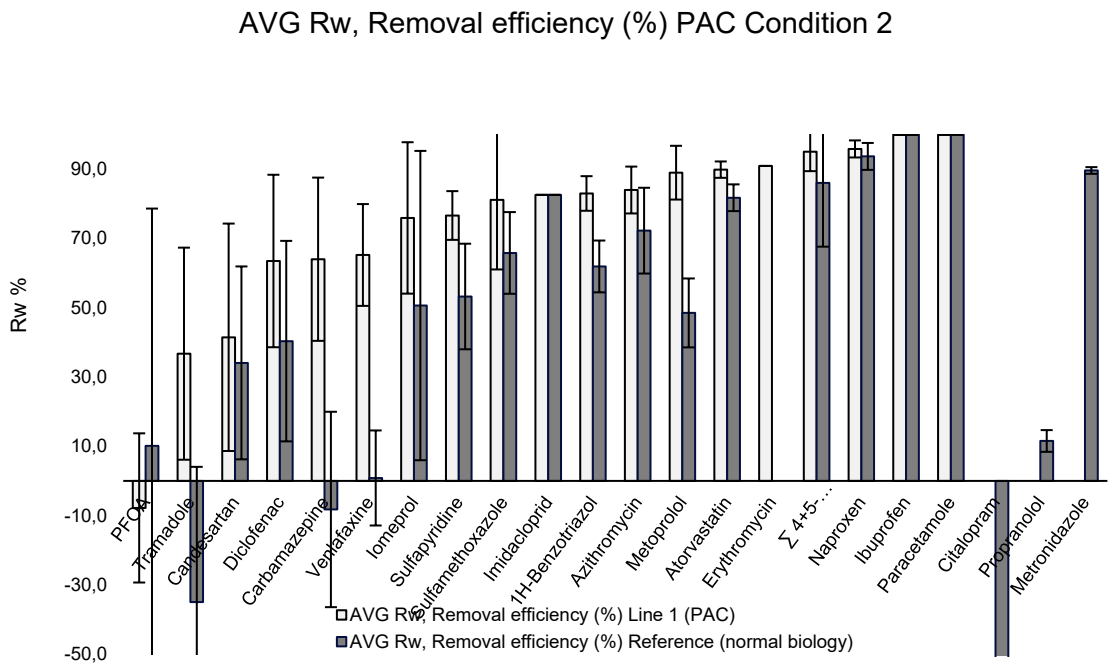


Figure 27 Compound specific removal efficiency (Rw) in percentage with standard deviations for 22/33 compounds that fulfilled the rules for removal percentage calculation during the second condition of PAC addition. (n=3). However, Rw for citalopram, propranolol, and metronidazole not calculable in experimental line. Results shown against reference line with no interference within the biology.

In summary the conventional biology removed between 45-54% during both conditions of PAC addition. Adding 5 mgPAC/l the test line managed to remove 29% more, reaching 73%. Doubling the PAC concentration, a removal of 74% (but only a 20% further reduction compared to conventional biology). For some molecules (benzotriazole, metoprolol, naproxen, PFOA), the reference line was more effective in removing them during campaigns at 10 mg/L than at 5 mg/L, which negatively influences the gain in removal obtained with 10 mg/L com-pared to 5 mg/L. By not taking into account these molecules which "distort" the results, a gain in removal of +35% is obtained with 5 mg/L and +45% with 10 mg/L. All in all, it ca be cocluded that there is an optimum PAC dose to remove certain molecules.

9.3 **Result evaluation based on PNEC value after PAC addition**

During sampling, after successfully adding PAC into the process tank of the experimental line (Line 1 during biological performance assessment at Brædstrup WWTP), it once again became clear, that at Brædstrup WWTP the same handful of compounds seems to be the hardest to remove and thereby getting below safe environmental guidelines ($PNEC_{freshwater}$). During 1.5 year of surveillance at Brædstrup the biology seems to have difficulty with reducing especially atorvastatin (beta-blocking agent, average inlet conc. 3,445 ng/l ±2284), azithromycin (antibiotic, average inlet conc. 322 ng/l ±229), diclofenac (anti-inflammatory, average inlet conc. 911 ng/l ±470), venlafaxine (antidepressant, average inlet conc. 357 ng/l ±334), and finally candesartan (blood pressure regulator, average inlet conc. 425 ng/l ±330). These are all well below the total average raw wastewater concentration of 6,877 ng/l across all 36 compounds studied.

From Figure 28A, 5 mgPAC/l got atorvastatin and azithromycin below the respective $PNEC_{freshwater}$ However, diclofenac and venlafaxine are not removed to safe concentration limits. Even though no significant difference in inlet concentrations on the specific sampling conditions can be noticed, it can be observed that venlafaxine is the only substance below $PNEC_{freshwater}$ after

addition of 10 mgPAC/l (Figure 28B). During the first PAC sampling the average inlet concentration of venlafaxine was 230 ng/l and during the second sampling condition it was 247 ng/l.

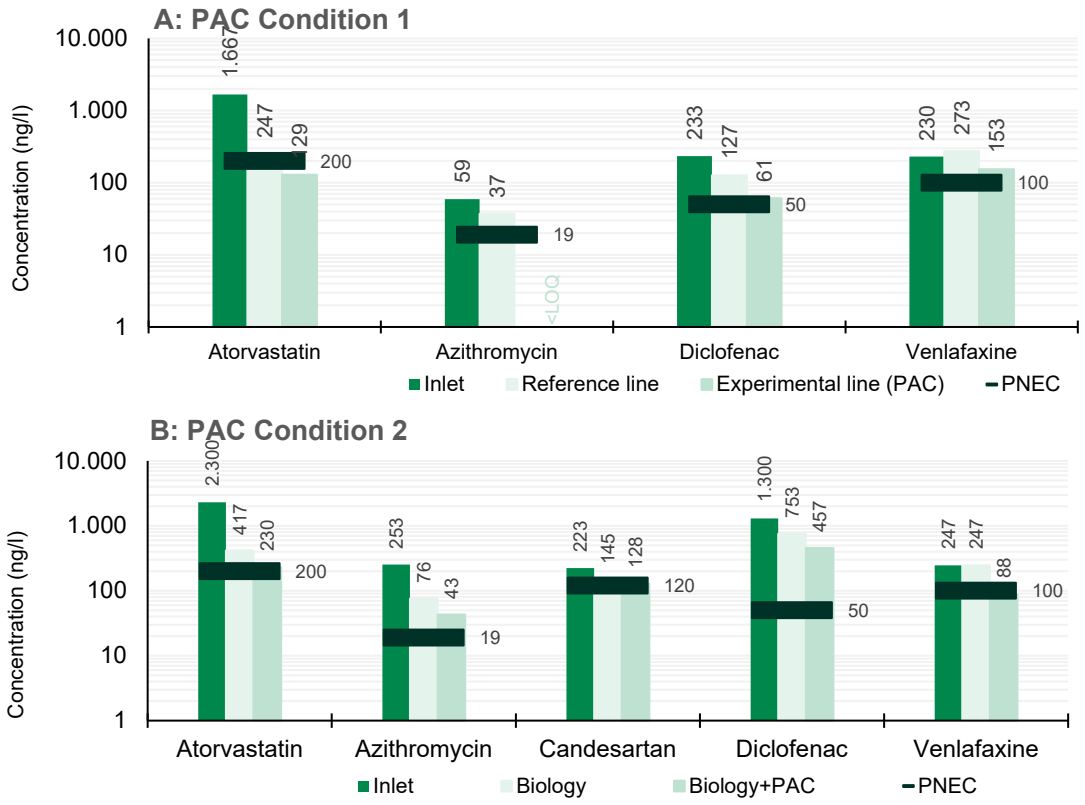


Figure 28 PNEC exceedance during the two conditions of Powder Activated Carbon (PAC) addition directly into the process tank of the experimental line. (n=3 for both conditions). Condition 1 = 5 mgPAC/l, Condition 2 = 10 mgPAC/l. Note logarithmic scale.

In summary one point PAC additions of 5 mgPAC/l and 10 mgPAC/l in aerated tank cannot stand alone to meet the PNEC values without complementary treatment.

10 Conclusions

33-36 selected compounds have been analysed in the inlet, outlet after conventional activated sludge treatment, and in the effluent after four different applications to one of the two lines at Brædstrup wastewater treatment plant (WWTP). The project has been ongoing for 18 months and a thorough monitoring of the biological performance have been performed. All sampling conditions consisted of three sampling days and all samples were 24-hours flow proportional samples. The study started in the autumn of 2018 (September 2018) and the experimental programme ended in the spring of 2020 (May 2020).

During baseline testing five pharmaceuticals were never above their Limit of Quantification (LQ) in the inlet of the plant. These being - the two antibiotics cefalexin and ciprofloxacin, the steroid medicament prednisolone and finally the antidepressant sertraline and psycholeptic zopiclone. Others were not above LQ in some days, but other days above, e.g. capecitabine and metronidazole. The top three most abundant pharmaceuticals (with concentration range) in the influent of the WWTP was paracetamol (7.100-160.000 ng/l), gabapentin (18.000-50.000 ng/l) and ibuprofen (9.000-39.000 ng/l). Even though, Brædstrup does not receive wastewater from hospitals, the two x-ray contrast media – iohexol and iomeprol - you would expect to find mainly in hospital effluents are found in the range 6.400-24.000 ng/l and 250-25.000 ng/l, respectively. Furthermore, a huge variation across the inlet concentrations of the compounds was found as in other studies - 11 ng/l for citalopram to 160.000 ng/l for paracetamol.

Especially, the ATC family of analgesics stand for a high percentage (46%) of the total load of substances. This can be explained by the very high inlet loads of paracetamol (an average inlet concentration of 111,304 ng/l, n=21). Contrary, the analgesics only stand for 10% in the effluent of Brædstrup WWTP which can be explained by the high biodegradation of paracetamol (an average effluent concentration of 435 ng/l, n=21).

The two separate treatment lines at Brædstrup WWTP, made it especially suited for direct comparison between conventional activated sludge (CAS) and CAS combined with multiple point ozonation or PAC addition. Thus, to say with certainty it is possible to compare the two lines, since the lines do not have the same configuration, several comparisons were made. The ratio of Line 1 (L1) versus Line 2 (L2) was 1.1, hence no significant difference in outlet concentration level is observed. Comparing the two lines removal capabilitie, we see an average ratio of 0.9, thus again supporting similarity across both lines.

Effluent is discharged to a small recipient nearby, which then is connected to a larger stream, Gudenåen. PNEC for 27 out of 36 micropollutant were available and 11 had inlet concentrations above their respective PNECs. However normal biology could remove 5 of those below their PNEC in both lines, but 6 were still above in the effluent of the lines and thereby posing a threat to the recipient waterbody. These compounds were Azithromycin (antibiotic), diclofenac (anti-inflammatory), Sulfamethoxazole (antibiotic), Venlafaxine (antidepressant), Candesartan (blood pressure medicament) and the antiandrogen Bicalutamide. No dilution has been used in this report. Thus, the outlet concentration is directly compared to $PNEC_{freshwater}$.

Ozonation conditions

In July 2019, the first full-scale trial with multiple ozonation finished and 3 days of sampling finished. After multiple point ozonation with an mixed liquor dose of 3.98 mgO_3/l and a tertiary dose of 7.20 mgO_3/l only 4/30 compounds were above detection limit. These being bicalutamide, iomeprol, ∑4+5-Methylbenzotriazol and 1H-Benzotriazol, none of which were above their subsequent $PNEC_{freshwater}$.
Contrary, after second ozonation condition were the mixed liquor dose was 7.20 mgO_3/l and tertiary dose 3.98 mgO_3/l multiple compounds could be detected (10/32). The full-scale multiple ozonation removed to a large extent all the organic micro pollution below detection limits.

Removal rate (Rw%) during the first condition of Multiple point ozonation could be calculated for 21/30 compounds, corresponding to roughly 70% of the investigated substances. Contrary, it was not possible to calculate Rw% for the rest, or about 30%. As seen with the concentrations during baseline testing, we observed a high removal difference pattern for each substance. Removal efficiencies from negative to almost 100% were noticed, depending on the compound and the sampling day. The mixed liquor ozonation was performed on full wastewater volume while the tertiary ozonation was conducted in a side stream (with a 100% treatment in those 10 m^3/h). Some of the hard-biodegradable compounds, such as venlafaxine, tramadol, and carbamazepine, already showed a higher removal after the mixed liquor ozone dosage. E.g. Venlafaxine were negatively removed (-9%) in the reference line at the same days, while an mixed liquor dose of 3,98 mgO_3/l in the mixed liquor got it removed by 45%. Adding the tertiary ozonation with a dose of 7.20 mgO_3/l remarkable 99% was removed. The average removal of pharmaceuticals during the first condition of multiple ozone was after mixed liquor ozonation 78% and adding tertiary ozonation step a 93% removal was observed. In the same period, July 2019, the conventional biology within the reference line removed 63%. Switching the ozone dosages around a total average removal of 86% was seen (79% mixed liquor). The small extra removal seen in the process tanks and the lowered overall removal support an optimal division between the dosages as in the first condition, i.e. having a low mixed liquor dose and a high tertiary dose.

After the first condition of ozonation 9/30 substances were above $PNEC_{freshwater}$ in inlet. However, as seen during the baseline study, some compounds might be above $PNEC_{freshwater}$ in raw wastewater, but get nearly or completely removed during the treatment line. Multiple point ozonation removed all harmful substances below $PNEC_{freshwater}$ and the effluent of the full-scale multiple ozonation line did not pose any threat to the environment. Both ozonation experiments have shown promising removal down to safe environmental concentrations making sure no ecological negative effects is discharged to the recipient. Further, it can be observed, that biological removal seems to battle with certain compounds namely the anti-depressant venlafaxine, the anti-inflammatory diclofenac and the antibiotic azithromycin.

Objective of the ecotoxicity study was to qualify and quantify the impact of ozonation compared to the reference line effluent. Based on these findings an evaluation of the effect of ozonation – whether if negative effects could be seen after treatment with ozone. The ecotoxicological studies were only done at the end of the first condition of ozonation. Ozonation did not increase the ecotoxicity, contrary a slightly positive effect of ozonation was observed.

Because antibiotic resistance, and not only organic micropollution (especially pharmaceuticals), causes an environmental negative effect in waterbodies as well, a part of this study was also to figure out the amount of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG). Five antibiotic targets were selected based on their coverage of different antibiotic bacteria classes:
sulfamethoxazole (sulfamides), trimethoprim (diaminopyrimidines), ciprofloxacin (fluoroquinolones), ofloxacin (fluoroquinolone) and the Extended Spectra Beta Lactamase resistance, ESBL (β-lactam family - cephalosporine resistance). Furthermore, different genes conferring resistance to β-Lactam Antibiotics (blaKPC), quinolones (qnrS), sulfonamides (sul1), as well as

a class 1 Integrase (intI1) were screened, quantified, and normalized against the total bacteria population using 16SrRNA gene quantification.
Compared to the reference line, the additional log-removals induced by the multipoint ozonation dose towards total culturable bacteria resistant to CIP, OFL, TMP and SMX were respectively of about +0.3 to +0.8 after mixed liquor ozonation (whatever the applied dose) and +0.4 to +1.2 after tertiary ozonation with the highest dose. When decreasing the dose applied for the tertiary ozonation (October conditions), no benefits of the tertiary disinfection was observed towards the removal of ARB. Unlike the inactivation of culturable bacteria, the efficiency of ozonation on the removal of ARG was less noticeable (from 0 to 2.7 log removal in gene copies), suggesting the inability of ozonation to completely compromise the DNA integrity, either due to a less active oxidizing fraction (especially in the mixed liquor) or due to the inability of the quantitative PCR approach (based on the amplification of small DNA sequence within antibiotic resistance genes) to distinguish between damaged and undamaged DNA (Stange, Sidhu, Toze, & Tiehm, 2019). Nevertheless, the qPCR approach remains to date the most well-adopted method for quantifying abundance and treatment efficiency towards ARG.

Powder activated carbon conditions
20/33 compounds in raw wastewater was detected more than once at the three samples taken during first PAC condition. During the second condition 27/33 compounds was more than once detected above their limit of quantification. The total average detected concentration during first condition was 7,744 ng/l and 8,464 ng/l in second condition. Compared with the average effluent concentrations of 347 ng/l for PAC condition 1, and 520 ng/l for the second condition. At the same time, the average effluent concentration in March 2020 was 615 ng/l and in May it was 1,032 ng/l.

Removal efficiency during the first condition of PAC addition were calculated for 18/33 investigated compounds, or about 55% of the investigated substances. A high removal difference pattern for each substance was observed.

PAC had a positive removal effect on the antiepileptic carbamazepine from 4% by biology to 61.2% when adding 5 mgPAC/l. Some did also seem not to be affected by the PAC, e.g. metronidazole (69% by conventional biology to 74.9% after sorption to PAC. The average removal of pharmaceuticals during the first condition of PAC was 73% and the conventional biology within the reference line removed 45% in March 2020. Removal efficiency during the second condition of PAC addition were calculated for 22/33 investigated compounds, or about 66% of the investigated substances. In the experimental line a total removal efficiency of 74% was reached. The reference line removed in May 2020 54% in average by biology.

This study has shown that the biological treatment at Brædstrup WWTP do not remove all substances below safe limits, but adding an extra treatment step of PAC it is possible to increase the removal efficiency and by multiple point ozonation, get all substances below safe environmental concentrations (PNEC levels) and furthermore increase the disinfection effect on the discharge.

In summary below the removal rates at different treatments:

	Biology at Brædstrup WWTP	Ozonation condition 1		Ozonation condition 2		Powder Activated Carbon condition 1	Powder Activa Carbon conditi
		Mixed liquor ozonation	Multiple point ozonation	Mixed liquor ozonation	Multiple point ozonation		
Average removal (%)	55%	78%	93 %	79%	86%	73%	75%

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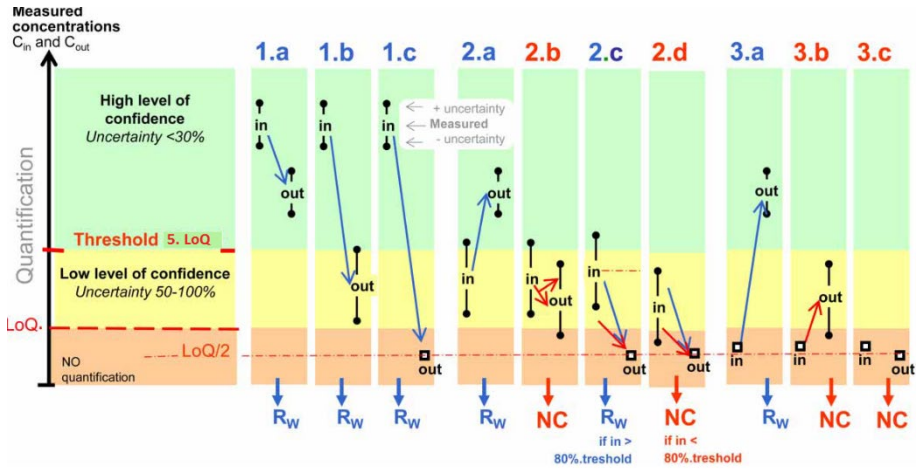
Appendix A: Removal efficiencies evaluation rules

The field of micropollutant removal by the treatment processes requires the use of special precautions for calculating their removal efficiencies. A rule of calculation of the elimination efficiencies (Rw) that integrates the global uncertainty (sampling + analysis) is applied to obtain results so-called "consolidated" elimination efficiencies.
Calculations account for measurement uncertainty of the micropollutant concentration, which will differ depending on concentration level and sample type.
In this study, a new ruleset is used for calculating pharmaceutical removal efficiencies in WWTPs proposed by Jean Marc Choubert et al. in cooperation between Irstea, UR MALY and SUEZ, CIRSEE.

The threshold is defined (depending on LoQ – Limit of Quantification) for each micropollutant: 5 × LoQ for influent and secondary effluent. Above the thresholds, we have defined a high level of confidence (noted 'high level'), where micropollutant concentrations were determined with a low level of uncertainty ranging between 20% and 30%. Below these thresholds, there is a low level of confidence (noted 'low level') which corresponds to measurement uncertainty ranging between 50% and 100%.

- [High level]: Max 30% error for C > 5 x LoQ
- [low level]: 50% < error < 100% for LoQ < C < 5 x LoQ

Figure 6 shows the different situations in which removal yields are obtained in a "robust" way. This approach allows us to go further than simple arithmetic calculations that do not consider the uncertainties on the results.



NC : non-calculable; LQ : limit of quantification; Rw : removal in the liquid phase

Figure 6 shows 10 different cases depending on influent concentration (Conc. in) and effluent concentration (Conc. out), LoQ and threshold.

Possible efficiency calculation (Rw): the concentration differences between the input and the output of tertiary processes are interpretable with regard to the admitted uncertainty on the concentration result.

Cas 1.a: the inlet and outlet concentrations are in the high level;

Cas 1.b: the inlet concentration is in high level and the outlet concentration is in low level;

Cas 1.c: the inlet concentration is in high level and the outlet concentration is less than LQ. In this case, the outlet concentration is replaced by a value equal to LQ / 2 to calculate the yield;

Cas 2.a: the inlet concentration is in low level and the outlet concentration is in high level;

Cas 2.c: the input concentration is in the low level but remains greater than 4 × LQ, and the output concentration is less than LQ. In this case, the output concentration is replaced by a value equal to LQ / 2 to calculate the yield;

Cas 3.a: the inlet concentration is lower than LQ and the output concentration is in the high level. In this case, the input concentration is replaced by a value equal to LQ / 2 to calculate the yield.

Non-calculable efficiency (NC): the differences in concentrations between the inlet and the outlet of the tertiary process are not interpretable because of the admitted uncertainty:

Case 2.b: the inlet and outlet concentrations are in low level;

Case 2.c: the inlet concentration is in low level but less than 4 × LQ, and the output concentration is less than LQ;

Case 3.b: the inlet concentration is lower than LQ and the outlet concentration is in the low level.

Case 3.c: inlet and outlet concentrations are both under LQ

When the efficiency is calculable, it is calculated with the formula $Rw\ (\%) = \frac{C_{in} - C_{out}}{C_{in}}$, except for the special cases mentioned above (where one of the two concentrations is replaced by LQ / 2).

Enclosed you find the Jean -Marc Choubert et al. article describing the background and detail procedure for Rw calculation.

Removal of micropollutants by application of multiple point ozonation and powder activated carbon

Appendix 1 – Effect of ozonation and PAC conditions on micropollutant removal

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Removal of micropollutants by application of multiple point ozonation and powder activated carbon

Appendix 2 – Removal of micropollutant from sludge liquid and solid phases

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Contents

Highlights

- Similar micropollutant concentrations were found for both treatment lines of the Brædstrup WWTP in the liquid and solid phases of the sludge.
- An ozone dose of 4 mgO_3/L reduced micropollutants concentrations in both the liquid (16%) and solid (45%) phases of the sludge, for a total reduction of 38%.
- An increase to a dose of 7 mgO_3/L had no additional effect on micropollutants concentrations in the liquid phase, in contrast to the solid phase where a reduction of 64% was achieved, for a total reduction of 43%.
- These results suggest that a dose of 4 mgO3/L lead to a significant removal of micropollutants from wastewater and could reduce the ozone dose needed for the tertiary ozonation.
- A dose of 7 mgO3/L provides a minor gain for micropollutants removal and seems interesting only to reduce the concentrations of micropollutants found in the solid phase of the sludge.

1. Introduction

One of the main objectives of this project is to assess whether ozone injection in mixed liquor in activated sludge process can reduce micropollutant concentrations in both the liquid and solid phases of the sludge, and to study the impact of the applied mixed liquor ozone dose on this expected reduction. Similar sampling campaigns were carried out as described in detail in Appendix 1 chapter 5.

1.1 Sampling campaigns

Similar sampling campaigns were carried out as described in detail in Appendix 1 chapter 5. Due to lack of analytical methodology and laboratories limitation, it was not possible to quantify all selected compounds in liquid and solid phases of the sludge.

1.2 Micropollutants analyses

For each campaign, 30 organic micropollutants were studied in the WWTP inlet (liquid and solid phases), the activated sludge recirculation pipe of each line (liquid and solid phases) and the WWTP outlet (only the liquid phase, the TSS in the WWTP outlet are considered negligible) as shown in *Table **1***.

The analyses were performed by the Institute of Energy and Environmental Technology (IUTA, Duisbourg, Germany). Only 11 micropollutants could be searched in the solid phase with the analytic method used (capecitabine, carbamazepine, citalopram, clarithromycin, diclofenac, erythromycin, ibuprofen, metoprolol, naproxen, propranolol and tramadol).

Table 1. Use, molecular weight (MV), ozonation constant rate (kO3, pH 7 and 20°C), hydrophobicity (log KOW), corrected hydrophobicity at pH = 7 (log D), charge at pH = 7, typical removal in conventional activated sludge (CAS, from Margot et al., 2015) and limit of quantification for the 30 micropollutants studied.

Micropollutant	Use	MW (g/mol)	k_{O3} (L/mol/s)	Log K_{ow}	Log D (pH = 7)	Charge (pH = 7)	Typical removal in CAS (%)	LOQ (ng/L)
Iomeprol	Contrast medium	777.1	0.5				34	50
1H-Benzotriazole	Corrosion inhibitor	119.1	2.3×10^2	1.26	1.25	-1	26	10
∑ 4+5-Methylbenzotriazole	Corrosion inhibitor	133.1	7.8×10^2				30	10
Dimethylbenzotriazole	Corrosion inhibitor	147.2						10
Atorvastatin	Anti-cholesterol	558.6						10
Azithromycin	Antibiotic	749.0	1.1×10^5	2.44	0.52	2	39	10
Bicalutamide	Antiandrogen	430.4	1.1×10^1					10
Candesartan	Angiotensin blocker	440.5	5.0×10^5					10
Capecitabine	Chemotherapy	359.3	1.0×10^1	0.56				10
Carbamazepine	Anticonvulsant	236.3	3.0×10^5	2.77	2.77	0	16	10
Cefalexin	Antibiotic	347.4					90	20

Ciprofloxacin	Antibiotic	331.3	1.9×10^4	-1.47	-1.47	0	69	10
Citalopram	Antide-pressant	324.4	1.0×10^3	3.50	0.98	1		10
Clarithromycin	Antibiotic	747.9	7.0×10^4	3.16			33	10
Diclofenac	Anti-in-flammatory	296.1	1.0×10^6	4.51	1.26	-1	20	10
Erythromycin	Antibiotic	733.9	1.0×10^5	3.06	1.20	1	45	20
Ibuprofen	Anti-in-flammatory	206.3	9.6	3.97	1.69	-1	80	10
Imidacloprid	Insecticide	255.7		-1.95	-1.95		0	30
Metoprolol	Beta blocker	267.4	2.0×10^3	1.89			25	10
Metronidazole	Antibiotic	171.2					45	10
Naproxen	Anti-in-flammatory	230.3	2.0×10^5	3.18	0.18	-1	40	30
Ofloxacin	Antibiotic	361.4	2.0×10^6		-0.05	-1	58	10
Paracetamol	Analgesic	151.2	3.1×10^6	0.91	0.91	0	100	10
Prednisolone	Steroid	360.4	1.0×10^2					10
Propranolol	Beta blocker	259.3	1.0×10^5	3.48	-0.09	1	28	10
Sulfa-methoxazole	Antibiotic	253.3	5.5×10^5		-0.11	-1	44	10
Sulfapyridine	Antibiotic	249.3						10
Tramadol	Analgesic	263.4	4.2×10^4	3.01			33	10
Venlafaxine	Antide-pressant	277.4	3.2×10^3				40	10
Zopiclone	Sedative	388.8						10

2. Results

2.1 Baseline

2.1.1 Liquid phase

Of the 30 micropollutants studied during Baseline tests, 10 were not detected in the liquid phase of the sludge for both lines (dimethylbenzotriazole, capecitabine, cefalexin, ciprofloxacin, ibuprofen, imidacloprid, metronidazole, ofloxacin, prednisolone and zopiclone). The average concentration for each micropollutant quantified as well as the average concentration and the sum of the concentrations of all micropollutants are presented for both lines in Figure 1. The average concentration for line 1 is 576 ± 240 ng/L, which is very close to 575 ± 226 ng/L for line 2. The same result is obtained for the sum of the concentrations which is 11420 ± 4995 ng/L for line 1 and 11030 ± 4657 ng/L for line 2.

These results show that the two lines have similar micropollutants levels in the liquid phase of their recirculated sludge. The calculation of the concentration ratio of the two lines for each micropollutant, as well as for the average concentration and the sum of the concentrations (Figure 2), confirms this observation. This ratio is close to 1 for all micropollutants, except for paracetamol with a ratio of 3.42 which indicates that this micropollutant is found in higher quantities in the sludge from line 1 (499 ng/L vs. 146 ng/L).

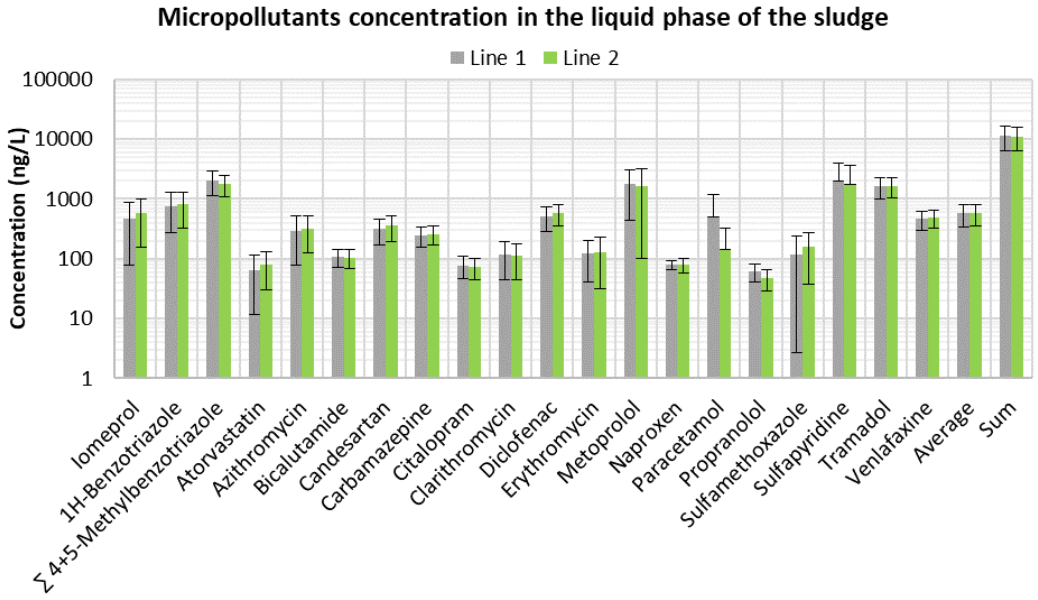

Figure 1. Average concentration of each micropollutant quantified in the liquid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for lines 1 and 2.

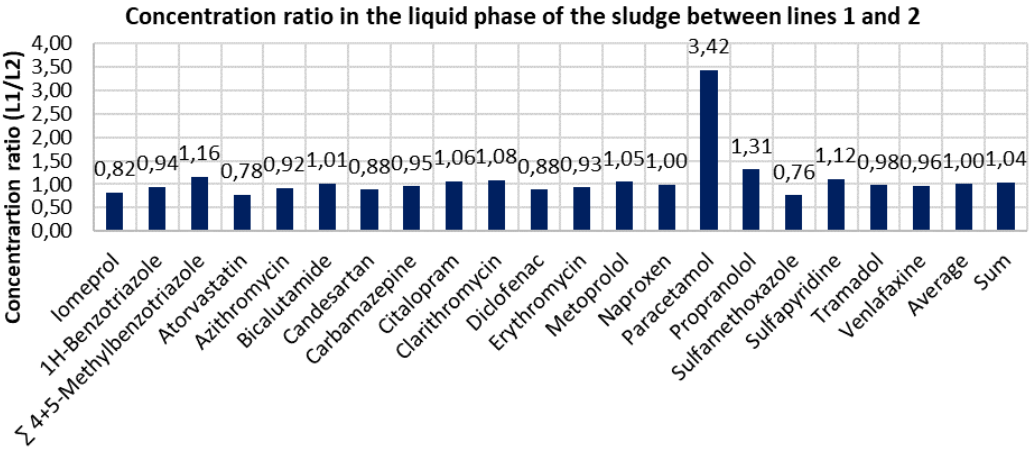

Figure 2. Concentration ratio in the liquid phase of the sludge between lines 1 and 2.

2.1.2 Solid phase

Of the 30 micropollutants studied during the 9 sampling campaigns, only 11 could be searched in the solid phase of the sludge for both lines (capecitabine, carbamazepine, citalopram, clarithromycin, diclofenac, erythromycin, ibuprofen, metoprolol, naproxen, propranolol and tramadol). Ibuprofen was not detected for both lines. The average concentration of each micropollutant quantified as well as the average concentration and the sum of the concentrations of all micropollutants are presented for both lines in Figure 3. The average concentration for line 1 is 420 ± 60 ng/g (ng of micropollutant per g of dry sludge), which is very close to 428 ± 60 ng/g for line 2. The same result is obtained for the sum of the concentrations which is 3488 ± 4670 ng/g for line 1 and 3325 ± 603 ng/g for line 2. The mixed liquor suspended solids concentration was in average 5.1 g/L for line 1 and 5.6 g/L for line 2.

These results show that the two lines have similar micropollutants levels in the solid phase of the sludge. The calculation of the concentration ratio of the two lines for each micropollutant, as well as for the average concentration (0.98) and the sum of the concentrations (1.04), confirms this observation (Figure 4). This ratio is close to 1 for all micropollutants, except for naproxen with a ratio of 0.01 which indicates that this micropollutant is found in much lower quantities in the sludge from line 1 than line 2 (0.7 ng/g vs. 85.2 ng/g). A significant variability is also observed for erythromycin (5.1 ng/g vs. 9.1 ng/g) and metoprolol (1638 ng/g vs. 1089 ng/g), but the concentrations of erythromycin can be considered negligible.

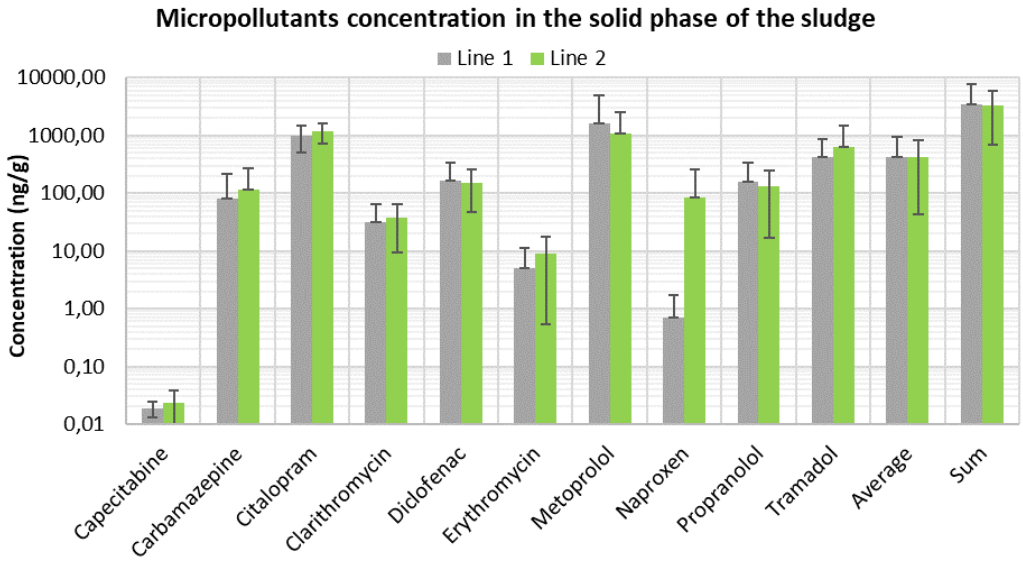

Figure 3. Average concentration of each micropollutant quantified in the solid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for lines 1 and 2.

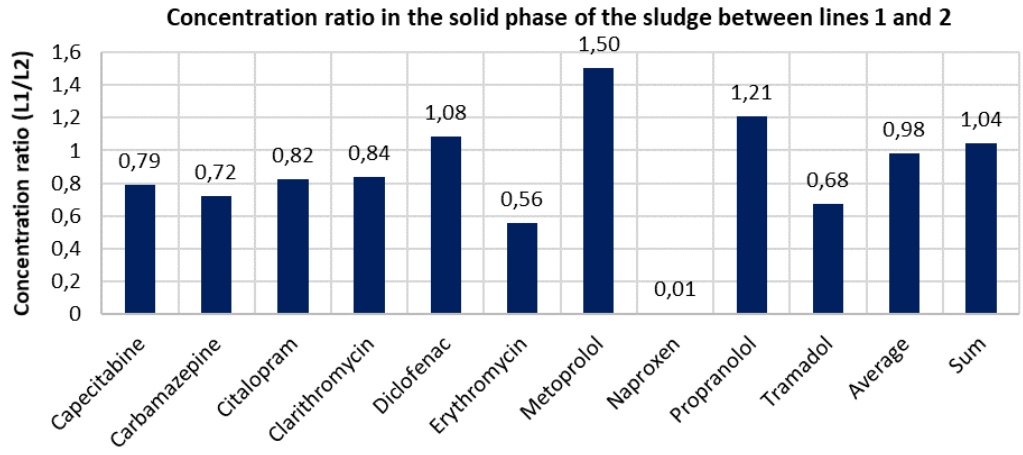

Figure 4. Concentration ratio in the solid phase of the sludge between lines 1 and 2.

2.1.3 Micropollutants total concentration and distribution into the sludge

The total concentration of micropollutants into the sludge was calculated for the 11 micropollutants analyzed in both phases and is presented in Figure 5 for line 1 and Figure 6 for line 2. The solid-phase concentrations (ng/g) were converted to ng/L using mixed liquor suspended solids concentrations (5.1 g/L for line 1 and 5.6 g/L for line 2). The average concentration for line 1 is 2645 ng/L, which is close to 2801 ng/L for line 2. The same result is obtained for the sum of the concentrations which is 21 998 ng/L for line 1 and 22 245 ng/L for line 2.

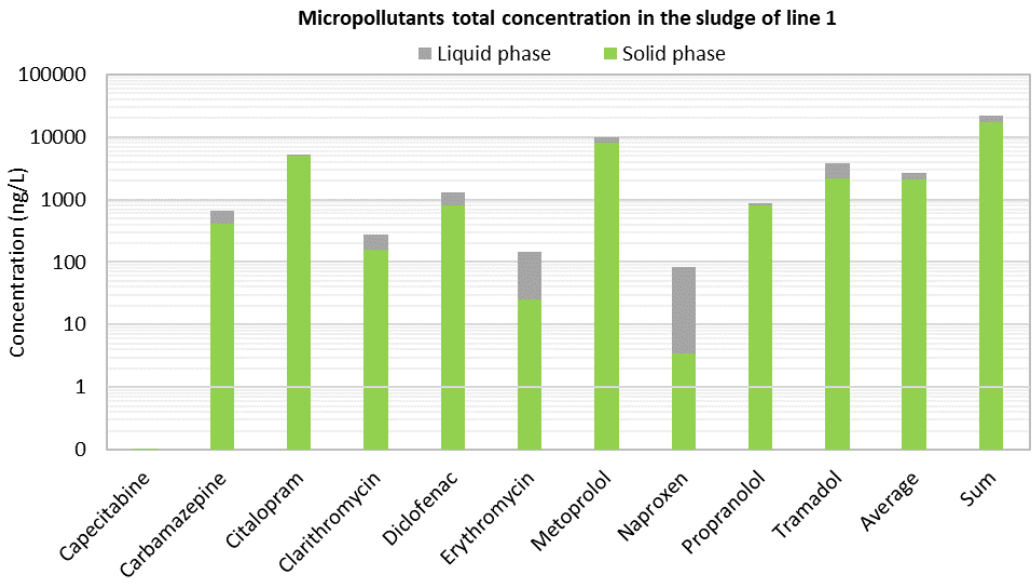

Figure 5. Micropollutants total concentration (liquid + solid phases) in the sludge of line 1.

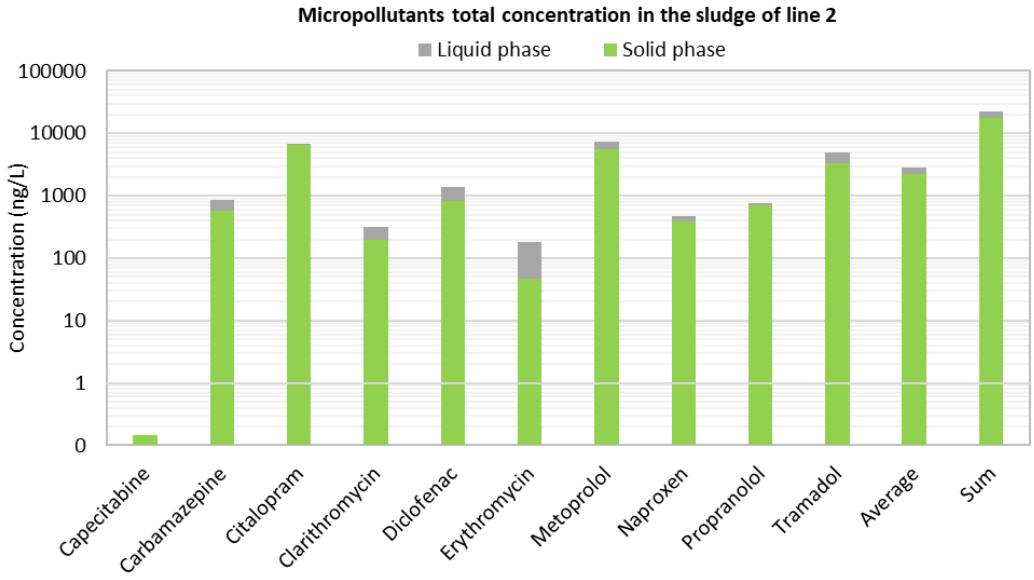

Figure 6. Micropollutants total concentration (liquid + solid phases) in the sludge of line 2.

The distribution of micropollutants into the sludge between the liquid and solid phases was also determined and is presented Figure 7 for line 1 and Figure 8 for line 2. The results are close for the two lines, although a variability of ± 10% is observed for most micropollutants. All micropollutants are mainly found in the solid phase except for erythromycin presents in both phases and naproxen for line 1 presents mainly in the liquid phase.

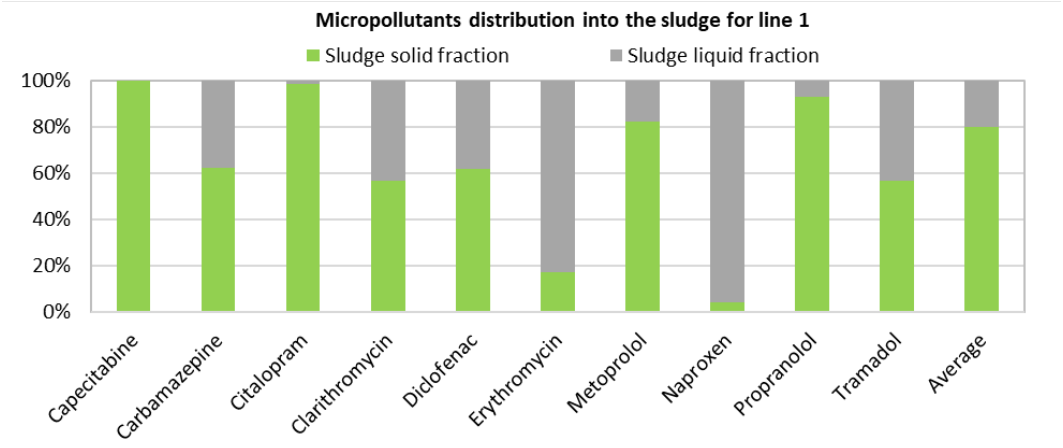

Figure 7. Micropollutants distribution into the sludge between the solid and liquid phases for line 1.

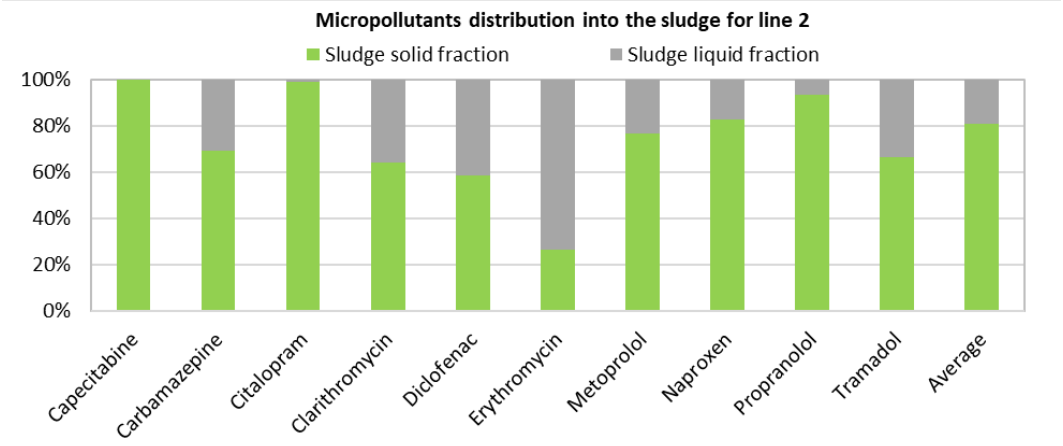

Figure 8. Micropollutants distribution into the sludge between the solid and liquid phases for line 2.

2.2 First ozone condition

2.2.1 Liquid phase

Of the 30 micropollutants studied during the 3 sampling campaigns, 10 were not detected in the liquid phase of the sludge for both lines (dimethylbenzotriazole, capecitabine, cefalexin, ibuprofen, metronidazole, ofloxacin, paracetamol, prednisolone, sulfamethoxazole and zopiclone). Imidacloprid was quantified in the reference line (line 2) but not in the experimental line with ozonation (4 mgO_3/L line 1). The average concentration of each micropollutant quantified as well as the average concentration and the sum of the concentrations of all micropollutants are presented for both line in Figure 9. The average concentration for line 1 is 545 ± 26 ng/L, which is higher than 465 ± 1 ng/L for line 2. The same result is obtained for the sum of the concentrations which is 9817 ± 473 ng/L for line 1 and 8687 ± 1058 ng/L for line 2.

The calculation of the concentration ratio shows that despite a value close to 1 for the average concentration (1.17) and the sum of the concentrations (1.13), there is a high variability for each micropollutant (Figure 10). A ratio of 4.26 is obtained for iomeprol (5533 ng/L for line 1 vs. 1300 ng/L for line 2) and 2.99 for atorvastatin (127 ng/L vs. 42 ng/L), while for most micropollutants it is lower than 1 which indicates that ozonation decreased their concentrations. The very high concentrations of iomeprol in line 1 (5533 ± 603 ng/L), which represents more than 50% of the sum of the concentrations, has a high impact on calculations and counterbalance the positive

effect of ozonation observed for most micropollutants. The origin of the high concentration difference between the two lines for iomeprol is under study, but several hypotheses could be envisaged:

- An analytical problem.
- A difference in operation between the two lines.
- Transformation of iomeprol metabolites or conjugates/complexes into the parent molecule after reaction with ozone.
- Release of iomeprol from suspended particles to the liquid phase attacked by ozone.

Not including iomeprol, a ratio of 0.58 (252 ng/L vs. 435 ng/L) is obtained for the average concentration and 0.55 (4284 ng/L vs. 7829 ng/L) for the sum of the concentrations, which corresponds to a reduction of micropollutants concentrations by more than 40% and illustrates that ozonation is an effective process to remove micropollutants from the liquid phase of sludges.

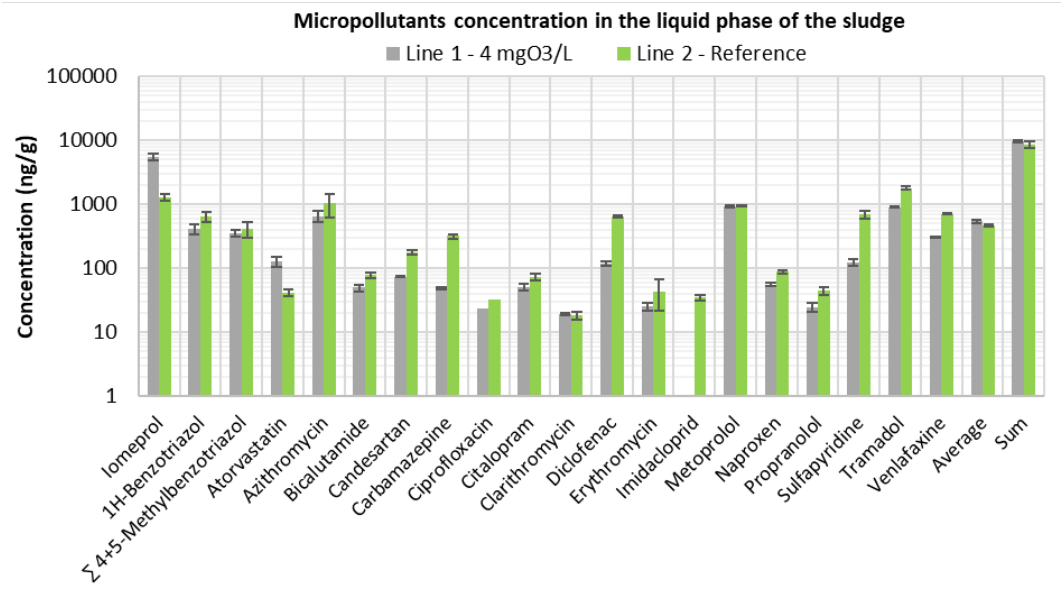

Figure 9. Average concentration of each micropollutant quantified in the liquid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for lines 1 (ozonation, 4 mgO3/L) and 2 (no ozonation).

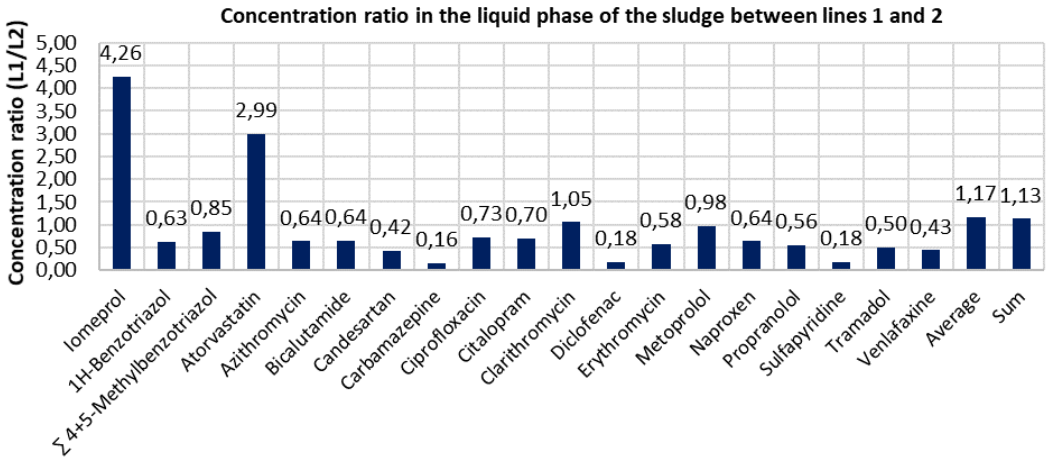

Figure 10. Concentration ratio in the liquid phase of the sludge between lines 1 (ozonation, 4 mgO3/L) and 2 (no ozonation).

2.2.2 Solid phase

Of the 30 micropollutants studied during the 9 sampling campaigns, only 11 could be searched in the solid phase of the sludge for both lines (capecitabine, carbamazepine, citalopram, clarithromycin, diclofenac, erythromycin, ibuprofen, metoprolol, naproxen, propranolol and tramadol). Capecitabine, clarithromycin and naproxen were not detected for both lines. The average concentration of each micropollutant quantified as well as the average concentration and the sum of the concentrations of all micropollutants are presented for both line in Figure 11. The average concentration for line 1 is 34.9 ± 16.4 ng/g, which is very close to 39.9 ± 20.8 ng/g for line 2. The same result is obtained for the sum of the concentrations which is 240.3 ± 116.4 ng/g for line 1 and 324.7 ± 156.8 ng/g for line 2. The mixed liquor suspended solids concentration was in average 5.1 g/L for line 1 and 5.06 g/L for line 2.

These results show that ozonation can reduce the concentration of micropollutants in the solid phase of the sludge. The calculation of the average concentration (0.87) and the sum of the concentrations (0.74) confirms this observation (Figure 12). However, a significant variability is observed for each micropollutant individually. A ratio of 1.98 is obtained for citalopram (103 ng/g for line 1 vs. 52 ng/g for line 2), 1.19 for erythromycin (2.5 ng/g vs. 2.1 ng/g), 2.49 for ibuprofen (2.6 ng/g vs. 1.0 ng/g) and 1.57 for propranolol (9.6 ng/g vs 6.1 ng/g), which indicates that ozonation has a detrimental effect on the concentration in the solid phase of the sludge for these 3 compounds. This increase of concentration can be considered negligible for erythromycin, ibuprofen, and propranolol but not for citalopram, and for the latter the origin of this increase is under study. For the other micropollutants, a decrease of the concentration in the solid phase of the sludge is observed after ozonation, and this decrease is very important for tramadol with a ratio of 0.38 (71.0 ng/g vs. 186.3 ng/g).

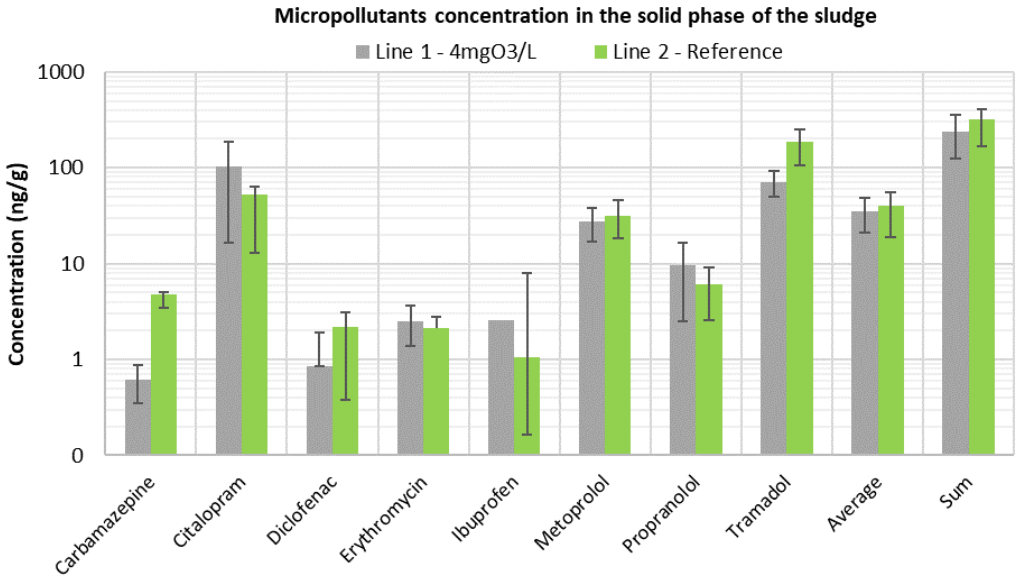

Figure 11. Average concentration of each micropollutant quantified in the solid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for lines 1 (ozonation, 4 mgO3/L) and 2 (no ozonation).

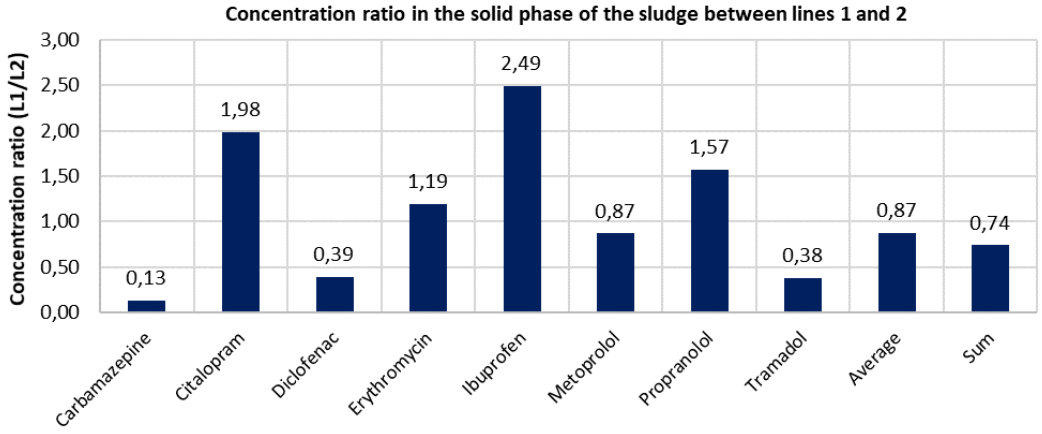

Figure 12. Concentration ratio in the solid phase of the sludge between lines 1 (ozonation, 4 mgO3/L) and 2 (no ozonation).

2.2.3 Micropollutants total concentration and distribution into the sludge

The total concentration of micropollutants into the sludge was calculated for the 11 micropollutants analyzed in both phases and is presented in Figure 13 for line 1 (4 mgO_3/L) and Figure 14 for line 2 (no ozonation). The solid-phase concentrations (ng/g) were converted to ng/L using the mixed liquor suspended solids concentrations (5.1 g/L for line 1 and 4.5 g/L for line 2). The average concentration for line 1 is 429 ng/L, which is lower than 623 ng/L for line 2. The same result is obtained for the sum of the concentrations which is 3 397 ng/L for line 1 and 5452 ng/L for line 2. The ozone injection decreased the concentration of all micropollutants except citalopram (576 ng/L for line 1 and 307 ng/L for line 2) while it has no effect for clarithromycin, ibuprofen, metoprolol and propranolol. The origin of this difference in behavior for citalopram is under study.

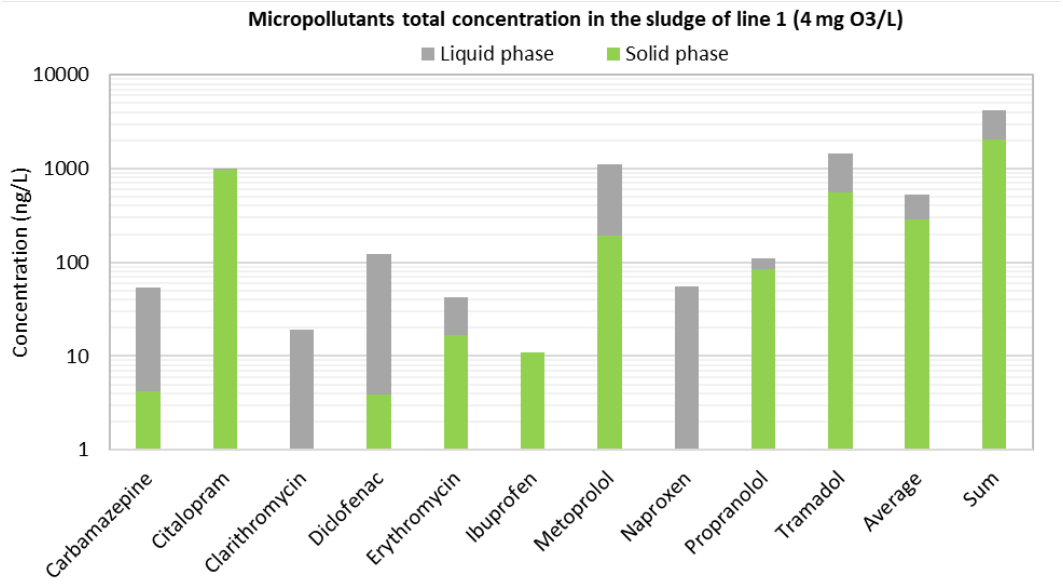

Figure 13. Micropollutants total concentration (liquid + solid phases) in the sludge of line 1 (experimental line, 4 mgO3/L).

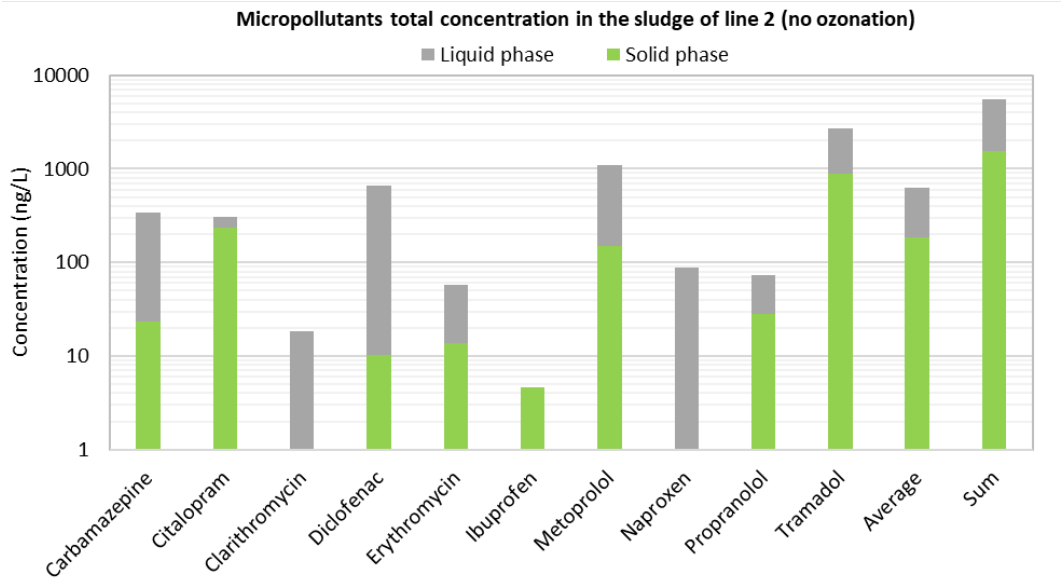

Figure 14. Micropollutants total concentration (liquid + solid phases) in the sludge of line 2 (reference line, no ozonation).

The distribution of micropollutants into the sludge between the liquid and solid phases was also determined and is presented Figure 15 for line 1 and Figure 16 for line 2. It can be observed that this time most micropollutants are mainly found in the liquid phase of the sludge for both lines, contrary to baseline results, except for citalopram and ibuprofen but also propranolol for line 1. The distribution of citalopram, erythromycin, propranolol and the average concentration into the solid phase of the sludge increases with ozone injection while it has no impact on other compounds. This result indicates that ozone is more reactive toward micropollutants in the liquid phase of the sludge as expected.

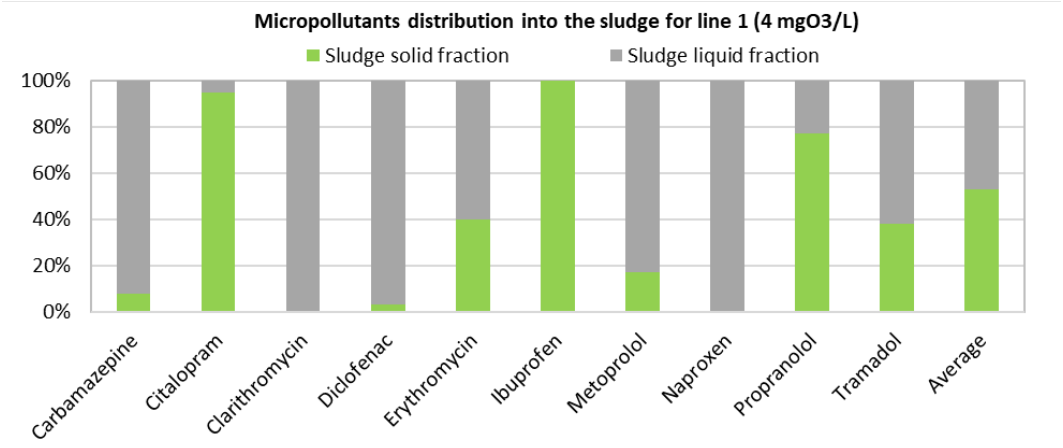

Figure 15. Micropollutants distribution into the sludge between the solid and liquid phases for line 1 (4 mgO3/L).

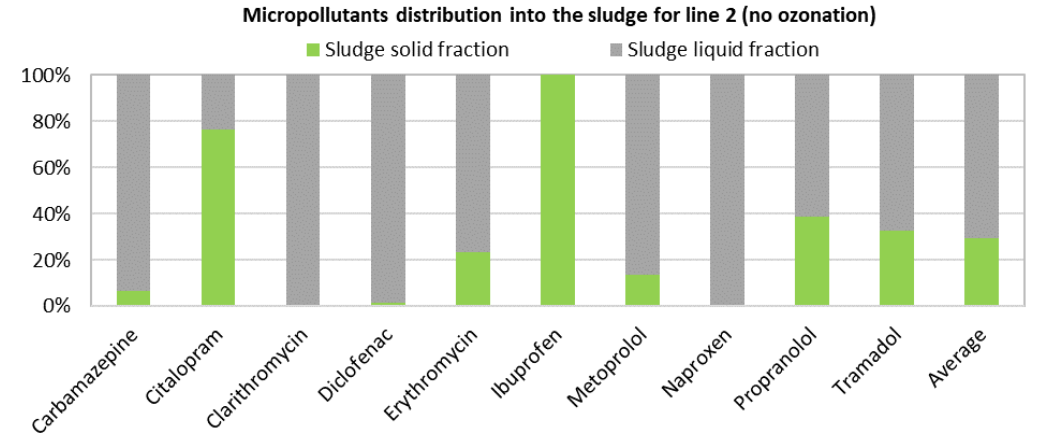

Figure 16. Micropollutants distribution into the sludge between the solid and liquid phases for line 2 (no ozonation).

2.3 Second ozone condition

2.3.1 Liquid phase

Of the 32 micropollutants studied during the 3 sampling campaigns (PFOA and PFOS in addition to the 30 previously studied micropollutants), 9 were not detected in the liquid phase of the sludge for both lines (capecitabine, cefalexin, ciprofloxacin, erythromycin, imidacloprid, metronidazole, paracetamol, prednisolone and zopiclone). Dimethylbenzotriazole, ofloxacin, sulfamethoxazole and tramadol were quantified in the reference line (line 2) although the concentration of these compounds were below limit of quantification in the experimental line with ozonation (7 mgO_3/L, line 1). The average concentration of each micropollutant quantified as well as the average concentration and the sum of the concentrations of all micropollutants are presented for both line in Figure 17. The average concentration for line 1 is 499 ± 294 ng/L, which is higher than 305 ± 73 ng/L for line 2. The same result is obtained for the sum of the concentrations which is 9264 ± 5771 ng/L for line 1 and 6024 ± 1611 ng/L for line 2.

The calculation of the concentration ratios confirms this observation with a value of 1.64 for the average concentration and 1.54 for the sum of the concentrations (Figure 18). However, a high variability is observed from one micropollutant to another. A ratio of 1.95 is obtained for iomeprol (6500 ng/L for line 1 vs. 3333 ng/L for line 2), 3.24 for azithromycin (793 ng/L vs. 245 ng/L), 1.61 for ibuprofen (295 ng/L vs. 183 ng/L) and 1.41 for naproxen (61 ng/L vs. 43 ng/L), while for the other micropollutants the ratio is close to 1. A significant reduction in concentrations after ozonation is only observed for Σ 4+5-methylbenzotriazole (ratio of 0.59, 147 ng/L vs. 247 ng/L), carbamazepine (ratio of 0.34, 25 ng/L vs. 72 ng/L) and diclofenac (ratio of 0.34, 96 ng/L vs. 283 ng/L).

The very high concentrations of iomeprol (6500) and azithromycin (793 ± 530 ng/L) in line 1, which represent a 70% and 9% of the sum of the concentrations, respectively, have a high impact on calculations and counterbalance the positive effect of ozonation observed for most micropollutants. The origin of the high concentration difference between the two lines for iomeprol and azithromycin is under study.

Not including iomeprol, a ratio of 1.08 (160 ng/L for line 1 vs. 148 ng/L for line 2) is obtained for the average concentration and 1.03 (2764 ng/L vs. 2688 ng/L) for the sum of the concentrations, indicating that ozone injection had no impact on micropollutants concentration in the liquid phase of the sludge.

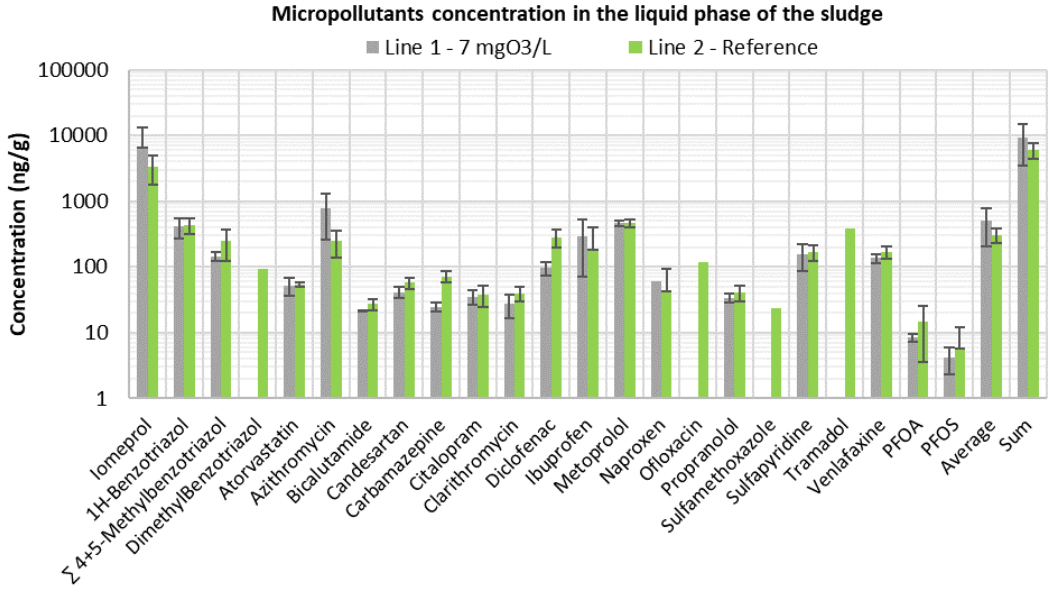

Figure 17. Average concentration of each micropollutant quantified in the liquid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for lines 1 (ozonation, 7 mgO3/L) and 2 (no ozonation).

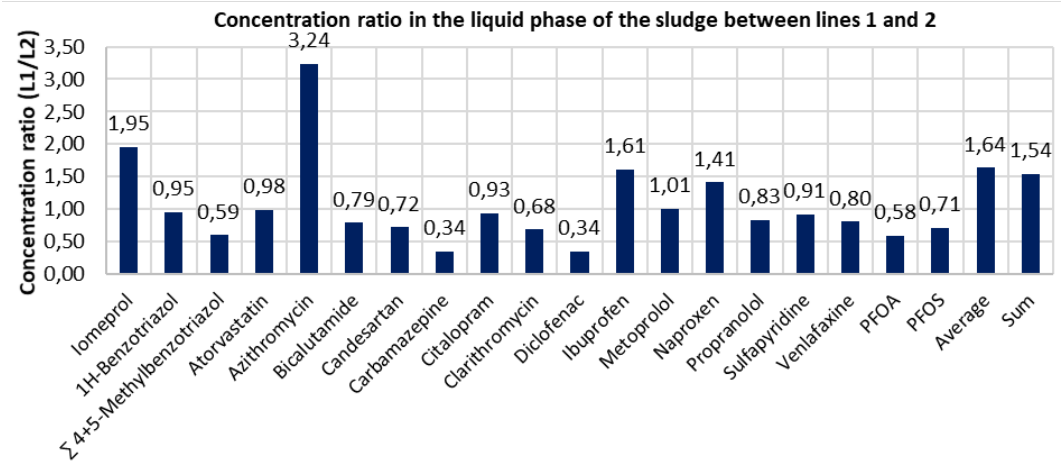

Figure 18. Concentration ratio in the liquid phase of the sludge between lines 1 (ozonation, 7 mgO3/L) and 2 (no ozonation).

2.3.2 Solid phase

Of the 30 micropollutants studied during the 9 sampling campaigns, only 11 could be searched in the solid phase of the sludge for both lines (capecitabine, carbamazepine, citalopram, clarithromycin, diclofenac, erythromycin, ibuprofen, metoprolol, naproxen, propranolol and tramadol). Capecitabine, diclofenac, ibuprofen, naproxen and tramadol were not detected for both lines. The average concentration of each micropollutant quantified as well as the average concentration and the sum of the concentrations of all micropollutants are presented for both lines in Figure 19. The average concentration for line 1 is 11.8 ± 6.9 ng/g, which is lower than 33.0 ± 21.5 ng/g for line 2. The same result is obtained for the sum of the concentrations which is 87.7 ± 44.0 ng/g for line 1 and 264.0 ± 171.9 ng/g for line 2. The total suspended solids concentration was in average 5.1 g/L for line 1 and 4.8 g/L for line 2.

These results show that ozonation can significantly reduce the concentration of micropollutants in the solid phase of the sludge. The calculation of the average concentration (0.36) and the sum of the concentrations (0.33) confirms this observation (Figure 20). A similar trend is observed for each micropollutant individually with ratios lower than 0.50 except for citalopram with a ratio of 2.10 (30.3 ng/g for line 1 vs. 14.5 ng/g for line 2), which indicates that ozonation has a detrimental effect on the concentration in the solid phase of the sludge for these 3 compounds.

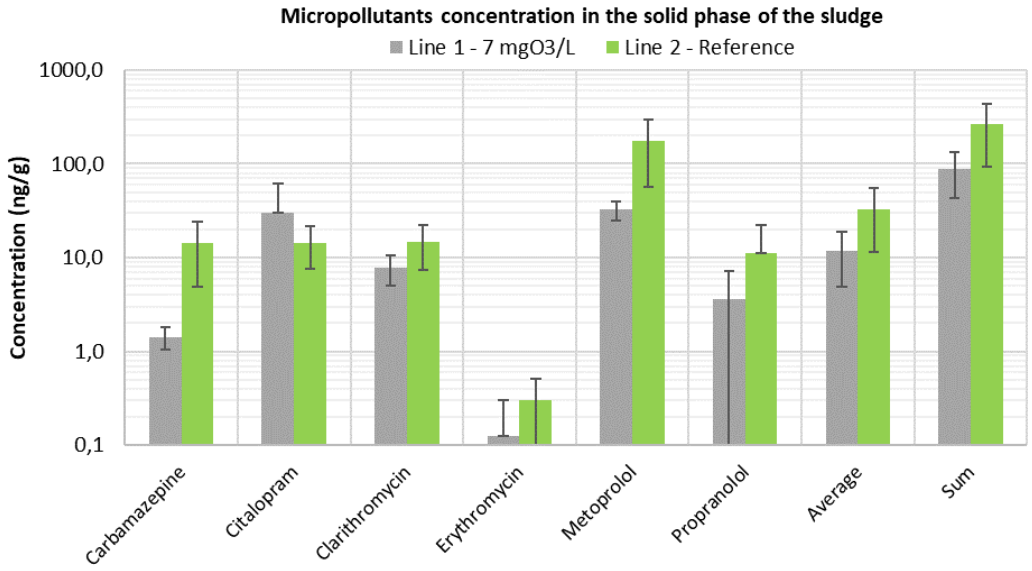

Figure 19. Average concentration of each micropollutant quantified in the solid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for lines 1 (ozonation, 7 mgO3/L) and 2 (no ozonation).

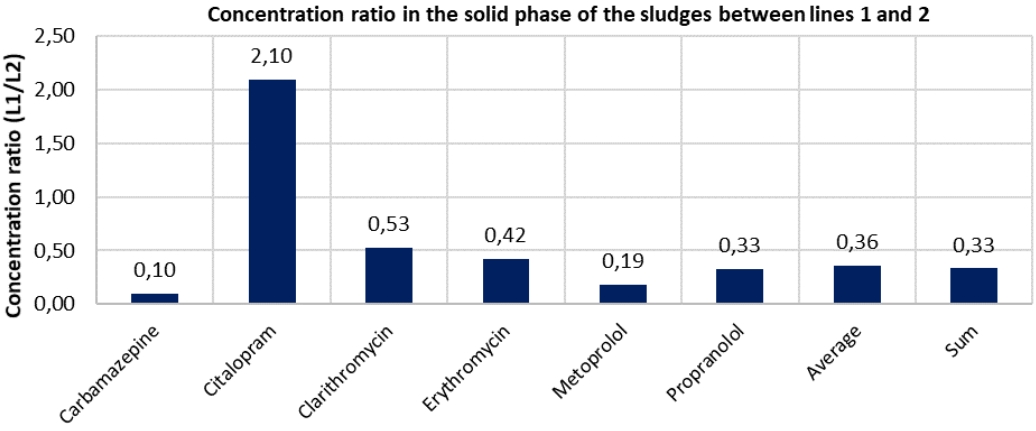

Figure 20. Concentration ratio in the solid phase of the sludge between lines 1 (ozonation, 7 mgO3/L) and 2 (no ozonation).

2.3.3 Micropollutants total concentration and distribution into the sludge

The total concentration of micropollutants into the sludge was calculated for the 11 micropollutants analyzed in both phases and is presented Figure 21 for line 1 (7 mgO_3/L) and Figure 22 for line 2 (no ozonation). The solid-phase concentrations (ng/g) were converted to ng/L using the total suspended solids concentrations (5.1 g/L for line 1 and 4.8 g/L for line 2). The average concentration for line 1 is 200 ng/L, which is lower than 318 ng/L for line 2. The same result is obtained for the sum of the concentrations which is 1 455 ng/L for line 1 and 2 561 ng/L for line 2. The ozone injection decreased the concentration of all micropollutants except citalopram, ibuprofen and naproxen. The origin of this difference in behavior is surprising, especially for naproxen that is reactive toward ozone ($k_{O3} > 10^5$).

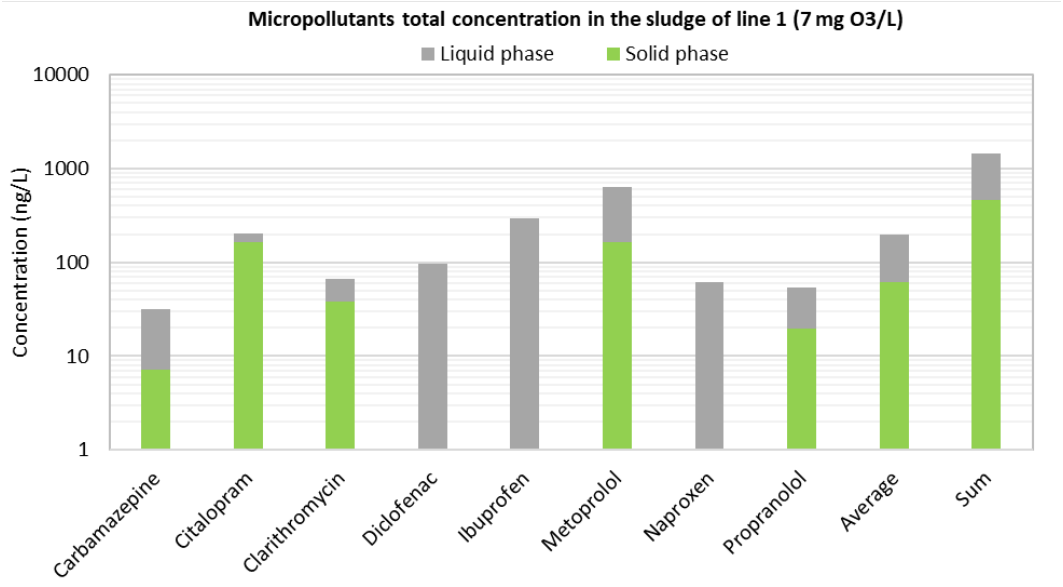

Figure 21. Micropollutants total concentration (liquid + solid phases) in the sludge of line 1 (experimental line, 7 mgO3/L).

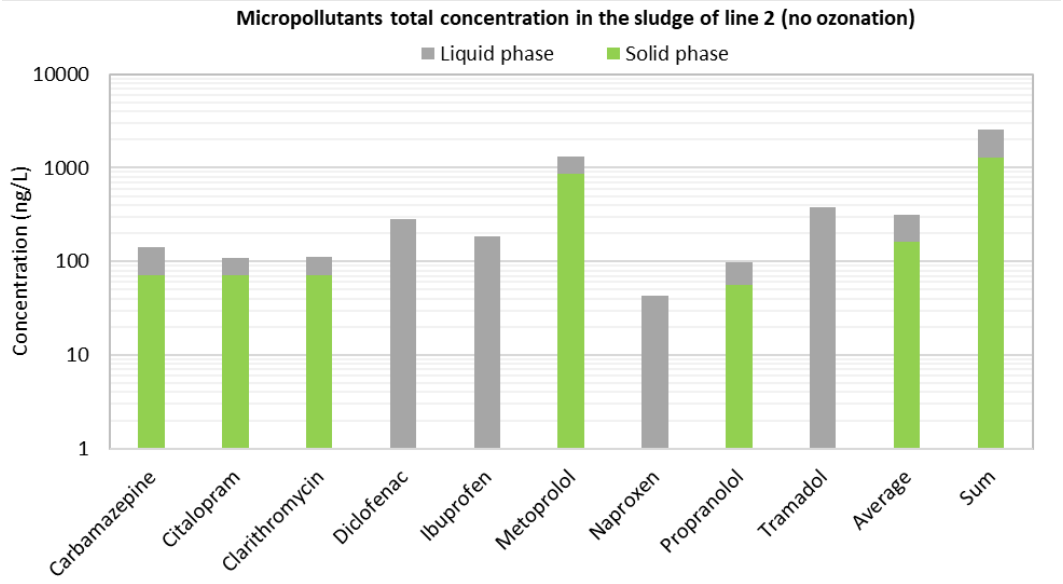

Figure 22. Micropollutants total concentration (liquid + solid phases) in the sludge of line 2 (reference line, no ozonation).

The distribution of micropollutants into the sludge between the liquid and solid phases was also determined and is presented Figure 23 for line 1 and Figure 24 for line 2. Contrary to the results obtained with a dose of 4 mgO_3/L, it can be observed that for most micropollutants, the percentage of distribution into the solid phase decreased after ozone injection. This result indicates that ozone was more reactive toward micropollutants in the solid phase of the sludge.

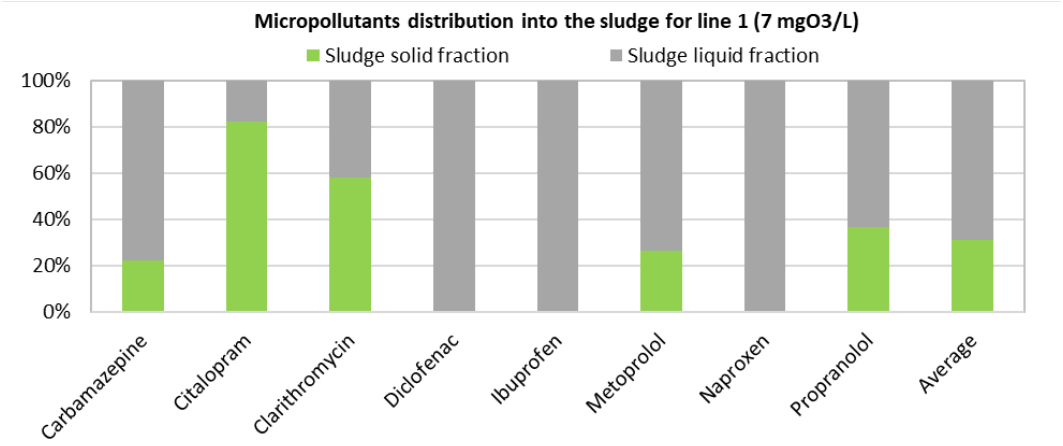

Figure 23. Micropollutants distribution into the sludge between the solid and the liquid phases for line 1 (7 mgO3/L).

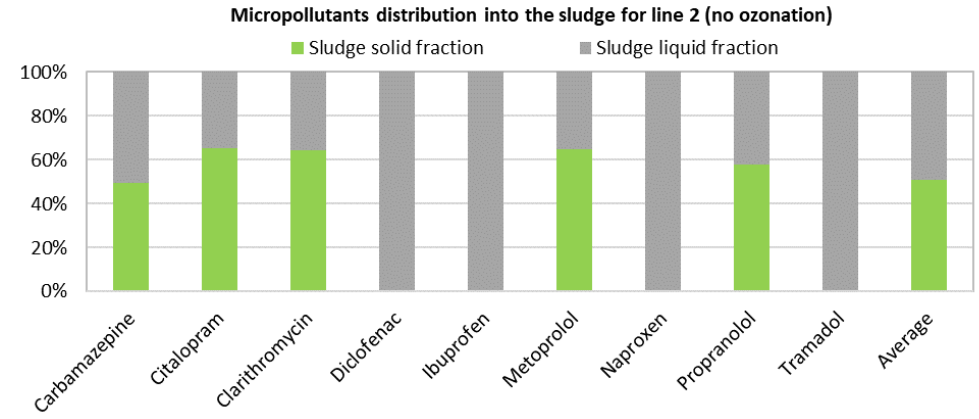

Figure 24. Micropollutants distribution into the sludge between the solid and the liquid phases for line 2 (no ozonation).

2.4 Micropollutants concentrations variability in the reference line

The results of the sampling campaigns without ozonation showed that the two lines studied at the Braedstrup WWTP have similar micropollutant concentrations in both the liquid and the solid phases of the sludge. In addition to reproducible results from one line to another, it is important to evaluate the evolution of the results on line 2, where there was no ozone injection, on the 3 series of campaigns conducted:

- Baseline, between September and November 2018.
- 1st ozone condition, July 2019.
- 2nd ozone condition, October 2019.

The concentrations of micropollutants in the liquid phase of the sludge are shown in Figure 25. It can be observed a decrease over time for the average concentration (from 560 ng/L to 436 ng/L and 311 ng/L), the sum of the concentrations (from 11198 ng/L to 8282 ng/L and 6526 ng/L) and each micropollutant. The origin of this decrease is under study, but it could be attributed to:

- An annual variability in concentration in the WWTP inlet or in performance of activated sludge. Additional data from previous years may or may not have supported this assumption.
- An increase in the performance of activated sludge over time.
- Lower concentrations in the WWTP inlet, due to a change in practice for drug use, to an increase of rainfall leading to dilution of domestic water loaded with micropollutants or to the connection to the network of non-domestic and low-concentration water.

The only exception is for iomeprol, whose concentration increases over time (from 577 ng/L to 1800 ng/L and 3350 ng/L). The origin of this increase is under study, but it could be investigated by checking with hospitals or radiology offices connected to the wastewater system.

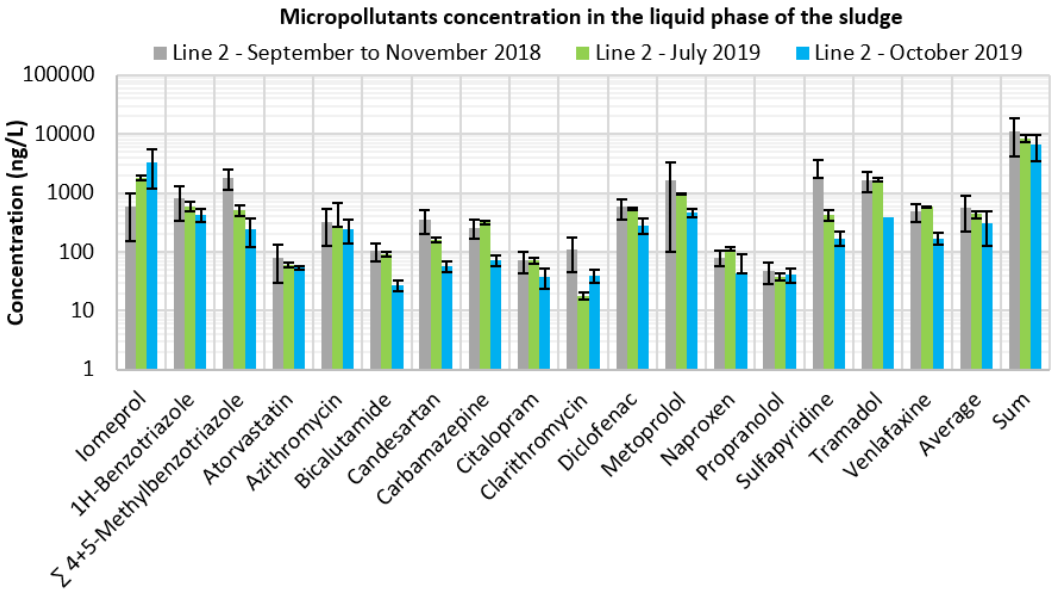

Figure 25. Average concentration of each micropollutant quantified in the liquid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for line 2 on the 3 series of campaigns conducted.

The concentrations of micropollutants in the solid phase of the sludge are shown in Figure 26. It can be observed a decrease over time for the average concentration (from 344 ng/L to 36 ng/L and 33 ng/L), the sum of the concentrations (from 3440 ng/L to 286 ng/L and 231 ng/L) and

each micropollutant. The origin of this decrease is under study, but it could be attributed to the assumptions previously presented.

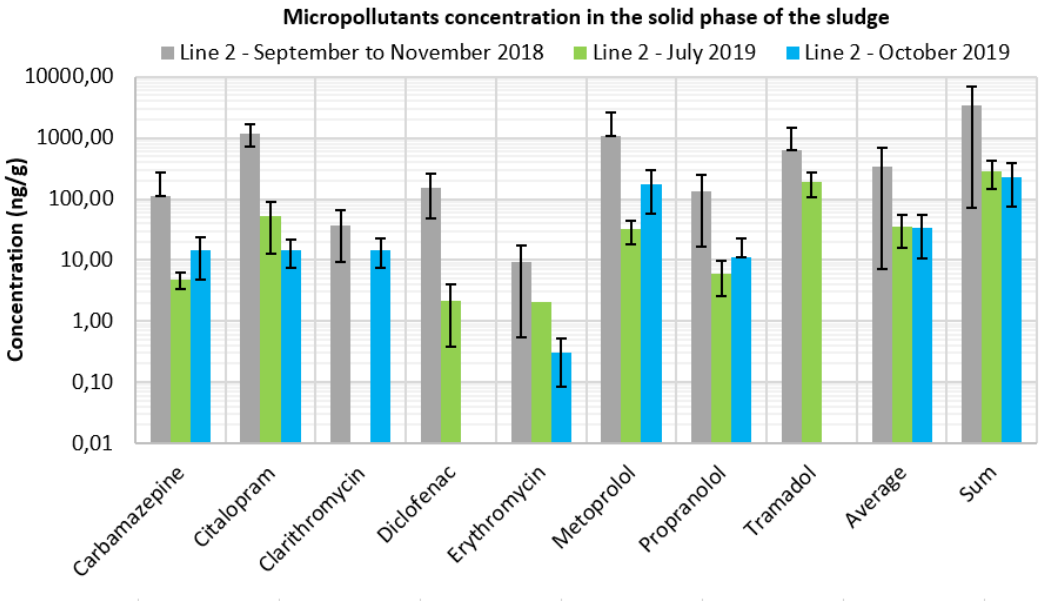

Figure 26. Average concentration of each micropollutant quantified in the solid phase of the sludge as well as the average concentration and the sum of the concentrations of all micropollutants for line 2 on the 3 series of campaigns conducted.

2.5 Influence of applied ozone dose on micropollutants levels in the sludge

Two ozone doses were injected into the activated sludge of line 1 to study the influence of this dose on the removal of micropollutants in both the liquid and solid phases of the sludge in comparison to line 2 (reference line without ozonation).

The removal for the different quantified micropollutants as well as the average removal and the removal for the sum of the concentrations for both ozone doses in comparison to the reference line are shown in Figure 27 for the liquid phase of the sludge. It can be observed that an increase of the ozone dose injected into the activated sludge from 4 mgO_3/L to 7 mgO_3/L seems to decrease the removal of micropollutants from the liquid phase of the sludge, with an average removal from 42 % to -14% and, a removal of 45% to -3% for the sum of the concentrations, respectively. The same result is obtained for each micropollutant except for Σ 4+5-methylbenzotriazole (from 15% to 41%), clarithromycin (from -5% to 32%) and tramadol (from 50% to 100%). This decrease of the removal of micropollutants with the ozone dose is surprising and contrary to what could be expected, especially for azithromycin and naproxen whose removal becomes negative with an increase of the ozone dose.

Those results are difficult to interpret, but a first possible explanation would be the lower concentrations of micropollutants encountered in campaigns with 7 mgO_3/L, which influence removal calculations. Moreover, a higher concentration of suspended solids was measured in the process tank during the 2nd ozone condition (5.67 g/L) than for the 1st ozone condition (5.14 g/L), as well as for chemical oxygen demand (1574 mg/L vs. 1349 mg/L, respectively) resulting in a higher competition between organic micropollutants and organic matter which could reduce the efficacity of the ozonation.

To conclude on these results on the liquid phase of the sludge, it would be preferable to estimate the performance of ozonation by comparing the concentrations at the inlet and outlet of the WWTP and not to rely on the concentrations in the sludge, which are only an intermediate stage. Nevertheless, it is important to study what happens in the liquid phase of sludge, as sludge is not completely dewatered when it is reclaimed.

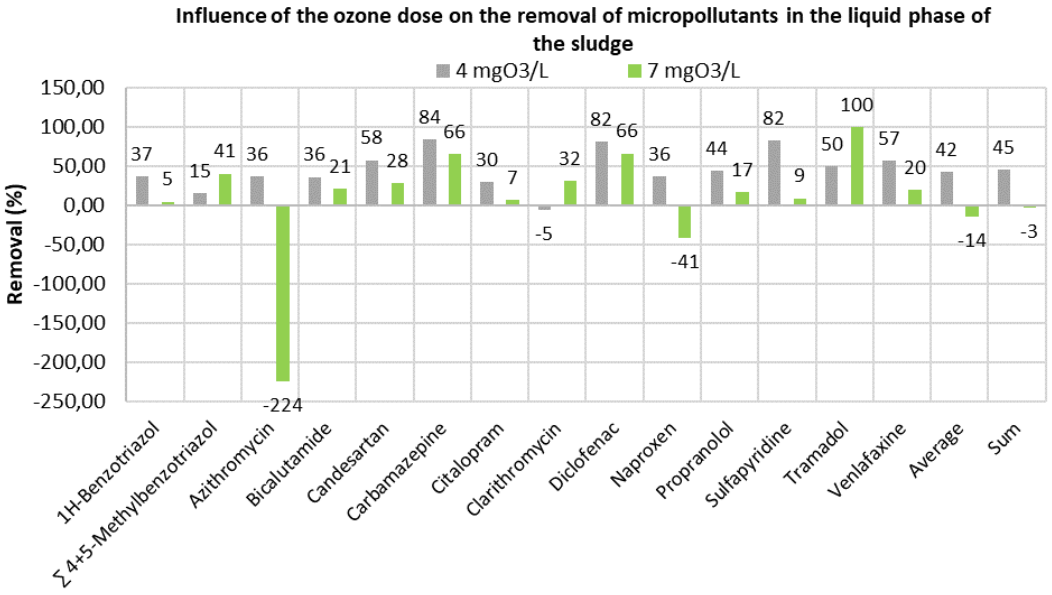

Figure 27. Influence of the ozone dose on the removal of micropollutants in the liquid phase of the sludge.

The removal for the different quantified micropollutants as well as the average removal and the removal for the sum of the concentrations for both ozone doses in comparison to the reference line are shown in Figure 28 for the solid phase of the sludge. In contrast to the liquid phase, it can be observed that an increase of the ozone dose injected into the activated sludge significantly increase the removal of micropollutants, with an average removal of 1% with 4 mgO_3/L and 61% with 7 mgO_3/L, and a removal of 16% and 64% for the sum of the concentrations, respectively.

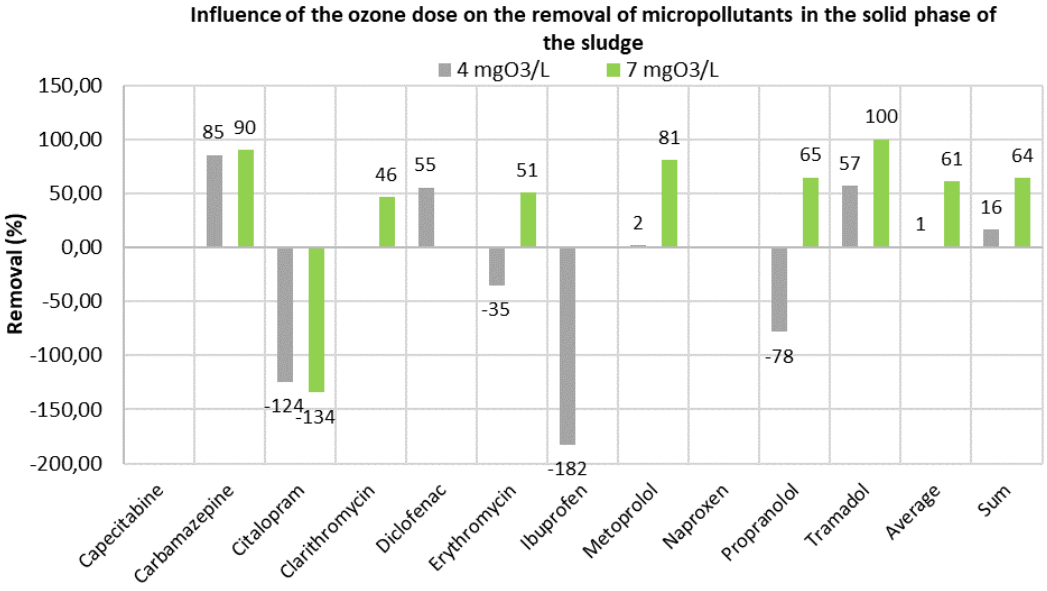

Figure 28. Influence of the ozone dose on the removal of micropollutants in the solid phase of the sludge.

The removal for the different quantified micropollutants in both phases as well as the average removal for both ozone doses in comparison to the reference line are shown in Figure 29 for the sludge. It can be observed that an increase of the ozone dose injected into the activated sludge significantly increase the removal of micropollutants, with an average removal of 31% with 4 mgO_3/L and 37 % with 7 mgO_3/L, and a removal of 38% and 43% for the sum of the concentrations, respectively. A decrease is obtained only for carbamazepine (from 85% to 78%), diclofenac (from 81% to -66%) and naproxen (from 36% to -41%).

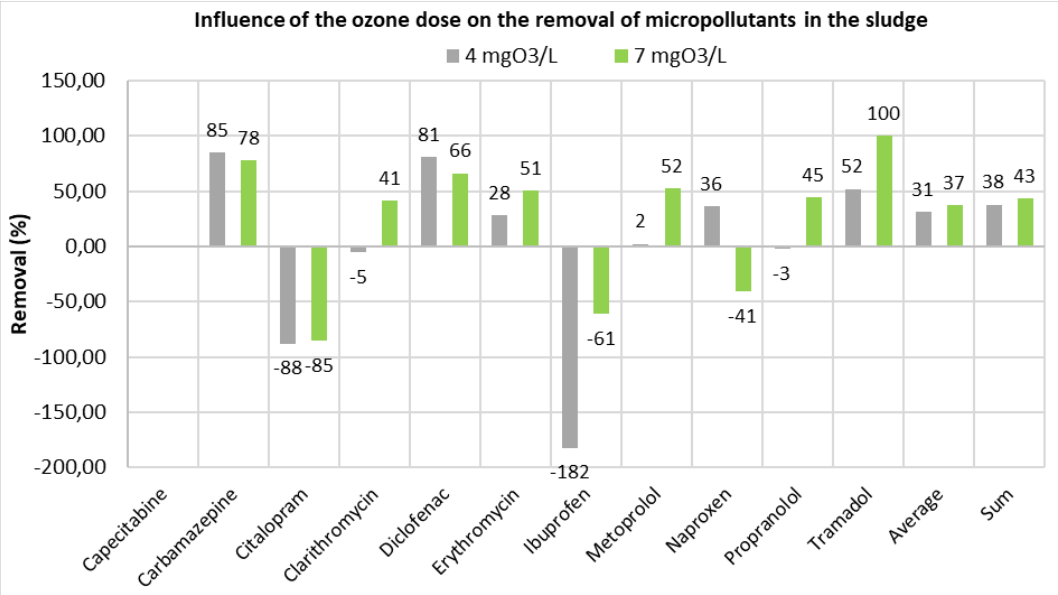

Figure 29. Influence of the ozone dose on the removal of micropollutants in the solid phase of the sludge.

3. Discussion

The results obtained show that an ozone dose of 4 mgO_3/L reduced micropollutants concentrations in the liquid and solid phases of the sludge (removal of 45% and 16%, respectively), for a total removal of 38%. An increase to a dose of 7 mgO_3/L had no additional effect on micropollutants concentrations in the liquid phase, in contrast to the solid phase where a removal 64% was achieved, for a total removal of 43% in both liquid and solid phases. These results indicate that a dose of 4 mgO_3/L is sufficient to treat only micropollutants in wastewater. To treat micropollutants in wastewater and produced sludge, a dose of at least 7 mgO_3/L is required.

The distribution of micropollutants into sludge changed with an ozone dose of 4 mgO_3/L, with a decrease of the distribution in the liquid phase, but with a dose of 7 mgO_3/L their percentage of distribution into the solid phase decreased. This shows that a high dose of ozone is needed to remove micropollutants from produced sludge.

A low dose of ozone is not sufficient to oxidize the micropollutants in the sludge due to the high competition in the liquid phase, the transferred ozone is rapidly consumed by the micropollutants and dissolved organic matter. As the ozone dose increases, there is enough transferred ozone to react with both the water phase and solid phase micropollutants.

Removal of micropollutants by application of multiple point ozonation and powder activated carbon

Appendix 2 – Effect of ozonation on organic micropollutants removal from activated sludge

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Removal of micropollutants by application of multiple point ozonation and powder activated carbon

Appendix 3 - Physical-chemical sludge characterization

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Contents

1. Sampling for physical-chemical sludge analysis

Samples were taken from Brædstrup WWTP and transported to Aalborg University for analysis at the following times:

- Week 37, 41 and 45 2018: baseline period
- Week 20 and 27, 2019: ozonation
- Week 41 2019: double dose ozonation
- Week 7 and 18 2020: PAC treatment

Samples were collected from the aerobic tanks of Line 1 (experimental line with ozonation and PAC treatment) and Line 2 (control line). The collected samples were characterized by the following analyses:

- Total solids (TS) and volatile solids (VS) (Danish standard 204, 1980)
- Suspended solids (SS) and volatile suspended solids (VSS) (Danish standard 207, 1985)
- Solid volume index (SVI) and dilute solid volume index (DSVI) as described in Bugge et al. (2013)
- Capillary suction time (CST) was measured by CST meter from Triton Electronics
- Floc size was measured with Coulter LS 13320
- Floc strength was measured with the method described in Jørgensen et al (2017)
- Oxygen uptake rate (OUR) was determined with the method described in Rudelle et al. (2013)
- Nitrification and denitrification rate was measured as described in van Loosdrecht et al. (2016)
- BioP capacity was measured by measuring the release of phosphate over 60 minutes of anaerobic treatment of aerobic conditioned sludge. The orthophosphate release was normalized with the VSS concentration to determine the released orthophosphate with the units *mg PO4-P / g VSS*.

2. Sludge dewaterability and settling properties

SS concentrations, CST, SVI and DSVI values measured on Line 1 and 2 samples are listed in Table A1.1 below.

Table A1.1: Measured CST, SVI, DSVI and SS concentration for Line 1 (experimental line) and Line 2 (control line) samples sampled during baseline campaign (week 37, 41 and 45, 2018), ozonation (week 20, 27 and 41, 2019) and PAC treatment (week 7 and 18, 2020). From week 27, SVI and DSVI was triplicated on each sample, hence a mean value is shown with a standard deviation.

		SS (g/L)	CST (s)	SVI (mL/g)	DSVI (mL/g)
Week 37 2018	Line 1	4.0	16.5 ±2.2	157	90
	Line 2	4.9	15.0 ±0.6	138	83
Week 41 2018	Line 1	4.9	14.4 ±0.4	125	80
	Line 2	6.8	15.2 ±0.3	103	60
Week 45 2018	Line 1	4.8	11.2 ±0.6	149	91
	Line 2	4.8	15.1 ±0.4	168	106
Week 20 2019	Line 1	5.4	16.9 ±0.6	106	73
	Line 2	4.8	16.7 ±0.7	169	93
Week 27 2019	Line 1	2.5	10.8 ±0.4	122 ±7	108 ±12
	Line 2	4.7	11.7 ±0.5	137 ±16	83 ±5
Week 41 2019	Line 1	5.2	13.9 ±1.1	59 ±3	26 ±3
	Line 2	4.3	13.6 ±0.7	147 ±18	45 ±3
Week 7 2020	Line 1	4.4	14.5 ±1.2	97 ±6	36 ±0
	Line 2	4.6	14.3 ±0.3	115 ±7	37 ±0
Week 18 2020	Line 1	5.4	15.0 ±0.9	38 ±4	22 ±2
	Line 2	5.6	17.3 ±0.7	37 ±8	18 ±4

The measured CST values are low (all CST < 20 s) for Line 1 and Line 2. Samples from line 1 and 2 had similar CST values, and no effect was observed on line 1 sludge samples during ozonation (week 20-41 2019) compared to Line 2 samples. Hence, CST measurements could not reveal changes in dewaterability of sludge by ozonation. It should be noted that CST values are already low, making it difficult to determine a change in CST.
However, SVI and DSVI values was significantly reduced after the onset of ozonation. This is confirmed by the analyses of sludge samples at AAU (Table A1.1 and Figure A1.1), as well as weekly analyses measured at Brædstrup WWTP (Figure A1.2). Figure A1.1 and Figure A1.2 shows the development of SVI measured at AAU and Brædstrup WWTP, respectively. Both show that Line 1 and Line 2 SVI values are similar during the baseline campaign (autumn 2018). As the ozonation condition is started in spring 2019, Line 1 SVI decreases compared to Line 2 SVI. After turning off ozonation in November 2019 and switching to PAC treatment, SVI is again similar between line 1 and 2. Hence, the results supports that ozonation promotes reduction of SVI and DSVI, i.e. better settling properties. The results also show that the effect is reversible, i.e. as ozonation is stopped, the sludge turns to have the same properties as the non-ozonated sludge from Line 2.

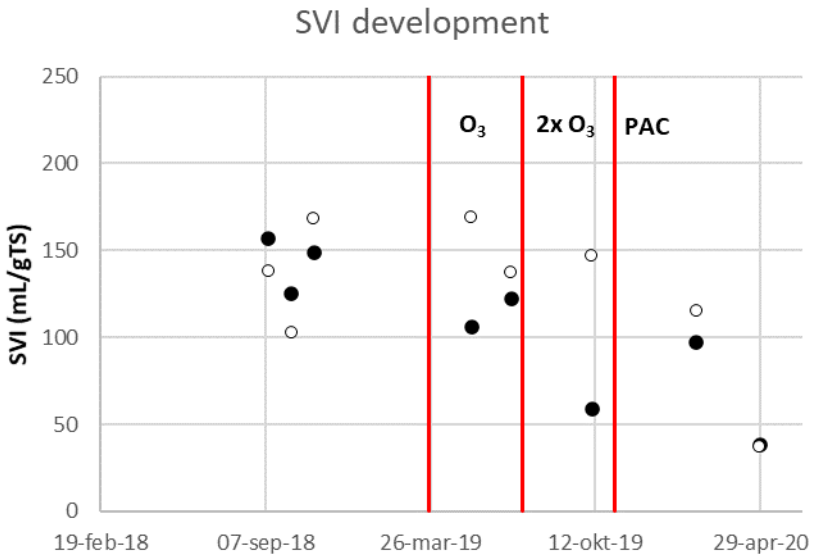

Figure A1.1: SVI of Line 1 (experimental with ozonation and PAC, □) and Line 2 (control, □) sludge samples measured at AAU.

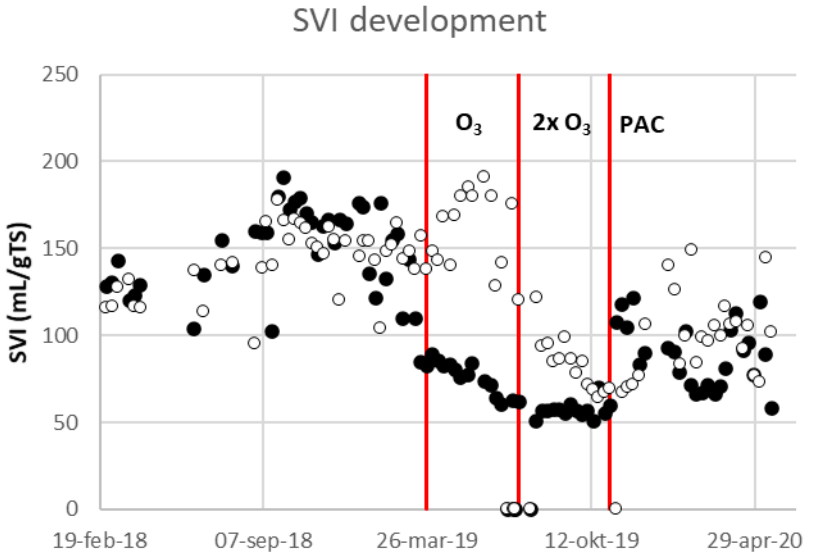

Figure A1.2: Development in SVI of Line 1 (experimental line, □) and Line 2 (control, □) measured weekly at Brædstrup WWTP during the baseline campaign, ozonation and PAC conditions.

3. Sludge floc properties

The sludge degree of flocculation was described by measuring the residual turbidity, shear sensitivity and floc sizes, as summarized in Table A1.2.

Table A1.2: Residual turbidity, flocs deflocculation potential, shear sensitivity and diameter measured for Line 1 (experimental) and Line 2 (control) during week 37-41 2018 (baseline), Week 20-41 2019 (ozonation) and week 7-18 2020 (PAC).

		Residual turbidity (L/g SS)	Deflocculation potential	Shear sensitivity	Floc diameter (µm)
Week 37 2018	Line 1	0.0031 ±0.0002	0.076	0.021	219.5 ±399.1
	Line 2	0.0027 ±0.0004	0.040	0.022	101.9 ±170
Week 41 2018	Line 1	0.0027 ±0.0003	0.051	0.036	119.3 ±192.4
	Line 2	0.0010 ±0.0002	0.018	0.027	79.98 ±130
Week 45 2018	Line 1	0.0016 ±0.0004	0.034	0.026	141.4 ±260
	Line 2	0.0033 ±0.0001	0.026	0.008	97 ±176.2
Week 20 2019	Line 1	0.0021 ±0.0003	0.063	0.022	198.5 ±398.8
	Line 2	0.0018 ±0.0004	0.039	0.021	115.5 ±248.4
Week 27 2019	Line 1	0.0026 ±0.0008	0.105	0.022	111.7 ±185.7
	Line 2	0.0008 ±0.0002	0.020	0.024	88.71 ±144.9
Week 41 2019	Line 1	0.0030 ±0.0012	0.034	0.041	102 ±182
	Line 2	0.0018 ±0.0007	0.022	0.021	119 ±222
Week 7 2020	Line 1	0.0275 ±0.0007	0.068	0.035	276.5 ±453.6
	Line 2	0.0200 ±0.0014	0.039	0.031	104.3 ±183.8
Week 18 2020	Line 1	0.0070 ±0.0008	0.080	0.014	105.6 ±157.9
	Line 2	0.0085 ±0.0021	0.039	0.009	116.9 ±242.1

Residual turbidity was determined by centrifugation (2200 rpm, 2min) of sludge samples followed by absorbance measurement at 650 nm wavelength in a spectrometer. This absorbance was normalized with the SS concentration and denoted the residual turbidity, which is proportional to the amount of non-flocculated matter in the aqueous phase of sludge. This did not show significant differences between the experimental line and the control line. Hence, a positive or negative impact of ozonation on degree of flocculation could not be concluded from the residual turbidity analyses. The floc strength were characterized by shearing 600 mL sludge samples in a baffle reactor with a paddle and taking samples over two hours and measuring their residual turbidity to determine the release of material to the aqueous phase by sludge deflocculation. Figure A1.3 shows the baffle reactor and paddle along with representative plots of Line 1 and Line 2 residual turbidities over time during shearing.

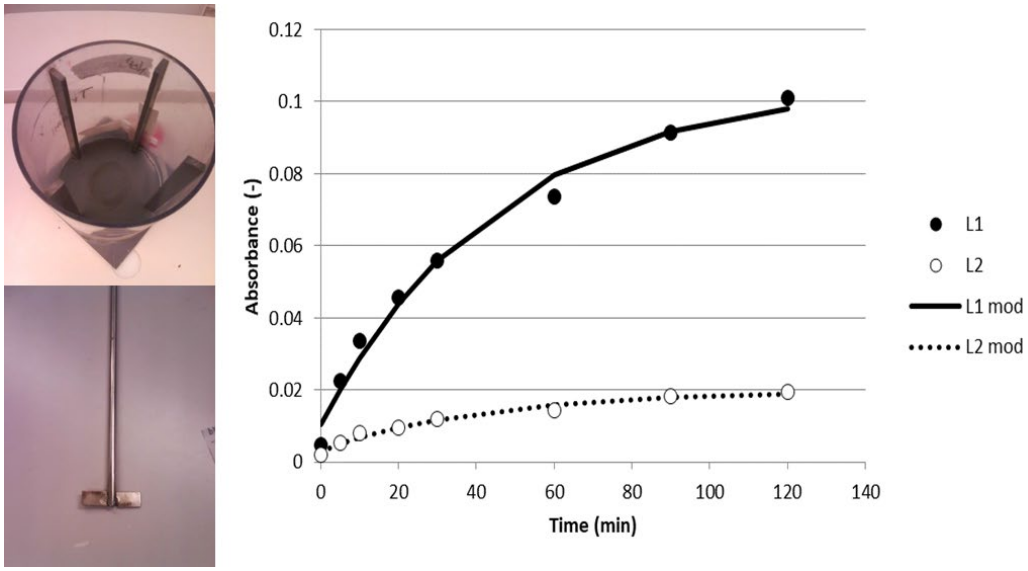

Figure A1.3: Baffle reactor and paddle for deflocculation (left) and measured development in residual turbidity over time during deflocculation by shearing (right) measured in Line 1 (L1, □) and Line 2 (L2, □).

The following model was fitted to the measured values to express the residual tudbidity as function of time.

$$\tau_t = \tau_0 + \tau_\infty(1 - kt)$$

In which τ_t, τ_0 and τ_∞ is the residual turbidity at time t, at start and at infinity (when no more release from sludge occur), while k is the rate of release, i.e. the floc shear sensitivity. There was not found any systematic difference in shear sensitivity between flocs in Line 1 and Line 2 sludge samples, and no effect of ozone or PAC treatment was found. The deflocculation potential is expressed as the sum of τ_0 and τ_∞. The deflocculation potentially was consistently larger for flocs in Line 1 compared to Line 2, but an effect of treatment by ozonation or PAC could not be concluded.

Figure A1.4 shows the mean diameters of sludge flocs in Line 1 and 2 over time.

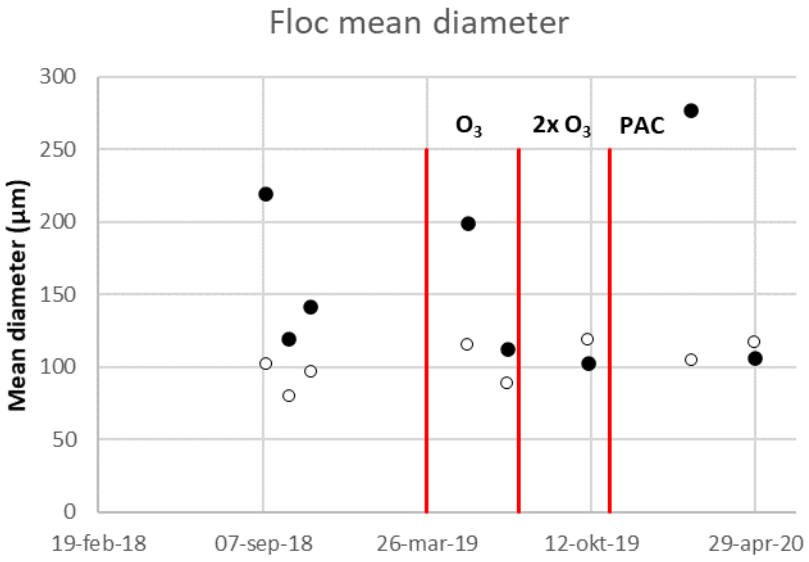

Figure A1.4: Mean floc diameters measured over time in Line 1 (experimental, □) and Line 2 (control, □) samples.

In the baseline campaign (autumn 2018) Line 1 flocs has consistently larger mean diameters than Line 2 flocs. This changed during 2019, where the mean diameters become more similar between Line 1 and 2 during the treatment with double dose of ozone, and remain similar as ozonation is switched off.

4. Sludge biological performance

Results of biological performance analyses are summarized in Table A1.3.

Table 3: Oxygen uptake rate (OUR), denitrification and nitrification rate and BioP capacity for samples from Line 1 (experimental) and Line 2 (control) sampled during baseline campaign (week 37, 41 and 45, 2018), ozonation (week 20, 27 and 41, 2019) and PAC treatment (week 7 and 18, 2020).

		OUR (mgO2/gVSS/hr)	Denitrification rate (mg/gVSS/hr)	Nitrification rate (mg/gVSS/hr)	BioP capacity (mg/gVSS)
Week 37 2018	Line 1	4.2	0.9	-	-
	Line 2	3.8	0.6	-	-
Week 41 2018	Line 1	3.1	0.9	1.1	8.3
	Line 2	0.9	0.6	0.4	18.0
Week 45 2018	Line 1	5.9	1.0	1.2	7.9
	Line 2	5.5	1.0	0.4	4.7
Week 20 2019	Line 1	5.7	1.6	1.0	13.9
	Line 2	5.3	1.4	0.7	18.9
Week 27 2019	Line 1	6.6	2.1	2.6	28.7
	Line 2	4.2	0.6	1.4	64.4
Week 41 2019	Line 1	4.6	0.6	1.0	15.6
	Line 2	6.3	0.6	1.1	14.5
Week 7 2020	Line 1	3.6	0.5	1.2	15.3
	Line 2	3.7	0.4	1.2	14.2
Week 18 2020	Line 1	2.3	1.5	1.7	30.0
	Line 2	2.1	0.8	0.8	15.2

Throughout the different condition, there were no systematic variations between oxygen uptake rates, nitrification and denictrification rates and BioP capacity. The development in OUR and BioP capacity is plotted in Figure A1.5, showing some seasonal variations of OUR samples (but no conclusive differences between Line 1 and 2 samples), while BioP capacity was similar thoughout the timeframe of analyses. Hence, there cannot be concluded any positive nor negative effect of ozonation on biological performance from the analyses of sludge biological performance.

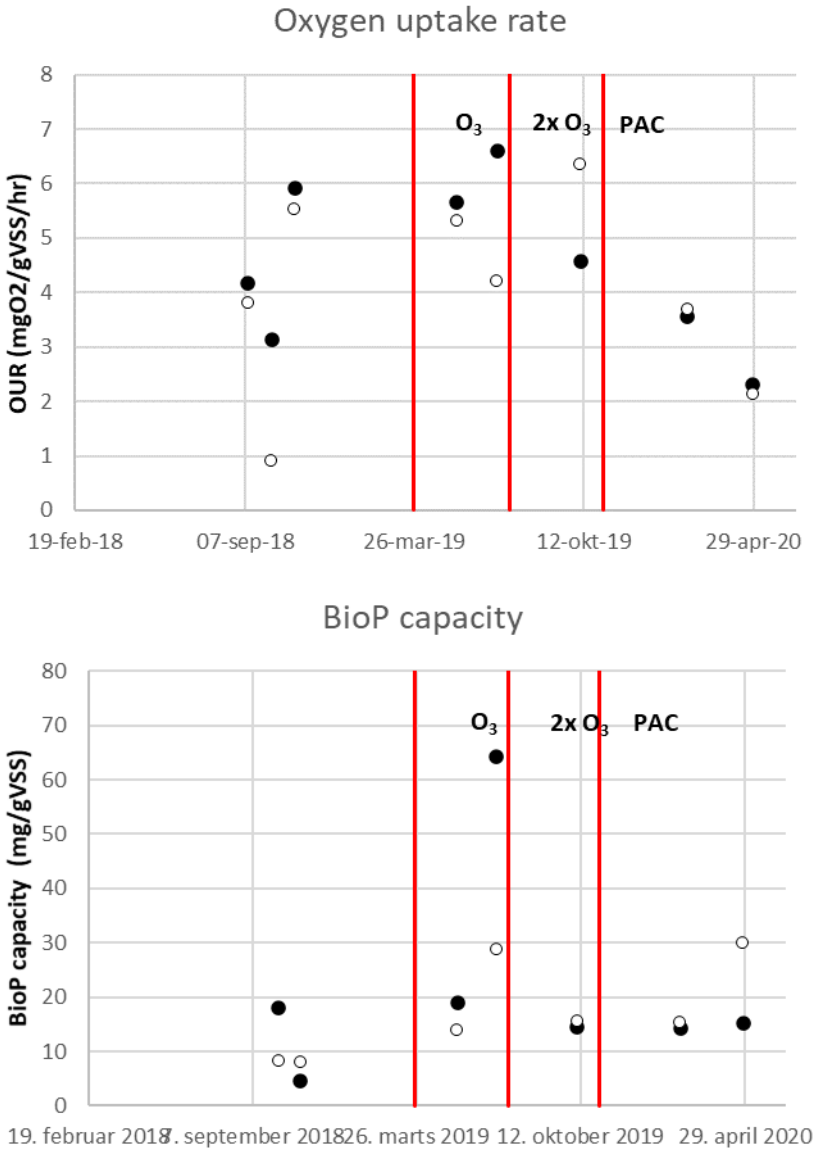

Figure A1.5: Oxygen uptake rate (OUR, top) and BioP capacity (bottom) of Line 1 (experimental □) and Line 2 (control □) sludge samples during baseline campaign, ozonation and PAC treatment.

The lack of influence of ozonation or PAC treatment on biological performance is supported by data for effluent quality. In Figure A1.6, effluent COD and total nitrogen (TN) concentrations are plotted over time. For both COD and TN concentrations, there was no systematic variations between the experimental and control line that would support any impact of ozonation or PAC treatment on the biological performance.

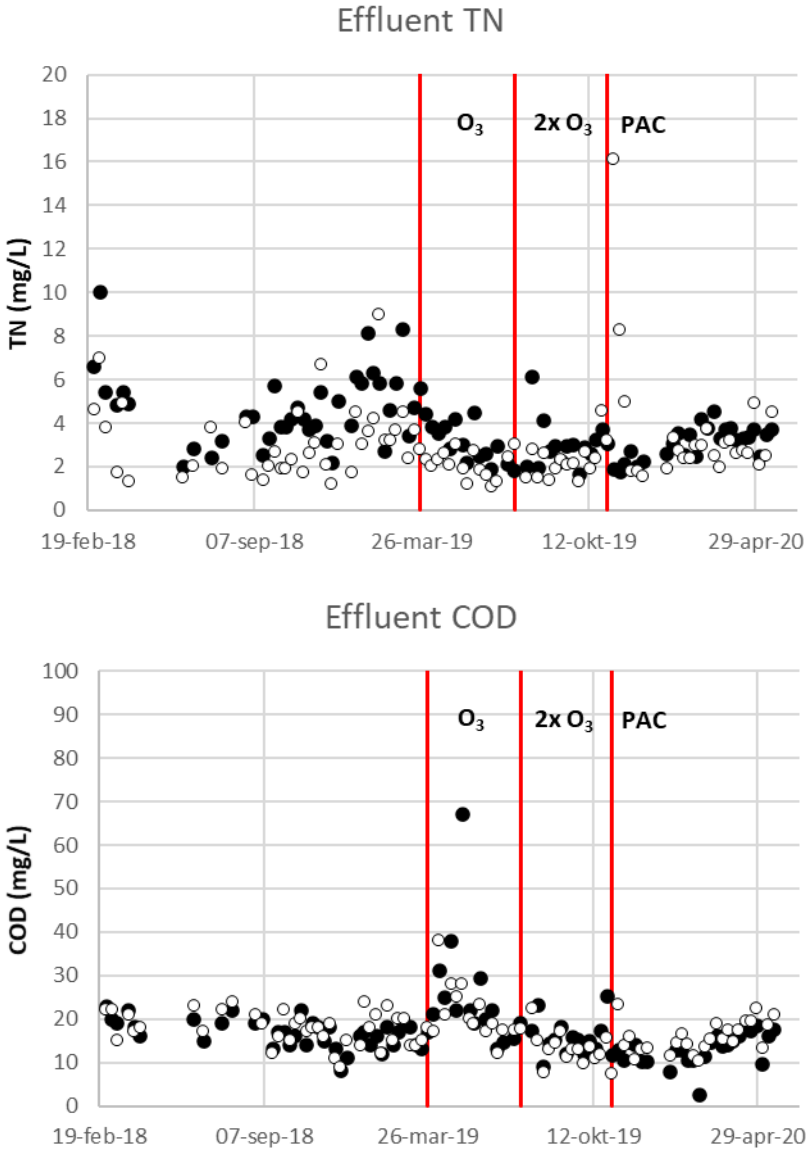

Figure 6: Weekly measurements of effluent TN (top) and COD (bottom) concentrations from Line 1 (experimental □) and Line 2 (control □) during the baseline campaign, ozonation and PAC tests.

5. Conclusions

- The onset of ozonation significantly reduces SVI and DSVI of sludge. This is a reversible process, i.e. after termnation of ozonation, the sludge SVI and DSVI increased again. Hence, the settling properties are enhanced by ozonation.
- CST measurements could not conclude any impact of ozonation of dewaterability. This may be because CST values are already low, i.e. dewaterability is high.
- No change in floc properties as a result of the treatments could be concluded.
- There was no influence on the biological performance by ozonation or PAC treatment in terms of both nitrification, denitrification and phosphorous and COD removal.

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Removal of micropollutants by application of multiple point ozonation and powder activated carbon

Appendix 3 - Physical-chemical sludge characterization

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Appendix 4 – Sludge characterization by light microscopy and 16S rRNA sequencing

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Contents

1. Biological effects from full-scale tests at Brædstrup WWTP.

1.1 State-of-the-art, literature review

The microorganisms in activated sludge and their soluble substrates may be affected by ozonation. The strong oxidation potential of ozone contributes to decomposition of suspended solids (SS) and extracellular polymeric substances (EPS), cell lysis, oxidation and mineralization of organic matter when it is applied to the activated sludge process (Barbarroja et al., 2019). In addition, ozonation directly affects the activity and mortality rates of the microorganisms depending on the species and their location in the sludge floc (Barbarroja et al., 2019). However, no adverse effect on the biological nitrification- or the phosphorus removal rates could be detected in a previous study by (Nilsson et al., 2014). Contrary to microorganisms related to nitrification- and phosphorus removal, the filamentous microorganisms are more exposed to ozonation (Böhler and Siegrist, 2004; Nilsson et al., 2019). Other reported effects of ozonation were elevated levels of free bacteria in the sludge and decreasing numbers of higher organisms such as protozoa and metazoa with increasing ozone dose (Nilsson et al., 2019).
The goal of this part of the project was to detect possible effects of ozon on the the microbial community, the protozoan and animals, and the sludge floc structure. This was done by light microscopy and DNA sequencing in the ozone and reference line.

1.2 Materials and Methods

1.2.1 Sampling overview

Fresh activated sludge was sampled from the aeration tanks of line 1 (experimental) and line 2 (control) every second week and shipped directly to the lab and analyzed by light microscopy within 48 hours. Weekly samples were stored at -20°C for later DNA extraction and microbial community analysis. Sampling started 13th September 2018 and ended 16th December 2019. Two ozone conditions were conducted in line 1, with a dosing of 3.98 mg/L ozone in the process tank from 25th Marts 2019 until 16th July where the dose was increased to 7.20 mg/L ozone in the process tank and continued until the end of November. Due to startup issues, a short period of on/off ozonation was experienced from February to March 2019. Figure 1 shows the timeline for biological assessment

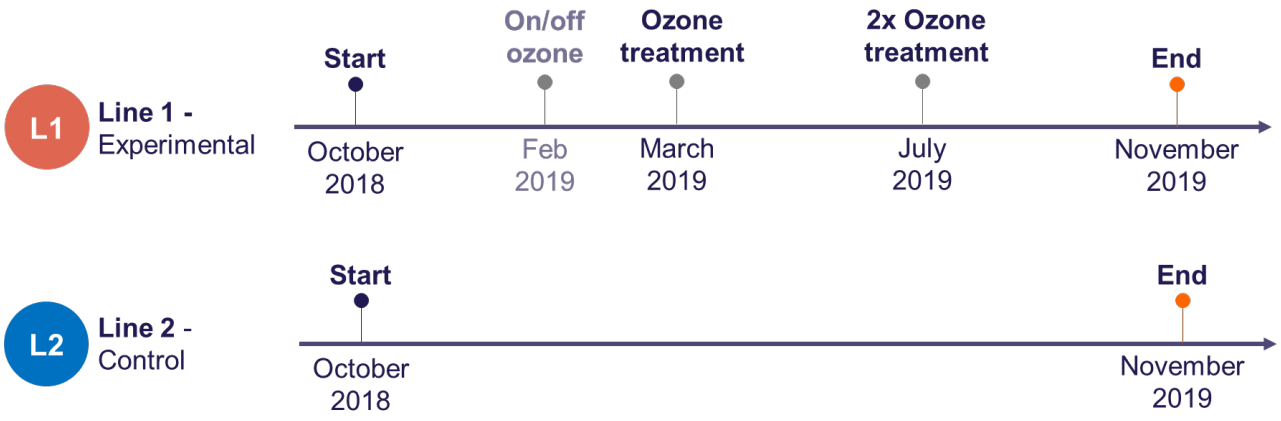

Figure 1 - Timeline of biological assessment at Brædstrup WWTP.

1.2.2 Light Microscopy Analysis

General sludge properties such as sludge floc size (small, medium and large), floc structure (open structure, bridging between flocs by filaments and no filament impact), floc properties (strength, form and shape), compactness (scale from 0 = diffuse to 1 = compact), firmness (scale from 0 = weak to 1 = firm), filament index (FI, range from 0–5, no filaments – very many filaments), presence of animals (range from 0-5, none – massive), presence of free cells (range from 0-5, none – massive) were recorded with light microscopy in accordance with procedures by Eikelboom (2000).

1.2.3 Microbial community analysis

A schematic overview of the procedure for DNA based microbial community analysis is seen in Figure 2.

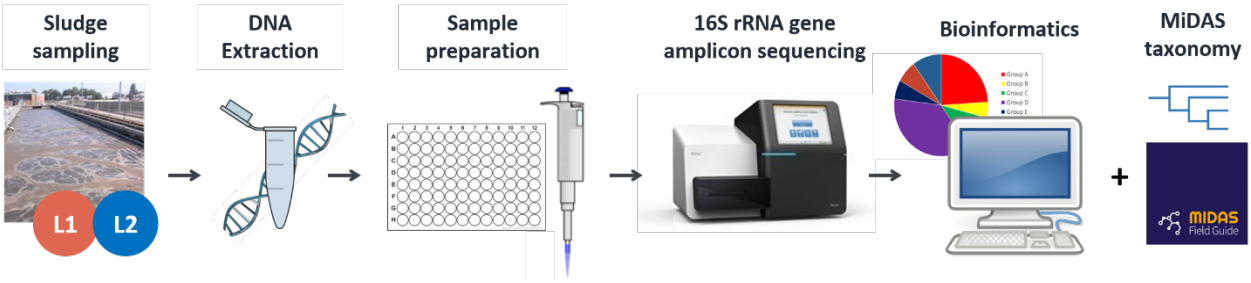

Figure 2 - Microbial community analysis practical overview.

DNA extraction, 16S rRNA V1-3 amplicon preparation and sequencing were performed as described in (Stokholm-Bjerregaard et al., 2017) and the protocols are available at www.midasfieldguide.org. The raw sequencing data was processed as described by (Dueholm et al., 2019) using MiDAS 3.5 taxonomy. Data processing was performed using R v.3.5.1 (R Core Team, 2019). In total, 118 samples were analysed and they had minimum 45,000 reads and were normalized to 100% and visualized using ggplot2 v.3.2.0 (Wickham, 2016) and amp_heatmap function in ampvis2 v.2.5.8 (Andersen et al., 2018). Principal Components Analysis (PCA) with hellinger transformation (Legendre and Gallagher, 2001) was used to visualize the development of community structure in the two lines over time. Prior to the analysis, ASVs (amplicon sequence variants) that were not present in more than 0.1% relative abundance in any sample, were removed. For alpha diversity analysis, the samples were randomly subsampled to 45,000 sequences per sample. Alpha diversity was calculated by amp_alphadiv function in ampvis2. Time series of selected microorganisms were plotted with the amp_timeseries function.

1.3 Results and Discussion - Light Microscopy Analysis

Figure 3 summarizes the observations of the light microscopy analysis. The two lines showed similar sludge characteristics during the baseline campaign and first ozone condition with only minor fluctuations. However, during second ozone condition the two lines became more distinct.

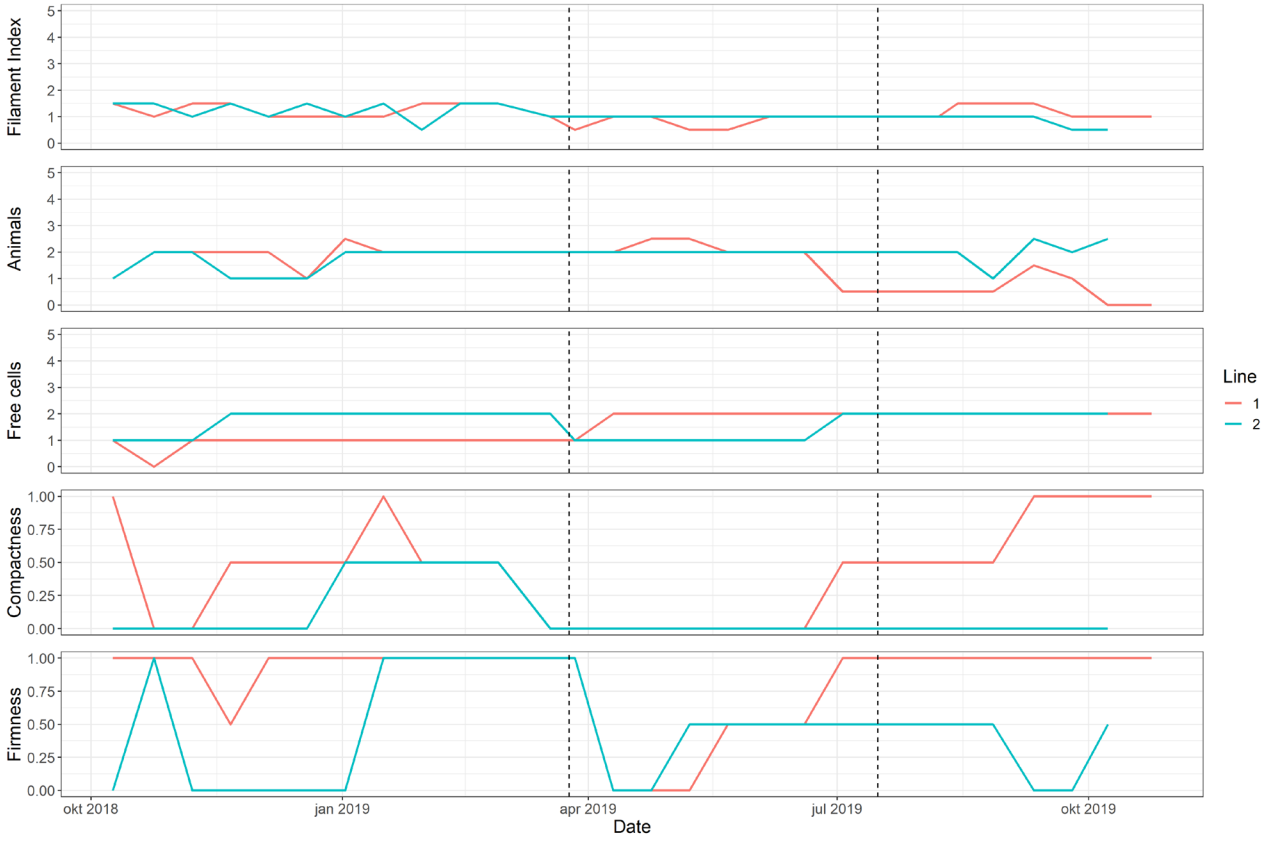

Figure 3 - Microscopic sludge investigation. Line 1 = experimental (red) and line 2 = control (blue). Dotted line represent start of ozonation condition 1 (3.98 mg/L ozone) and 2 (7.2 mg/L ozone). Filament Index, Animals and Free cells (scale from 0 = none, 5 = massive). Compactness (scale from 0 = diffuse to 1 = compact) and Firmness (scale from 0 = weak to 1 = firm).

1.3.1 Filament index

The filament index was low (around 1) in the whole experimental period indicating very few filamentous microorganisms and did not differ notably between the two lines.

1.3.2 Animals

Animals such as single-celled protozoa and multi-cellular metazoa are usually present in activated sludge (Eikelboom, 2000). They are much larger than the bacteria and possess a variety shapes and forms. Their biomass comprises, at most, a few percent in the treatment plants. Their presence can be good indicators for the process performance. For example, the attached species (like *Vorticella*) will appear at low sludge loading levels (long sludge age), whereas metazoa and testate amoeba appear at very long sludge ages. The testate amoeba *Arcella* with the characteristic yellow-brown round shape is a commonly occurring species in low-loaded activated sludge plants under nitrifying conditions (Eikelboom, 2000). The number of animals in the experimental line decreased drastically during second ozone condition with the higher ozone dosage (Figure 4). Only a few *Arcella* or no animals at all were observed during the condition.

1.3.3 Free cells

Free bacterial cells are supplied with the influent as well as from “erosion” of the sludge flocs in the aeration tanks. These cells are extensively consumed by the protozoa, in particular by the ciliates. If the sludge load rise, the size of the protozoa population may also increase. However, if the sludge age is short, the protozoa population cannot be maintained as they grow too slow. Even though almost no animals were present in the experimental line (line 1) in the second ozone condition, the level of free cells did not seem to increase compared to the control line.

1.3.4 Floc properties

The floc properties in regards to compactness and firmness in the two lines were quite similar until the second condition with the higher ozone dosage. The flocs in the experimental line (line 1) were more compact and firm compared to the control line. No clear differences were observed between the two lines during the conditions regarding floc size and shape (Figure 5 and Figure 6).

1.3.5 Attached growth

Another notable effect of the second ozone condition was the removal of attached growth present on the surface of filamentous bacteria (Figure 4). This phenomenon was previously reported by Nilsson et al. (2014) by fluorescence microscopy on sludge flocs exposed to increasing ozone concentrations (Figure 7).

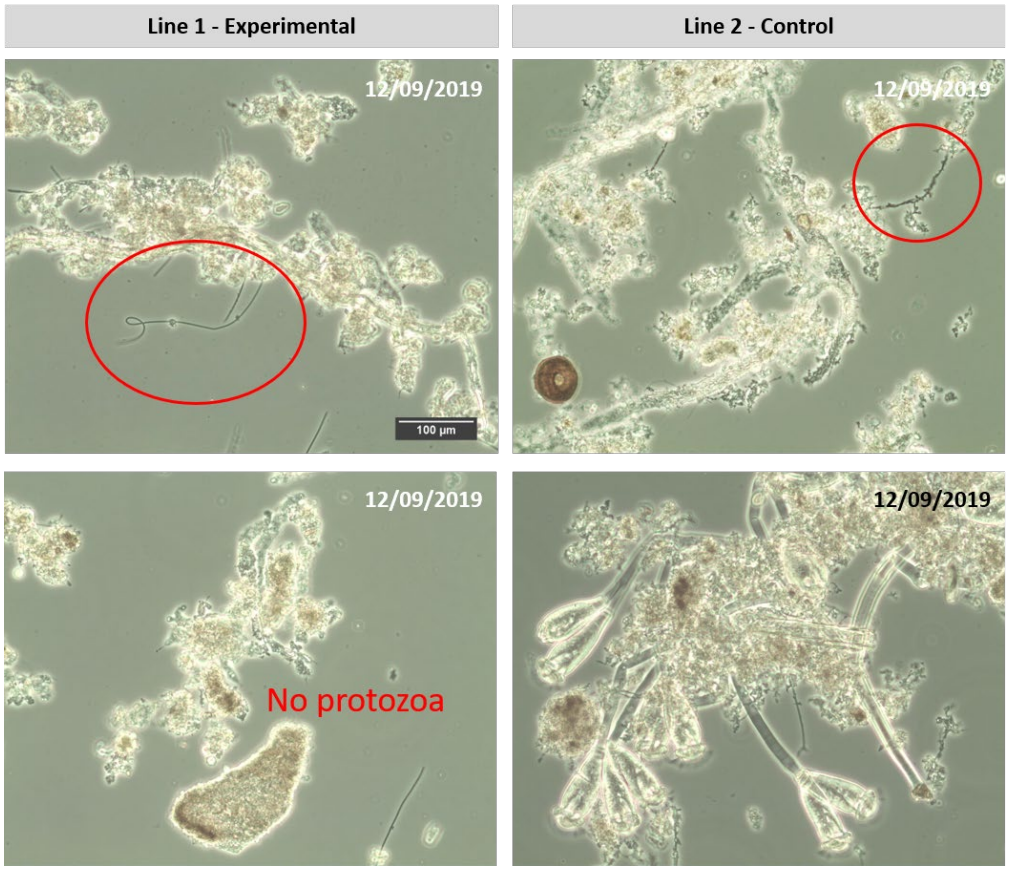

Figure 4 - Notable effects of second ozone condition. No attached growth on the filaments in line 1. No animals present in the experimental line 1, whereas the control line 2 had Vorticella, Arcella and Nematodes (not shown).

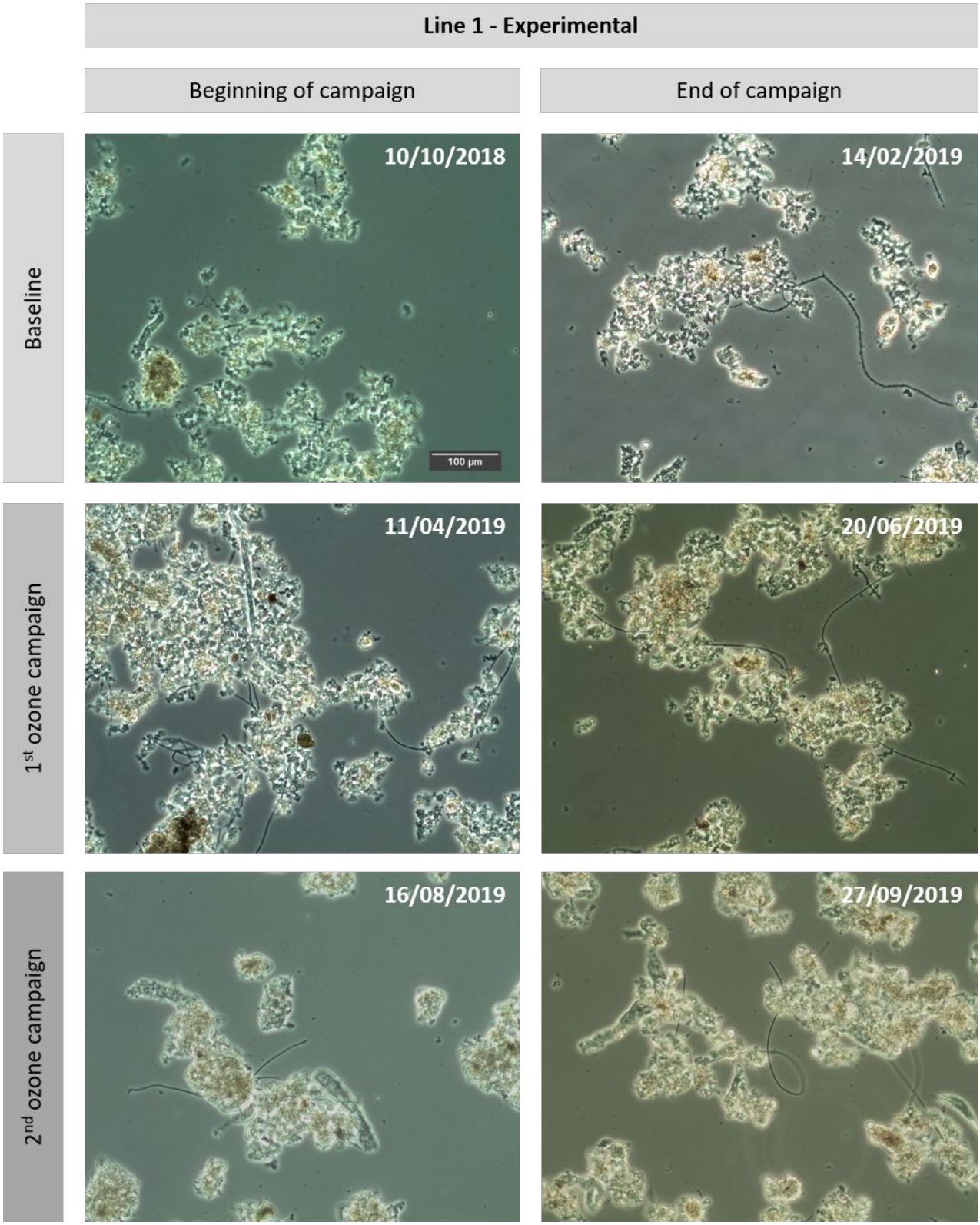

Figure 5 - Microscopic observation from Line1 - "Experimental". Development of floc structures during baseline campaign, ozonation condition and 2x ozonation condition.

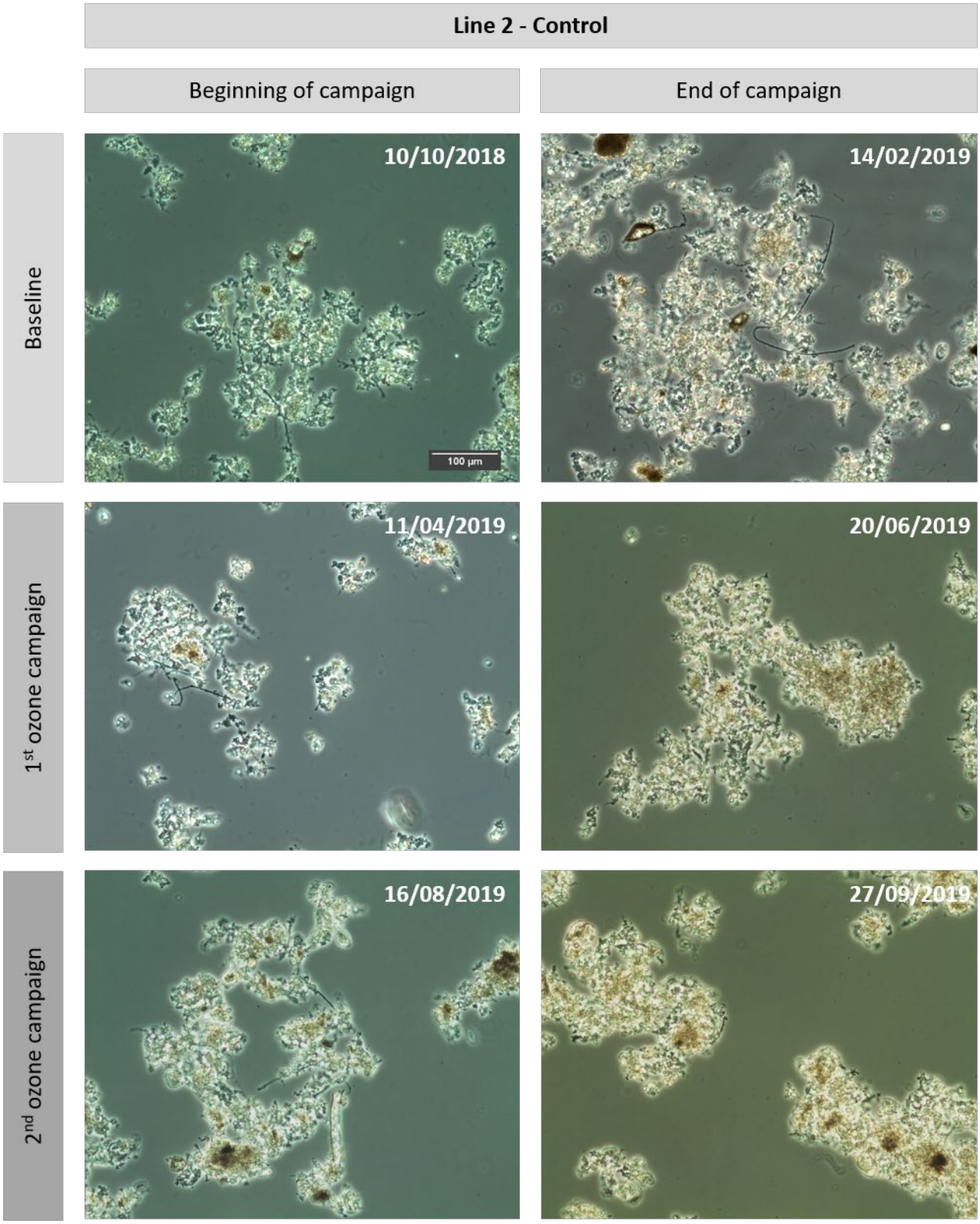

Figure 6 - Microscopic observation from Line2 - "Control". Development of floc structures during conditions in the control line.

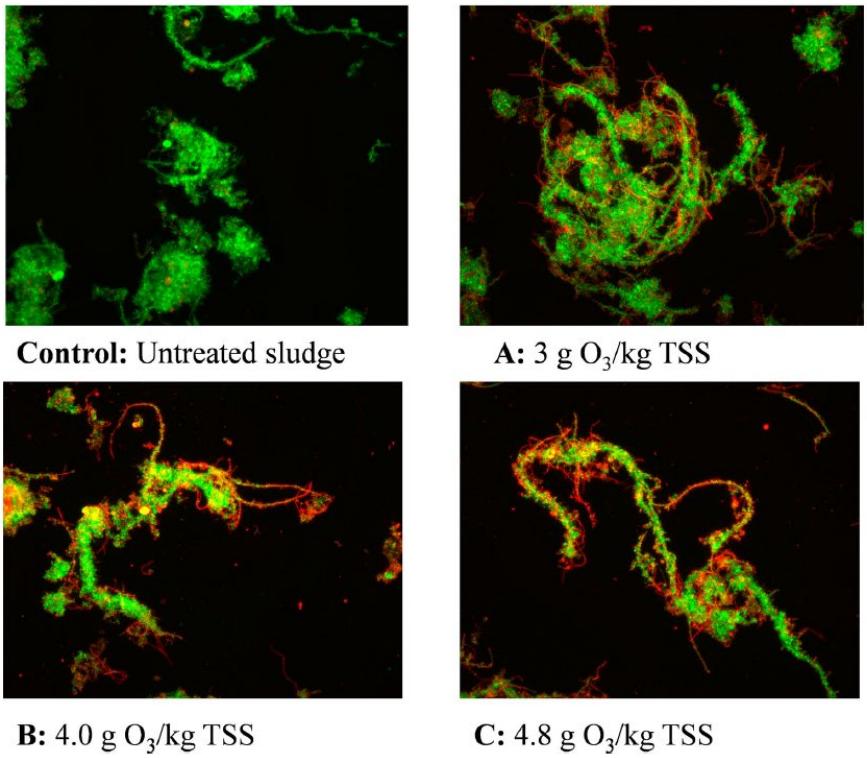

Figure 7 - From Nilsson et al. 2014: Live/Dead fluorescence microscopy images showing the most exposed bacteria, sitting on the outside of the sludge flocs, dies with the higher ozone dose (Green = live, Red = dead).

1.3.6 Conclusions - Light Microscopy Analysis

- High dose of ozone killed the metazoa and protozoa. However, their absence was not correlated with an increase of free cells in the sludge water phase.
- The attached growth present on the filamentous organisms protruding from the flocs disappeared during first ozone dosage.
- The compactness and firmness of the flocs improved during ozonation compared to the control line – especially during the high dosage condition.

1.4 Results and Discussion - Microbial community analysis: 16S rRNA amplicon sequencing

1.4.1 Overall microbial community structure

Brædstrup WWTP is a relatively small plant (16000 PE) with biological removal of C, N and partly P and it receives municipal and industrial wastewater. The community composition in Danish WWTPs is well described in plants with nutrient removal during the MiDAS project (Nierychlo et al., 2020a) and information about most species can be found at the web page (www.midasfieldguide.org). Phylum and genus classification are used throughout this report. Names containing the prefix “midas_” are unknown bacteria that are not found in the public taxonomic database (SILVA). The following letter after the prefix indicates at which taxonomic rank the bacteria could not be identified in the SILVA database (Kingdom, Phylum, Class, Order, Family, Genus, Species).

The 50 most abundant genera in the two lines of Brædstrup WWTP are listed in the heatmaps below (Figure 8 and Figure 9). The heatmap in Figure 8 shows microbial composition in the control line. Most of the abundant genera are well known from other WWTPs but some are unusual, those with midas_xx names. The heatmap shows that the microbial community experienced some seasonal dynamics over the year of sampling. The most abundant genus was *Trichococcus* and it increased in abundance during spring and summer. The second most abundant genus is likely filamentous and provides the backbone for the flocs. The third most abundant genus, the unknown midas _g_17, decreased in abundance during the spring and summer months. The nitrifier *Nitrospira* (NOB) was present in the normal abundance range of 0.5-3%. The lowest amounts was in summer, what is unusual. Ammonium oxidizing bacteria, AOB (*Nitrosomonas*) was present in low concentration outside top 50, more about that later. In the experimental line (Figure 9), the most notable change seems to be the that fourth most abundant genus, unknown midas_g_832, which was second most abundant in the control line, gradually disappeared from the line after the first ozonation. This species belongs to phylum Chloroflexi that contains a number of bulking filaments.

The ordination plot (Figure 10) shows how the overall community composition changed over time in the two lines. Each point represents the community composition in one sample, and the distance between the points represents the differences in microbial community composition between the samples. The figure shows that the two lines had very similar microbial communities until ozonation began in the experimental line (Line 1).

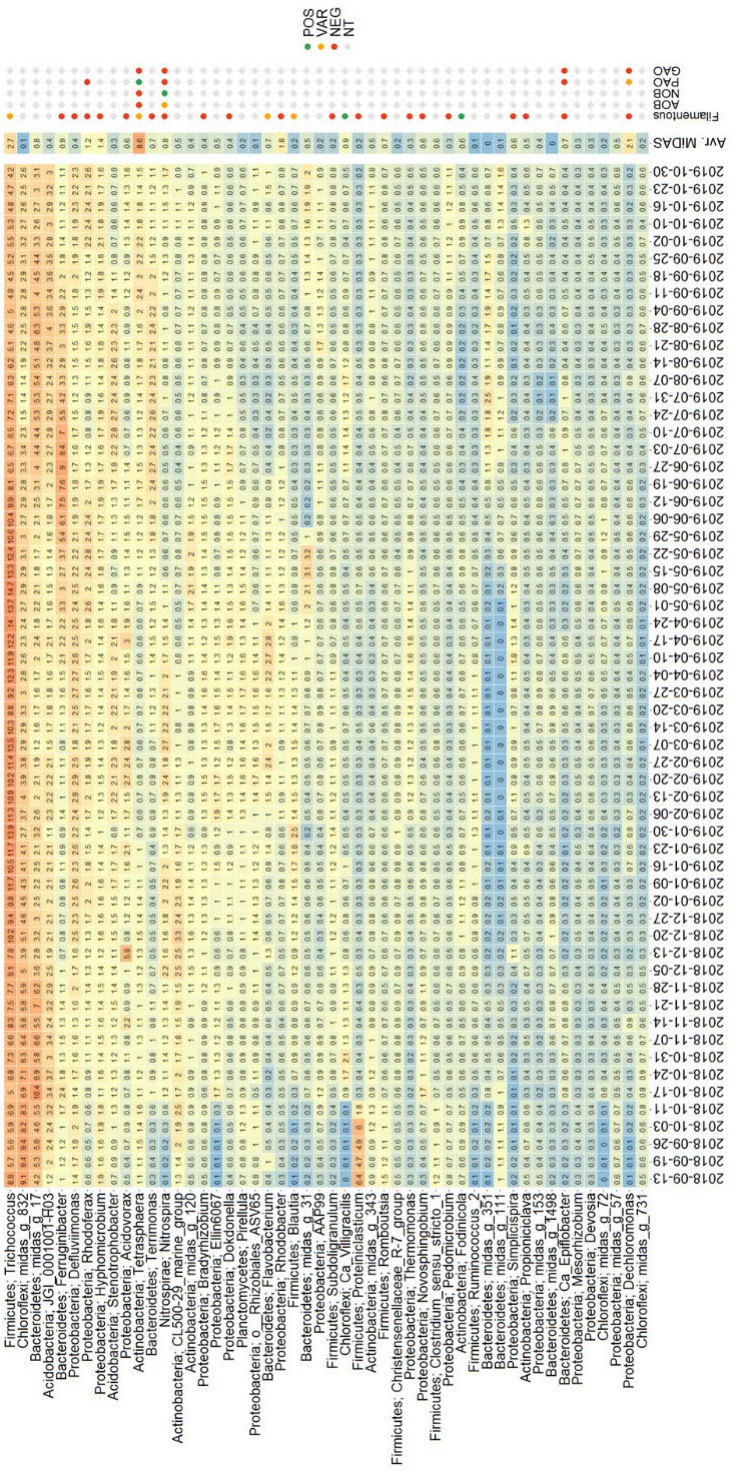

Figure 8 – Heatmap of top 50 abundant genera in Line 2 – Control. The names on the y-axis show the phylum and genus classification (separated by a semicolon ;) and are ordered by the abundance in decreasing order from the top. The relative abundances are indicated as a color gradient ranging from red (high) to blue (low). The last column presents the average abundance of the given microorganism in all Danish WWTPs with nutrient removal (members of the Danish microbial database of activated sludge project – MiDAS). Functional information about the genus is shown in the right panel, if known. AOB: Ammonia oxidizing bacteria, NOB: Nitrite oxidizing bacteria, PAO: Polyphosphate accumulating organisms, GAO: Glycogen accumulating organisms.

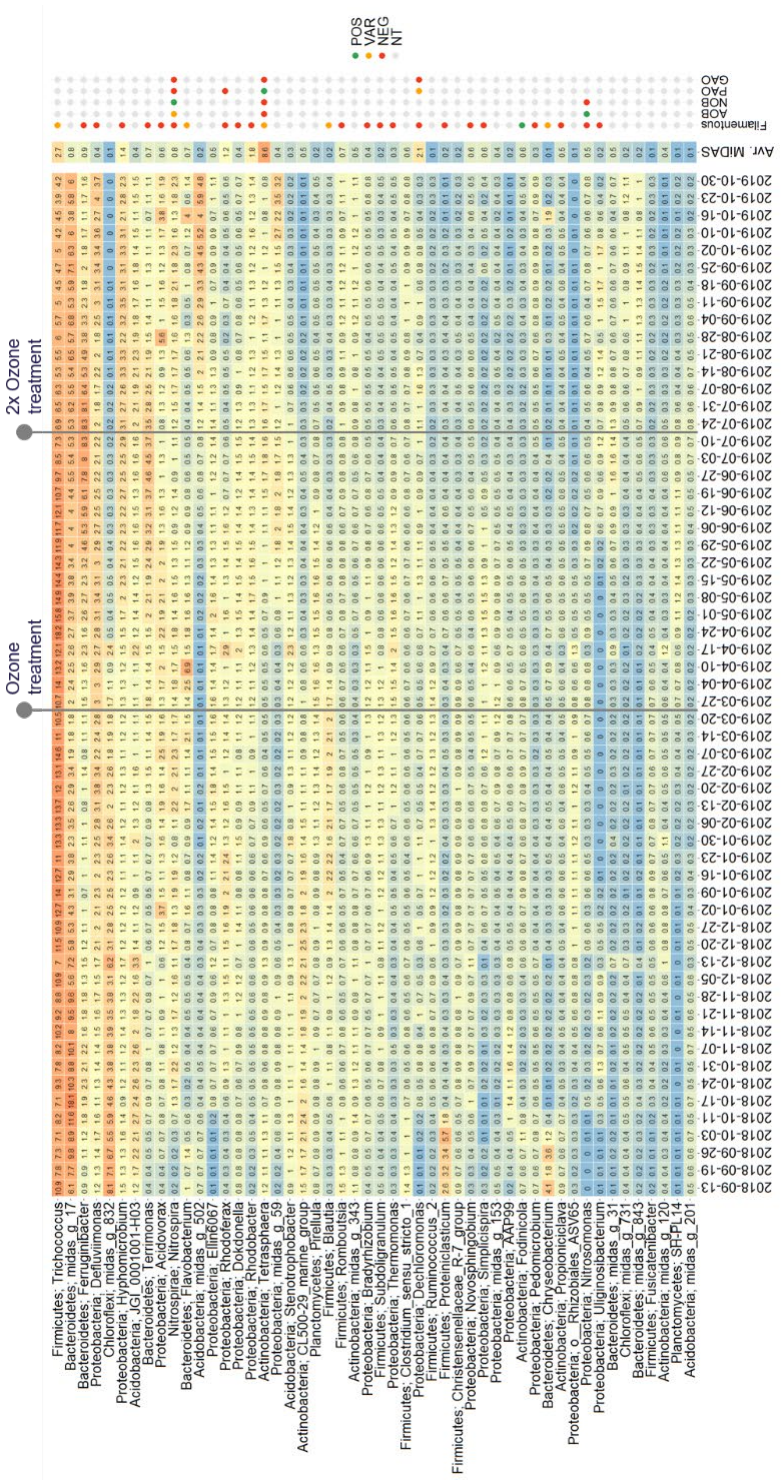

Figure 9 - Heatmap of top 50 abundant genera in Line 1 – Experimental. The names on the y-axis show the phylum and genus classification (separated by a semicolon ;) and are ordered by the abundance in decreasing order from the top. The relative abundances are indicated as a color gradient ranging from red (high) to blue (low). The last column presents the average abundance of the given microorganism in all Danish WWTPs (members of the Danish microbial database of activated sludge project – MiDAS). Functional information about the genus is provided if known. AOB: Ammonia oxidizing bacteria, NOB: Nitrite oxidizing bacteria, PAO: Polyphosphate accumulating organisms, GAO: Glycogen accumulating organisms.

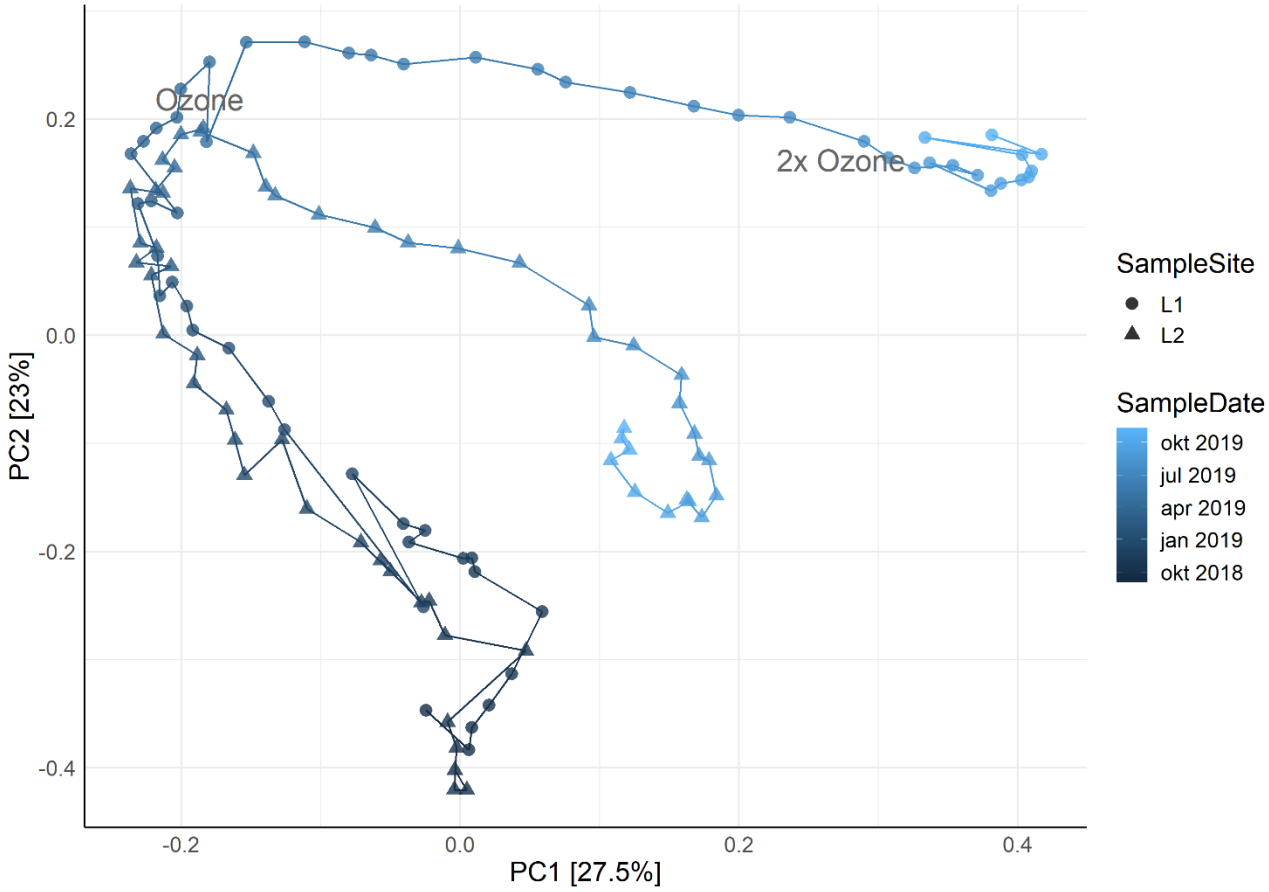

Figure 10 - Principal Components Analysis (PCA). Line 1 = experimental (•) and line 2 = control (∆). The relative contribution of each axis to the total inertia in the data is indicated in percent at the axis titles. The distance between the points represents the differences in microbial community composition between the samples.

1.4.2 Microbial diversity

The overall microbial diversity within samples was analyzed by alpha diversity, which is a measure for average species diversity within a particular area or ecosystem (Figure 11). The observed ASVs, which are unique sequences that can be treated as approximation of number of different species present, were used for the analysis. The number of ASVs in the two lines was similar during “baseline” campaign, however, less unique sequences were observed in the experimental line during the second ozone condition (Figure 11 - left). This means that some species died off or disappeared in the process tank during the second ozonation condition. Another alpha diversity measure, the Shannon diversity index, takes both species number and abundance into account and it showed lower diversity in the experimental line during the second ozonation condition compared to the control line (Figure 11 - right). This indicates that some species were removed by the ozonation. On a side note, the experimental line seems to have larger diversity during the On/Off ozone condition, however the “condition” might have too few samples (lasting less than a month) to be robust.

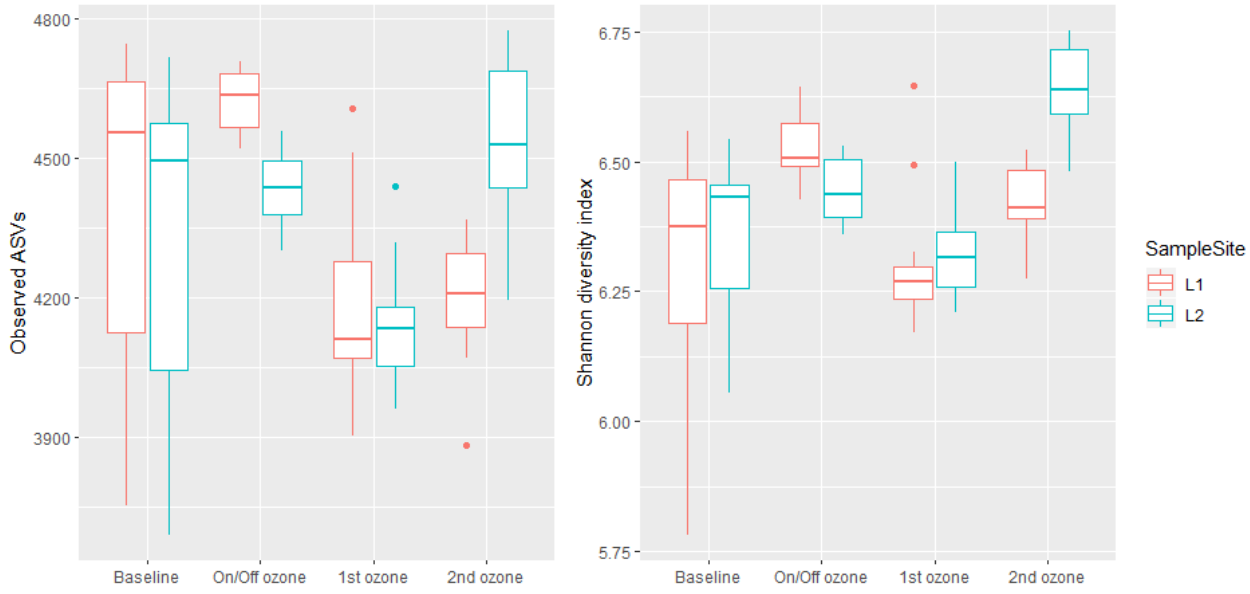

Figure 11 - Alpha diversity. Left: Boxplot of observed ASVs (unique sequences) in the two lines (line 1 – experimental (red) and line 2 - control (blue)) grouped by condition. Right: Boxplot of Shannon diversity index.

1.4.3 Nitrifiers

The main biological processes involved in nitrogen removal are nitrification (oxidation of ammonium to nitrate) and denitrification (reduction of nitrate to nitrogen gas). Little is known about the identity of denitrifiers, so here we focus on the nitrifiers: the group of known ammonia-oxidizing bacteria (AOBs, *Nitrosomonas*, *Nitrosospira*, and Comammox-*Nitrospira*) and the group of known nitrite-oxidizing bacteria (NOBs, *Nitrospira*, *Nitrotoga*). Brædstrup WWTP had one genus from each group (*Nitrosomonas* and *Nitrospira*) Figure 10 shows the timeline of the two nitrifiers in both lines during baseline and the two ozone conditions. Both *Nitrosomonas* and *Nitrospira* increased in relative abundance during first ozone condition. Taking into account that the numbers are relative abundance, it cannot be concluded that more nitrifiers were present in the experimental line during ozonation, however, it is clear that they did not disappear. Both *Nitrosomonas* and *Nitrospira* are known to be microcolony formers and might be located inside the flocs which may protect them from the ozone (Figure 16) (Dolinšek et al., 2013).

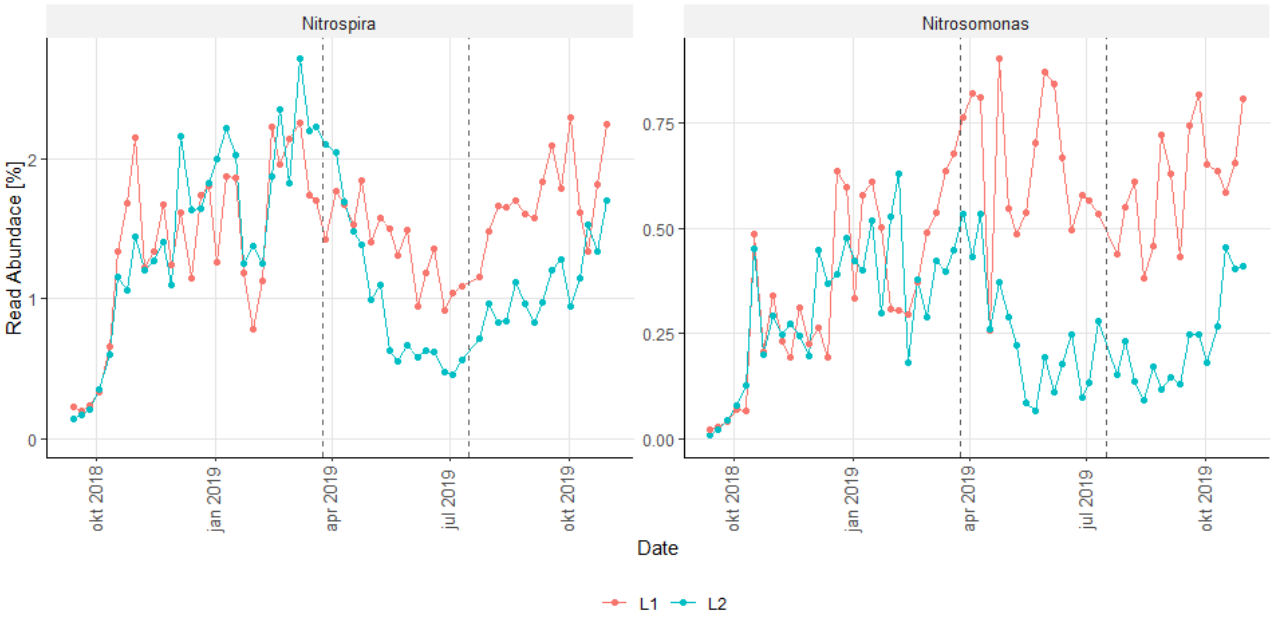

Figure 12 - Time series of known nitrifiers in line 1 – experimental (red) and line 2 - control (blue). The dotted vertical lines represent the start of the two ozone conditions. Note the Y-axis varies in the figures.

1.4.4 Filamentous bacteria

Filamentous bacteria are a normal component of the biomass of activated sludge and biofilms (Nierychlo et al., 2020b). While some filamentous bacteria have beneficial effects on floc structure, others can result in operational problems such as bulking or foaming. The figure below shows the timeline of known filamentous bacteria in Brædstrup WWTP in the two lines during baseline and the two ozone conditions. Only few filamentous bacteria were present and none known to be associated with filamentous bulking. The genus *Trichococcus*, which is known to be high in influent wastewater, showed no change in between the two lines. However, the genus *Ca.* Villigracilis (belonging to the phylum Chloroflexi) almost disappeared from the experimental line during the first ozonation condition. It could be speculated that *Ca.* Villigracilis is more disposed to the ozone from its position in the sludge floc and might not tolerate well. Despite its very low abundance below 0.1% the genus *Ca.* Amarolinea (also belonging to the phylum Chloroflexi) also seemed to be affected by ozonation and almost disappeared during second ozone condition. No clear pattern is observed for the remaining filamentous bacteria.

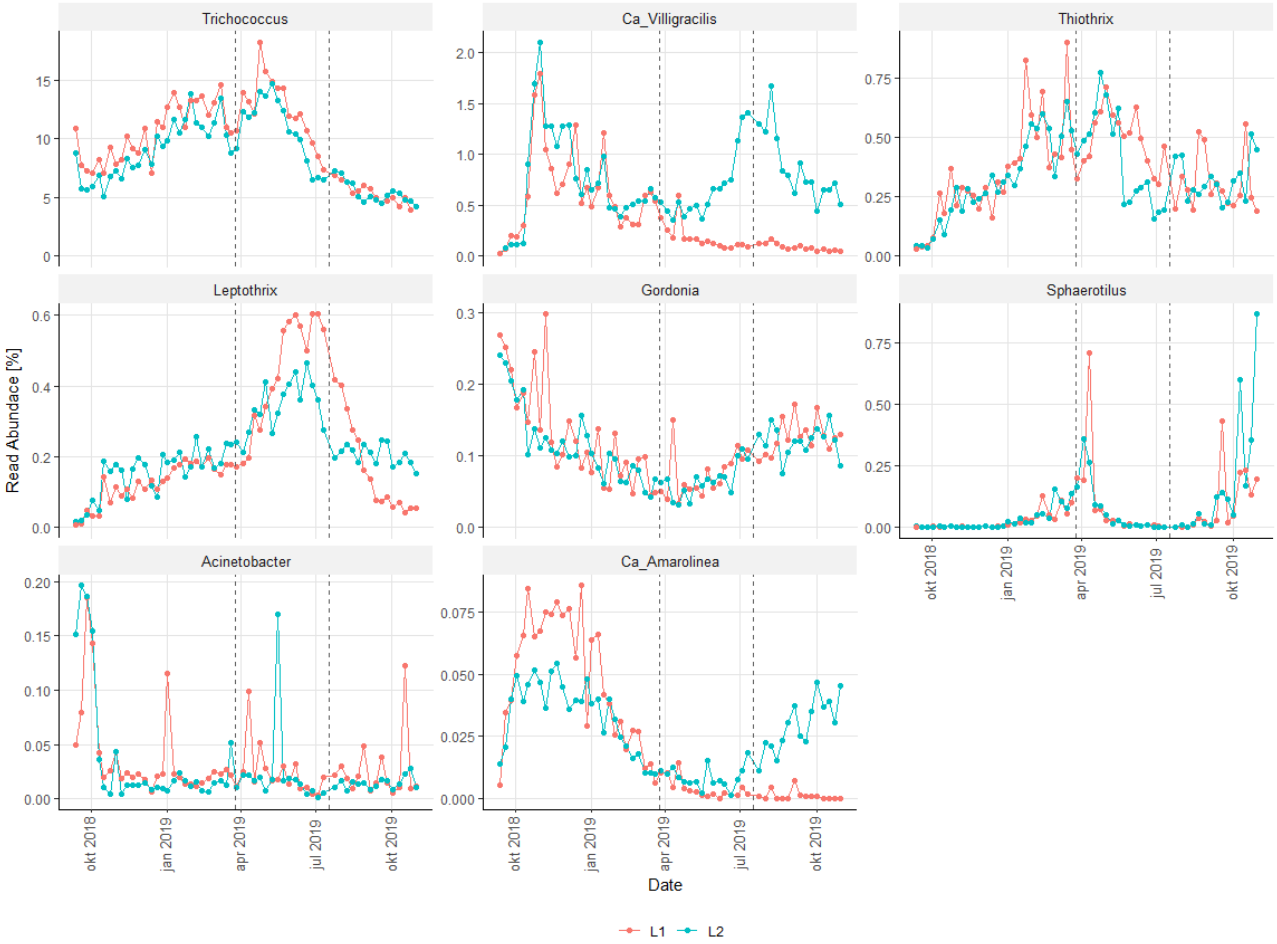

Figure 13 - Time series of known genera with filamentous morphology in line 1 – experimental (red) and line 2 - control (blue). The dotted vertical lines represent the start of the two ozone conditions. Note the Y-axis varies in the figures.

1.4.5 Polyphosphate accumulating organisms

PAOs belong to a group of bacteria that facilitate removal of phosphorus from wastewater by accumulating it within their cells as polyphosphate. Many PAOs also have denitrification properties and might therefor be present in Brædstrup WWTP, despite it is not EBPR configuration. The abundance of known and putative PAOs is shown below. The genus *Tetrasphaera* is the overall most abundant PAO in Danish WWTPs and can be present in up to 40% relative abundance, however in Brædstrup WWTP, it was below 2% and did not seem to be affected during the first ozone condition, whereas it drops in the experimental line during the second ozone condition. On the other hand, the genus *Dechloromonas* increased in abundance during the first ozone condition, but at the end of the second condition it droped again. An increase in relative abundance of *Dechloromonas* during ozonation seems plausible, since *Dechloromonas* is a well-known microcolony former and might be situated and protected from the ozone inside the flocs (Figure 16) (McIlroy et al., 2016). *Ca.* Accumulibacter showed similar behavior as *Dechloromonas*, however, it was less abundant and the difference between the lines were less distinct. The remaining PAOs showed no or little response to ozonation.

Figure 14 - Time series of known PAOs in line 1 – experimental (red) and line 2 - control (blue). The dotted vertical lines represent the start of the two ozone conditions. Note the Y-axis varies in the figures.

1.4.6 Highly abundant bacteria with no known function in activated sludge

Figure 15 shows the timelines of the highly abundant bacteria with no or little known functions in activated sludge. Interestingly, the midas_g_832 (Chloroflexi) decreased in abundance in both lines during baseline, however, it disappeared from the experimental line during the first ozone condition, similar to the two other known filamentous Chloroflexi described previously. Contrary to the unknown Chloroflexi, the potential denitrifier *Hyphomicrobium* increased in abundance compared to the control line during the first ozone condition. No clear pattern was observed for the others.

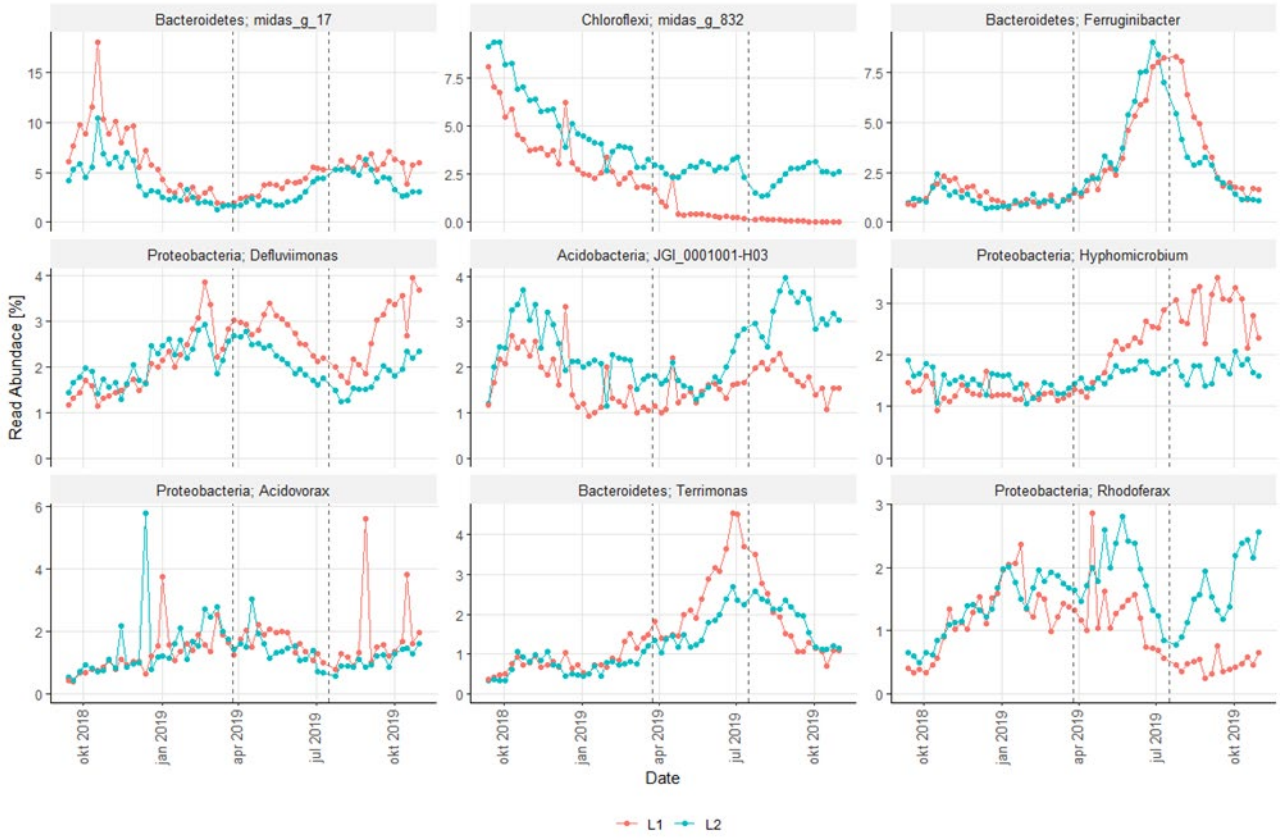

Figure 15 - Time series of other high abundant bacteria, with no/little known functions in activated sludge, in line 1 – experimental (red) and line 2 - control (blue). The dotted vertical lines represent the start of the two ozone conditions. Note the Y-axis varies in the figures.

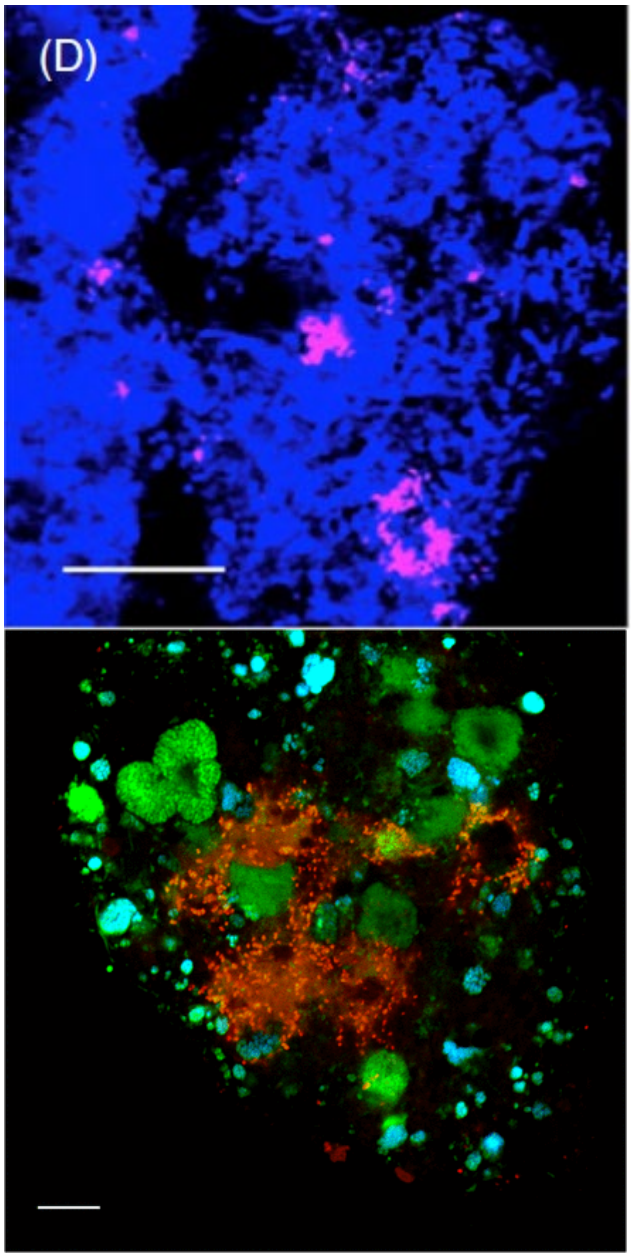

Figure 16 - Left: Fluorescence micrograph showing Dechloromonas (purple) and other bacteria (blue) in activated sludge, image from MiDAS fieldguide ("www.midasfieldguide.org," 2020). Right: Fluorescence micrograph showing Nitrospira (cyan), and other bacteria (green) detected by FISH in activated sludge (Dolinšek et al., 2013).

1.4.7 Conclusions - Microbial community analysis

- The analysis clearly demonstrates that the microbial communities in the two process lines were similar until the start of the first ozon condition. After the onset of ozonation, the community structure became gradually different in the two lines; however, no loss of any of the known process-critical genera was seen.
- The microbial diversity decreased during the second ozone condition (with high ozon doze), with less species observed compared to the baseline community structure.

- The nitrifier abundance were affected by ozonation. The AOB *Nitrosomonas* increased in relative abundance compared to the control line. Same observation for the NOB *Nitrospira*.

- Only few filamentous bacteria were present and none known to be associated with filamentous bulking. However, the genus *Ca.* Villigracilis almost disappeared during the first ozonation condition.

- The PAOs *Dechloromonas* and to some extend *Ca.* Accumulibacter increased in abundance during the first ozone condition compared to the control line.

- Some genera that increased in relative abundance after the onset of ozonation in the experimental line compared to the control line (e.g., *Nitrosomonas, Nitrospira and Dechloromonas*) have in common that they are recognized as microcolony formers and most often found to be sitting inside the sludge flocs. They are thereby considered good floc formers and seem to be protected from the ozone treatment.

1.5 Conclusions – Overall biological effects

- The higher organisms (animals) such as protozoa and nematodes disappeared during the second ozonation condition.
- The attached growth present on the filamentous organisms sticking out from the flocs disappeared during first ozone condition.
- The compactness and firmness of the sludge flocs improved compared to the control line during the second ozonation condition.
- The microbial communities in both process lines were similar until the start of the first ozonation condition. After the onset of ozonation, the community structure gradually became different in the two lines; however, no loss of any of the known process-critical genera was seen.
- The microbial diversity decreased during the second ozonation condition, as less species were observed.

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Removal of micropollutants by application of multiple point ozonation and powder activated carbon

Appendix 4 – Sludge characterization by light microscopy and 16S rRNA sequencing

The Danish Environmental
Protection Agency
Tolderlundsvej 5
DK - 5000 Odense C

www.mst.dk