Master's Thesis

Effect of ozonation to water quality in recirculating aquaculture system

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Recirculating aquaculture system (RAS) has been developed to produce aquatic food species faster and more flexible and environmentally friendly way than traditional aquaculture. As the water consumption in RAS is reduced to 5 %, the water must be purified to be habitable for the cultured species. Ozone is one of the water disinfection and treatment methods and it can potentially, disinfect the water and improve water by oxidising organic compounds. As the water quality varies greatly in different RAS, the required ozone dose must be chosen carefully, because dissolved ozone is very toxic to aquatic organisms and very low concentrations does not achieve desired effect. Therefore, residual ozone must be either destroyed or dose adjusted so that no residual ozone is left in water when it reaches the culture tank. The aim of this study was to determine optimal ozone dose for the Luke's experimental RAS platform in Laukaa. Ozone decomposition and dose was determined for the tank water (TW) and inlet lake water (LW) in the laboratory conditions and the effect of ozone to dissolved organic carbon (DOC), total nitrogen (TN) and pH were monitored. HPSEC-technique was used to track the molecular distribution of organic compounds by measuring the UV-254 absorbance and tryptophan-, tyrosine-, humic- and fulvic-like fluorescence for six different molecular size fractions within the water. Results indicate, that the most optimal dose for the LW was 1,07 mg of O_3/ mg of DOC and for TW 0,81 mg of O_3 / mg of DOC as it decreased the total fluorescence and absorbance by 78,0 ± 8,7 % (LW) and

77,3 ± 13,3 % (TW). Ozone decomposition was much faster in TW than in LW and decreasing temperature seemed to slow the process down.

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Kiertovesiviljelyjärjestelmä (RAS) on kehitetty tuottamaan ravinnoksi hyödynnettäviä vesieliöitä nopeammin, joustavammin ja pienemmällä ympäristökuormalla kuin perinteisessä vesiviljelyssä. Koska RAS:n tuloveden tarve on laskettu noin viiteen prosenttiin, järjestelmässä oleva vesi on puhdistettava viljellyille lajeille laadultaan sopivaksi. Otsonia voidaan käyttää veden desinfiointi- ja käsittelymenetelmistä, koska se voi mahdollisesti desinfioida ja parantaa veden laatua hapettamalla orgaanisia yhdisteitä. Koska veden laatu vaihtelee suuresti erilaisissa RAS-järjestelmissä, vaadittava otsoniannos on määritettävä huolellisesti, koska veteen liuennut otsoni on erittäin myrkyllistä vesieliöille. Siksi jäännösotsoni on joko tuhottava erillisellä käsittelyllä tai annos säädettävä siten, että vesi ei sisällä enää otsonia, kun se saavuttaa kasvatusaltaan. Tämän tutkimuksen tavoitteena oli selvittää tehokkain otsoniannos Luke:n kokeelliselle RAS-alustalle Laukaassa. Otsonin hajoamisnopeus ja annos määritettiin systeemin vedelle (TW) ja tulovedelle (LW) laboratorio-olosuhteissa. Otsonin vaikutusta DOC-, TN- ja pH-arvoihin tarkkailtiin. HPSEC-tekniikkaa käytettiin orgaanisten yhdisteiden hajoamisen seuraamiseen mittaamalla UV-254-absorbanssi ja tryptofaani-, tyrosiini-, humiini- ja fulvomainen fluoresenssi kuudelle erilaiselle veden sisältämälle molekyylikokofraktiolle. Tulokset osoittavat, että tehokkain annos LW:lle olisi 1,07 mg O3 / mg DOC ja TW:lle 0,81 mg O3 / mg DOC, laskien veden kokonaisfluoresenssia ja -absorbanssia 78 ± 8,7 % (LW) ja 77,3 ± 13,3% (TW). Otsonin

hajoaminen oli paljon nopeampaa TW:ssä kuin LW:ssä ja lämpötilan lasku näytti hidastavan prosessia.

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TERMS AND ABBREVIATIONS

ABBREVIATIONS

DAQ	Data acquisition program
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
HPSEC	High performance size exclusion chromatography
Luke	Natural Resource Institute Finland (Luonnonvarakeskus)
LW	Lake water
RAS	Recirculating aquaculture system
TN	Total nitrogen
TOC	Total organic carbon
TW	Tank water
UV	Ultraviolet
UV-254	UV-absorbance, λ = 254 nm

1 INTRODUCTION

As the world's population keeps rising, the demand for the food keeps rising too. For many countries and cultures, fish and other marine animals are an important source of proteins. Unfortunately, most of the world's fisheries are already under heavy fishing pressure which makes their further utilization very hard. This has been known for decades and for an increasing food production, aquaculture has seen a steep rise (Figure 1) in its popularity (FAO 2012).

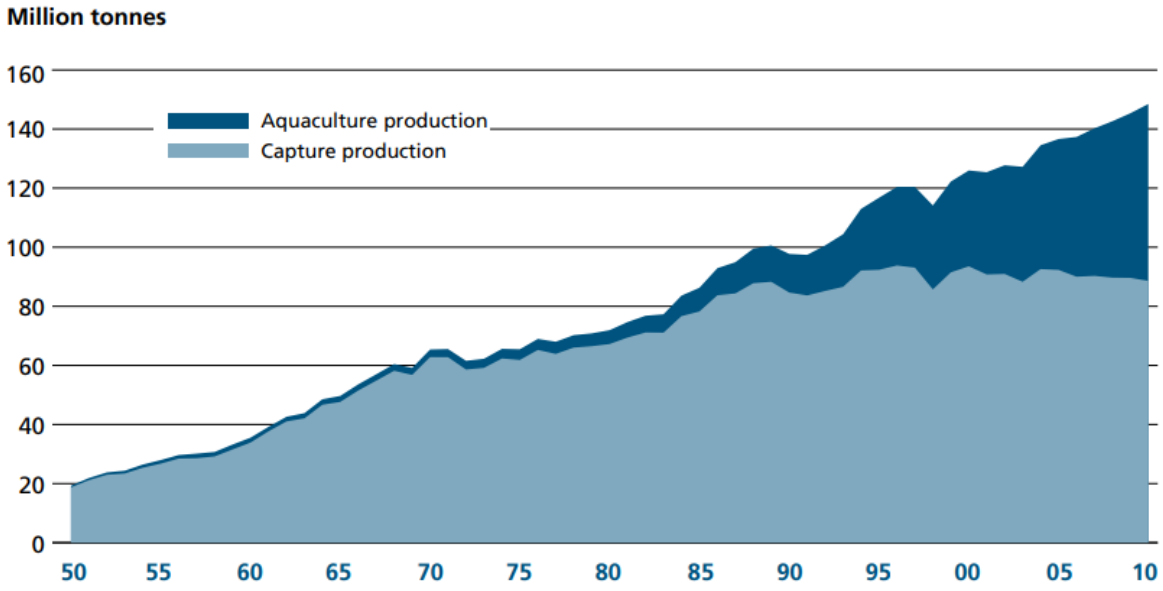

Figure 1. Production from aquaculture and fishing (capture) per year (FAO, 2012).

Aquaculture can be defined as controlled farming of aquatic organisms (including seaweed) in sea-, brackish- or freshwater. Even though aquaculture enables further usage of fish, molluscs, and other aqueous species with very efficient feed conversion rates, it does not come without problems. Traditional fish farm locations need to be selected carefully, their maintenance takes skill and knowledge, parasites and weather conditions can greatly affect the productivity, they can cause eutrophication in local water ecosystems (Honkanen & Helminen 2000) and huge amounts of water are daily required to keep the cultured species alive and growing.

To solve some of these problems a recirculating aquaculture system (RAS) has been developed. This system is highly automated and uses technology to maintain optimal growing conditions for the cultured species and most importantly, it recycles its water to minimize the water requirements and environmental load. When compared to regular flow-through culturing, RAS can reduce the water usage over 98 % (Masser et al. 1992). This water recirculation is achieved with many water purification processes during the circulation process. These processes include, solids removal, biological filtration, aeration, oxygen injection and water disinfection. Temperature and pH of water is usually maintained at some point of the cycle. The disinfection part of the purification process is usually done with UV-lights, ozone or both. It also varies where disinfection is placed or is circulating water, inlet water or both disinfected. UV is widely used as it is safe, easy to use a, cheap and efficient way getting rid of possible pathogens, but in the other hand it is mainly suitable for shallow water and only for one part of the recirculation cycle. Ozone requires knowledge and skill to use efficiently and it is toxic to fishes and other aquatic species in low concentrations. At the same time, unlike UV, ozone improves water quality by oxidizing organic material (Spiliotopoulou et al. 2018). The toxicity of ozone first led to decreased popularity, but new research and increased knowhow led to increase interest to its usage during the last decade (Powell & Scolding 2018).

ozone has been used in water purification has been used for a long time, as it has been used in drinking water purification plants around the world for many decades before to produce drinking water. For RAS, ozone was neglected at first, due to the fear of ozone killing the cultured species. By determining the right ozone dose for the system and planning the position of the ozone injection to the water correctly, there is no danger for residual ozone to get to the culture tanks. Ozone has been recorded to greatly increase water clarity, remove harmful nitrogen compounds and intensify biofilters performance (conversion of ammonium-ion first to nitrite and then to nitrate), decompose large organic molecules to smaller more bioavailable ones and reduce the number of fish disease outbreaks by killing and deactivating pathogens from inlet- and circulating water (Bullock et al. 1997,

Summerfelt et al. 1997). Ozone has also sparked interest in aquaculture as being one of the most efficient methods to remove taste and odor causing compounds from water such as methylisoborneol and geosmin (Westerhoff et al. 2006).

To ensure safe and robust treatment, it is vital to define the ozone demand and ozone kinetics of the specific water matrix to achieve the desired goals and to avoid ozone overdose. Different ozone dosages were applied to water in freshwater recirculating aquaculture systems (RAS). Experiments were performed to investigate ozone kinetics and demand, and to evaluate the effects on the quality of process water (TW, tank water) and the make-up water (LW, lake water), particularly in relation to the molecular size distribution and spectroscopic properties (absorbance and fluorescence) in organic matter. This thesis aimed at predicting a suitable ozone dosage for water treatment based on ozone demand via laboratory batch studies. These ozone dosages will be applied and maintained at these levels in pilot-scale/ full scale RAS demonstration station in Laukaa to verify predictions of optimal ozone doses. Selected water quality parameters were measured, including amount and molecular size distribution and spectroscopic (absorbance/fluorescence) properties organic compound concentration changes during.

Hypothesis are that ozone would decompose faster and achieved ozone doses would be smaller in circulation water than in inlet water. Water in RAS-systems is very concentrated with compounds that biological process produce and these compounds usually contribute to decomposition of ozone. Even though the effect of similar doses can be more noticeable in circulation water than in inlet water, circulating water is more concentrated with dissolved organic matter and nitrogen compounds that are oxidized easily. Decreasing temperature should reduce the decomposition rate and increase the max amount of dissolved ozone.

Thesis was performed in cooperation with LUKE. Samples were gathered during summer 2019 from Laukaa fish farm, tests and ozonation were carried out in laboratory at University of Jyväskylä.

2 THEORETICAL BACKGROUND

As this thesis contains three sub-regions when it comes to theory, they will be presented here separately. First will be basic introduction to RAS, then common water quality parameters and finally principle and application of ozonation.

2.1 Recirculating aquaculture system

The basic idea of a recirculating system is to provide a nearly closed, optimum environment for the reared species. Advantages of conventional flow-through cultivation include a significantly reduced inlet water requirement (1-10% of the flow-through farms requirements), reduced nutrient release to the environment, year-round growth and flexibility in farm location where climate and water resources might be limiting factors (Masser et al. 1992). However, advanced automation and machining bring with it the need for energy, high capital and expertise. Closed circulation also presents challenges in the use of potential chemicals, in the fight against pathogens and in maintaining good water quality. Challenge also comes from the accumulation of fish secretions and uneaten feed that must be dealt with properly. The systems may vary slightly in their structure and components, but in general, they include a culture basin, solid filtration, biofilter (conversion / removal of nitrogen compounds), aeration and oxidation, water addition and disinfection (Timmons & Ebeling 2013). Figure 2 illustrates a typical structure of a RAS, including all its basic components. The functioning and the purpose of each individual system component is explained in more detail in the following sections. The experimental RAS platform in Laukaa will be used as an example.

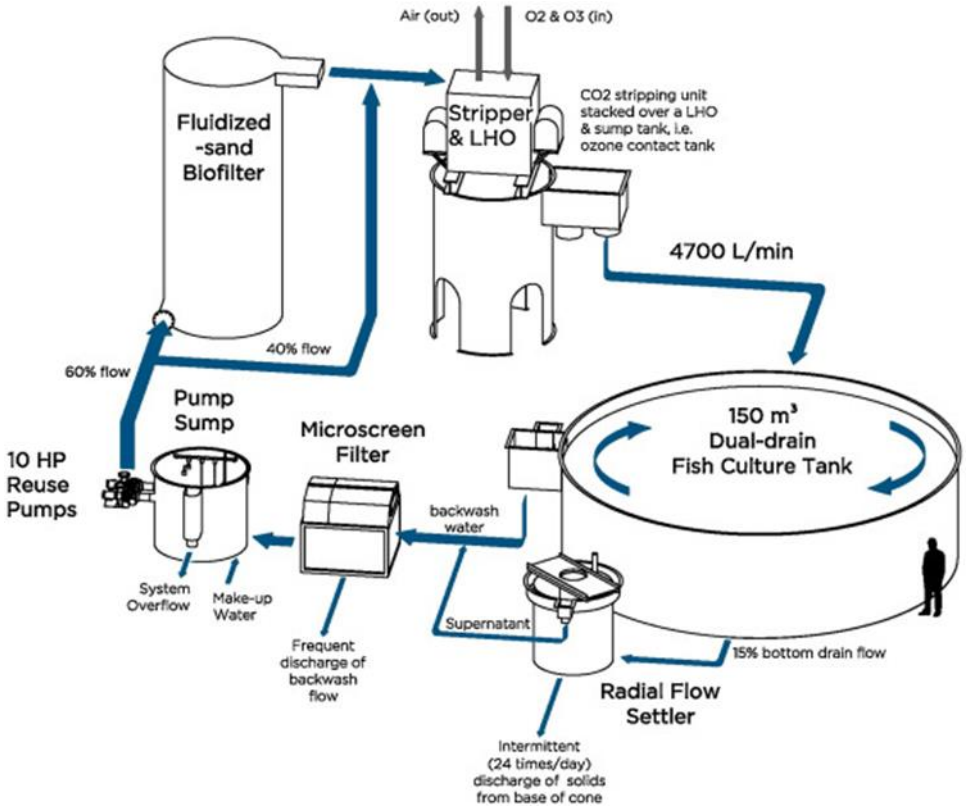

Figure 2. Typical structure of a recirculating aquaculture system. Specialty being usage of fluidized-sandfilter as biolfilter. (Davidson et al. 2016).

2.1.1 Solids removal

Solids removal is usually performed first, because the solids in the water can interfere with the operations of the biofilter. The solids mainly consist of fish faeces, undigested feed and from dead and living bacterial biomass. Most of the particles are less than 100 µm in size and can be divided into size classes on the basis of their size, where more than 100 µm can be removed by sedimentation using a vortex clarifier/settler. Particles of 1-100 µm are partially colloidal, their removal often involves foaming, where bubbles are produced in the water, particles get trapped to them and are carried to the top of the clarifier (flotation). The formed foam is then removed from the top. Particles smaller than this are either completely colloidal, that is, they are either in the middle of a homogeneous and heterogeneous solution or directly dissolved (Timmons & Ebeling 2013).

In Laukaa's RAS, solids removal consists two parts. First, water is led to a vortex clarifier (Figures 3A and 3B), which removes larger solids particles. The water then continues to the self-cleaning drum filter (Figure 3C). Used filter material removes particles up to 60 µm. The resulting sludge is collected to a sludge basin.

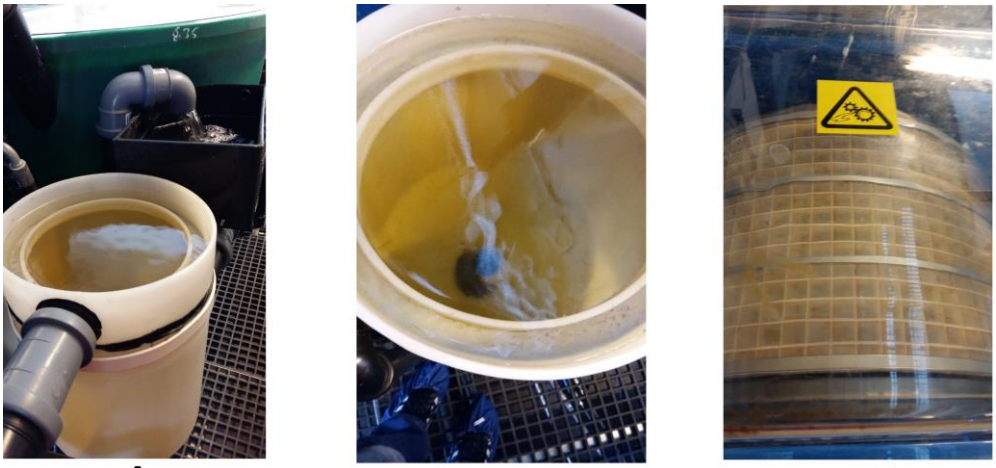

Figure 3. The vortex clarifier (A & B) Water enters the clarifier below surface and causes a small flow, while larger solids particles sink to the bottom of the clarifier. Water is collected from the surface into a drum filter (C).

2.1.2 Biofilter

The biofilter is one of the most important parts of the system as it converts nitrogen compounds that are harmful to fish into less harmful ones. Completely nitrogen removal can also occur in a biofilter, but it usually requires certain conditions to be effective (like anoxic environment), so this is less common in recirculating systems (Rijn 2013).

Fish are ammoniotelic animals, which means the end product of their metabolism is ammonia (NH_3). When dissolved in water, it forms an equilibrium with ammonium ion (NH_4^+), that depends heavily on pH. For example, when pH is around 8, NH_3 is about 4,6 % from all ammonia in water, but when pH changes to 7,25, the NH_3-percentage drops to 0,46 %. The unionized ammonia is more toxic to fish (concentrations of 0,06-0,23 mg / l being already harmful and recommended upper value being around 0,0125 mg / l) than its ionized form (0.08-2.2 mg / l

harmful levels for most of the fish and recommended levels below 0,05 mg / l) and explaining why is it important to prevent the pH of the system from rising too high. Toxicity of course depends slightly on tolerance of species (Miller et al. 1990, Timmons & Ebeling 2013). If these ammonium-compounds are not removed from the water, they will build up and eventually cause the fish to die (Timmons & Ebeling 2013). The biofilter contains nitrification bacteria that utilize less oxidized nitrogen compounds in its metabolism. First, they convert it to nitrite (NO_2^-) and further to nitrate (NO_3^-). Nitrite is still somewhat toxic, but nitrate is relatively harmless to fish, so moderate amounts of nitrate can be present in the water without problems: lethal doses can exceed 1000 mg / l. However, to control the nitrate levels and prevent it from concentrating too much, small amount of replacement water is introduced into the system (Timmons & Ebeling 2013).

A typical biofilter is usually bucket-shaped container (Figure 4) with growing medium for bacteria (Figure 4B). The water is brought into the filter from the bottom, which maximizes the time that water spends in the filter. The biofilter may also have aeration, whereby the growth media are in constant motion (Figure 4C, moving bed reactor), but they may also be stationary (Figure 4A, fixed bed reactor).

One solution for the biofilter and solids removal-hybrid is a sandfilter. Sand is acting as solids filter and removes solid particles depending to grain size and structure of filter. If filter is properly aerated/oxygenated it can also act as a biofilter when bacteria begins to grow on the sand granules and. Sandfilters are needed to be cleaned occasionally to prevent them from clogging and in some cases filter material needs to be changed. Cleaning usually is done by channeling water from the opposite direction to the filter, so the sludge is carried to the top and is then collected and removed. During this cleaning sandfilter is uncapable to function, so backup filter is recommended to be installed to the system for the maintenance breaks. (Timmons & Ebeling 2013)

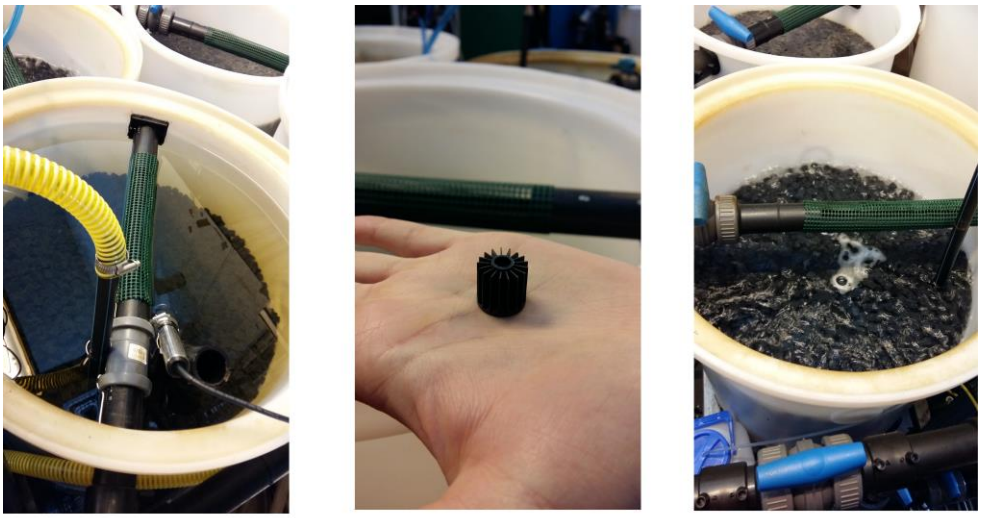

Figure 4. Biofilters at Laukaa fish farm. Fixed bed biofilter (A), a bacterial growth medium (B) and a moving bed biofilter (C).

The bacteria in the biofilter are very susceptible to possible disturbances and especially the pH and oxygen concentration of the system must be monitored and adjusted properly. If the pH drops too much, the nitrogen compounds may form nitric acid (HNO_2), which is very harmful to bacteria. If the amount of oxygen is too low, the nitrogen compounds cannot be effectively oxidized. The solids in the water can act as another source of energy for the bacteria and this can lead to inefficient nitrification. Another bacterial species can also destroy the slow growing nitrifying bacterial strain of the filter if the conditions are wrong (Martins 2010). Reproduction of the bacterial strain of the biofilter usually takes a couple of weeks to a month before it returns to adequate levels. During this time inlet water amount must be increased, possible backup system started, chemicals used, or the system must be shut down and fish moved away. In each case the result will most likely be significant economic loss.

2.1.3 Disinfection

Water disinfection is commonly carried out with UV light or ozonation. Chemicals are not recommended as they are difficult to remove from the system and may primarily affect the performance of the biofilter bacteria. Some exceptions though

exist like hydrogen peroxide H_2O_2 that has same oxidising effect as ozone and peracetic acid (CH_3CO_3H) that has been under study during recent years for its disinfecting and possible water quality improving features (Schmidt et al. 2006, Liu et al. 2018). Disinfection is usually placed in the system as the last part before the culture tank, and it would be optimal if it also disinfected the new water that comes to the RAS.

UV light is a common way to disinfect water. It is an inexpensive, easy and safe way to dispose disinfectants, which is why many facilities have come to use it. However, in recent decades, ozonation has become more widely used due to increased research and know-how, particularly due to its water quality-enhancing properties. Both disinfection methods can also be used at the same time to ensure the best possible efficiency, but even one of them can ensure sufficient water quality (Powell & Scolding 2018).

2.1.4 Aeration, oxygen injection, pH adjusting and temperature

Fish and the biofilter naturally consume oxygen from water for their vital functions while releasing the carbon dioxide. There is not enough time and gas-liquid interface for oxygen to dissolve efficiently to water during the cycle naturally, so it must be added artificially. An effective and much used way of doing this is the aeration cone. The tapered tank is sprayed with water from above and pure oxygen from below to form bubbles that create large quantities of reaction area for oxygen to dissolve (Figure 5). It is also viable to create water droplets to air which is like a reverse situation. The cone-like shape creates different velocities where liquid moves fastest in the top and slows downwards which gives more time for gas exchange. The cone is also pressured which according to the Henry's Law, intensifies the dissolving of gas to the liquid as the pressure rises. (Timmons & Ebeling 2013)

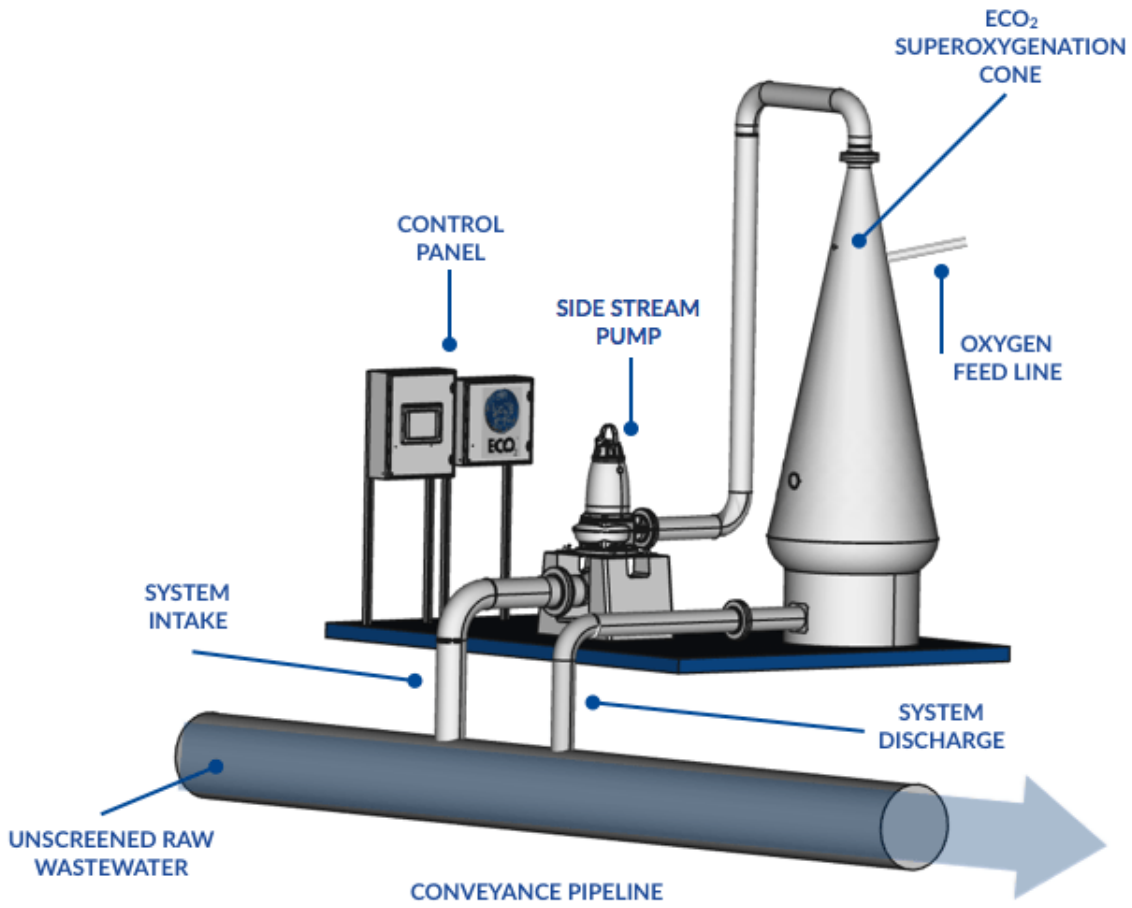

Figure 5. Speece cone or aeration cone / column. Common water oxidation equipment (ECO2 2017).

There are many different ways to adjust the pH of water, and from the system's point of view, it is usually chosen that is most adequate and easy to implement. In Laukaa, for example, the adjustment is made by dispensing lye (NaOH) into water using a pump and a pH-sensor. When the pH drops, enough sensor will detect this and start a pump that pumps the lye into the circulation until the pH has risen sufficiently.

The temperature is constantly monitored by devices and the heaters keep the temperature appropriate. The cultured species determine how warm the water should be. If the circulation system is large enough, heating may not be necessary, because the biological processes, like fish organ functions in the circulation system produces heat, which thus warms up their environment. This is the case, for

example, with the Finnforel's RAS-facility in Varkaus that produces little under 1 million kilos of fish per year.

2.2 Water quality

Several substances and compounds are dissolved in the water of a recirculating system, which can be determined and studied by various parameters. This section introduces the most important and common parameters used in water quality monitoring.

2.2.1 Natural organic matter (NOM)

Natural organic matter (NOM) consists of a large number of different compounds that are dissolved in water, in the form of particles or colloids. It comes from the metabolism of living organisms, their dead remains and compounds that are still degraded in the environment. Organic materials absorbed into inorganic compounds also belong to NOM. Natural material can leech from the soil with rainwater and diffuse from sediment into water bodies (Krasner 1996). NOM compounds have been extensively studied, but due to their large number and complexity, they are still relatively poorly known. In RAS, inlet water, which often comes from the lake or the sea, therefore naturally contains NOMs, but is also excreted into water as a result of fish metabolism (faeces), inedible feed and dead microbes (Timmons & Ebeling 2010).

The molecules of NOM are either hydrophobic or hydrophilic. Hydrophobic includes polar or weakly polar long chain carbon compounds, humic and fulvic acids, polysaccharides and hydrophilic polar smaller molecules such as proteins and amino acids. NOM can be divided into humus and non-humus parts. Most NOM are hydrophobic humus compounds consisting of fulvic- (95%) and humic acids (5%), but the composition depends on the source where it originates (Ghabbour & Davies 1998). For example, NOM from soil usually contains more aromatic structures than NOM from water and NOM from peat lands usually contains a lot of low molecular weight fulvic acids (Goel et al. 1995).

Humic compounds are typically large molecules with high UV absorption and aromaticity. The most typical functional groups are phenol and carboxylic acid and the typical molecules are various carbohydrates and amino acids (Ghabbour & Davies 1998). Humic compounds can be divided into three groups according to their solubility in aqueous acidic solutions. Fulvic acids dissolve at any pH (Figure 6A). Humic acids are soluble to water in higher pH (Figure 6B) and humin substances are not soluble in water at all (Stumm & Morgan 1981). It has traditionally been thought, that when compared to fulvic and humic acids, humins are low in number and less known and studied (Zularisam 2005). Studies have also found that humus compounds are not as distributed in molecular weight and dispersion as previously thought (Chin 1994).

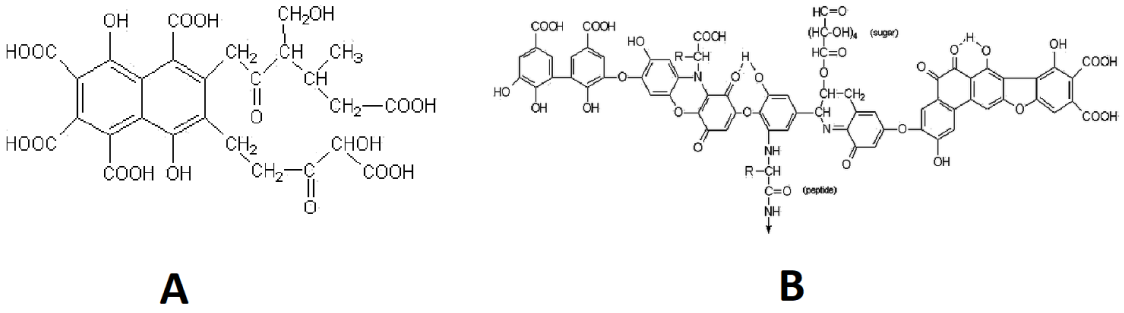

Figure 6. Model structures of fulvic acid (A) and humic acid (B) (Rudolf et al. 2006).

Humic substances themselves are not very dangerous or toxic compounds, but they change the color, smell and taste of water. The compounds can also serve as food for microbes, which increases their growth. They can also absorb organic and inorganic pollutants and, for example, chlorination of humic water can produce organochlorides which are carcinogenic and toxic to humans and organisms (Zularisam 2005).

Humic compounds can be removed from the water in various ways, for example by coagulation (Matilainen 2010), but since this study does not focus on the direct removal of humic compounds and does not occur in the recirculation system, it is left unexplained in more depth here. However, in the presence of a strong oxidant

such as ozone, large humus molecules can degrade to form smaller molecules that are more readily available as microbial food sources (Wang et al. 2008), which in turn enhances biofilter function (Wang et al. 2008, Timmons & Ebeling 2013).

2.2.2 Water quality parameters

This section introduces commonly used parameters to evaluate the quality of natural organic matter, as well as ways to determine that from water. All parameters are generally expressed with unit mg / l.

BOD (Biochemical Oxygen Demand) refers to the amount of dissolved oxygen in water that is needed to oxidize organic matter by aerobic organisms in certain temperatures. A very similar parameter, COD (Chemical Oxygen Demand), tells us how much different chemical reactions in water can consume dissolved oxygen. DOM (dissolved organic material) means the amount of organic matter dissolved in water, and DOC (dissolved organic carbon) is the amount of organic carbon when the dissolved organic matter is decomposed completely to CO2 by burning it in high temperature and presence of catalyst.

BOD can be measured for example with method where dissolved oxygen-probe is enfolded with biofilm membrane. This in practice constructs now a BOD-electrode that is just inserted into water (Strand & Carlson 2015). For COD-measurements there are several ways and many of them include addition of oxidizer and its consumption monitoring. More modern way is to use UV to photolyse the sample's compounds and produce known number of free radicals. Luminol is then added and it scavenges the radicals producing light at the process. This light production is then monitored (Su et al. 2007).

High Performance Size Exclusion Chromatography (HPSEC) has been found to be a good way to determine the amount and apparent molecular size distribution of water-bioavailable DOM (Ignatev & Tuhkanen 2019, Chin 1994) by monitoring the change in UV absorption and in protein-like fluorescence due to ozonation. In HPSEC, water-soluble substances are passed through a column with the aid of an

eluent, leaving the smallest molecules trapped in the pores of the column. Larger molecules, due to their size, pass through the column faster, thus having a lower retention time. Tryptophan and tyrosine fluorescence measure the absorbance of proteinaceous compounds. UV-254 fluorescence can be used to determine the aromaticity of compounds, since non-aromatic compounds have low absorption of UV-254 and high aromatic ones (Ignatev & Tuhkanen 2019).

NT (total nitrogen) is the amount of nitrogen in the water with nitrogen compounds. It is especially important for the RAS because, as stated earlier, nitrogen compounds (NH_4^+, NO_2^- & NO_3^-) accumulate in the system due to fish metabolism and are harmful to them at concentrations too high. The concentration of nitrogen in water can be measured, for example, by persulfate oxidation, in which the nitrogen is converted into ammonium ion form by reduction of nitrates and nitrites under basic conditions with Devarda alloy. They are then oxidized again by the addition of, for example, potassium persulfate, whereby the amount of nitrogen can be calculated from the consumption of persulfate (Raveh & Avnimelech 1979). TN can also be analysed with a dedicated analyser. For example, Shimadzu TOC-L organic carbon analyser (measures both DOC and TN at the same time) combust samples in very high temperature in the presence of catalyst. Formed carbon dioxide and nitrogen oxides are then measured (Shimadzu N.T.).

2.2.3 Pathogens

Naturally, many different pathogens are present in the water (bacteria, viruses, fungi & protozoans). Heterotrophic bacteria can use organic compounds that are present in water for their metabolism and energy production. They are not directly harmful but can reduce the water quality and overall hygiene. Pathogens can be abundant in natural lake waters, and it is important to remove these before leading water into the system. In RAS nitrification bacteria are abundant due to biofilter functioning, but other types of bacterial presence in larger abnormalities is undesirable as it can be affecting biofilter's functioning and fish welfare (Martins 2010). When killed, pathogens increase the amount of NOM in the water. Quantifying pathogens from water is challenging, but however ozonation is already

known to effectively reduce pathogen amount or at least deactivate them and thus reducing the possible decease outbreaks. (Timmons & Ebeling 2013).

2.3 Ozonation

Ozone is oxygen's three-atom allotrope O_3 (Figure 7) that occurs in nature to a small extent throughout the atmosphere and is concentrated in the stratosphere where it absorbs most of the ultraviolet radiation emitted by the sun. Ozone is formed by the UV molecule O_2 from the oxygen molecule, whereby the radiation is decomposed into individual oxygen atoms, which then combine with the complete oxygen molecules to form ozone and when electrical charges are discharged, such as lightning. After a thunderstorm, you can smell a recognizable pungent odor of ozone. (Oyama 2000)

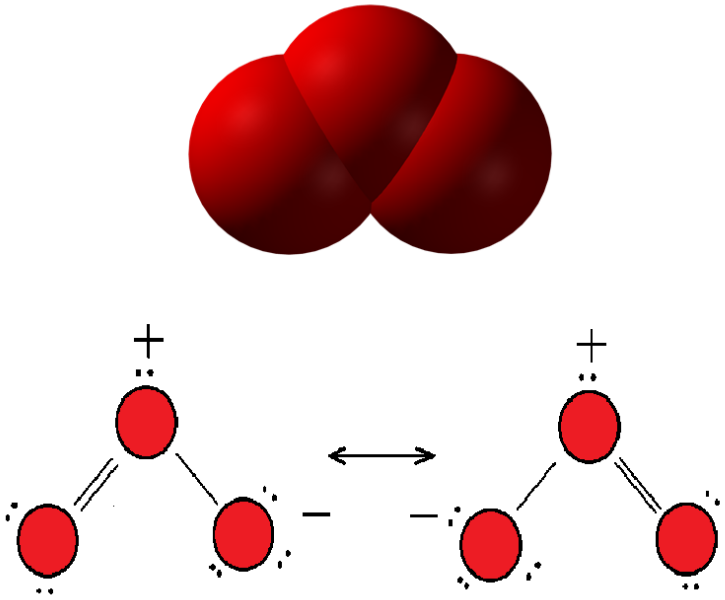

Figure 7. The ozone molecule above and below its resonance structures.

Ozone is a powerful oxidant and is capable of oxidizing almost all organic matter, as well as many metals (except precious metals) to their highest oxidation state. The high oxidation potential is due to its unstable resonance structure (Figure 7), which readily releases one oxygen atom, resulting in a much more stable O_2-molecule

(Oyama 2000). Today, this high oxidation potential of ozone is used in many industrial processes and especially for various disinfection purposes (Powell & Scolding 2018). However, it is harmful to organisms, damaging their respiratory organs. Increased levels of ground-level ozone, particularly in urban areas due to traffic, industry and other atmospheric pollution, can cause serious health problems (Gryparis 2007). Ozone reactivity also prevents its storing because, even at low concentrations, as a liquid (30%) or as a gas (less than 10% already hazardous), it becomes highly explosive, though slightly depending on storage method and solution / gas mixtures (Waller & McTurk 2008). As a gas, the ozone's color is light blue, as liquid dark blue (-112 ° C) and as solid dark purple (-193.2 ° C).

2.3.1 Use of ozone in water purification

Ozone has been used in water purification since the early 20th century, which means it is not a new technology. Because of the complexity of ozone chemistry in water, and potential to cause problems when not used right, cheap and easier to use chlorine supplanted it for a long time. When knowledge and skills increased and usage of chlorine was found to be problematic, ozone has become more common and nowadays many water treatment plants in Europe use ozone as one of their purification steps. (Powell & Scolding 2018)

Ozone is a powerful but selective oxidant and therefore its usage in water purification requires knowledge. Ozone improves clarity, smell and taste of water by oxidizing various organic compounds and breaking them down, which is a very desirable reaction in water purification. Ozone's selectivity can be seen as an advantage as less selective oxidiser's efficiency could be easily spent to less efficient reactions (Hoigne 1988), though sometimes it may be necessary to produce enough ozone to form an OH-radical, a very strong and non-selective oxidiser. Excess ozone is rapidly degraded to oxygen and no harmful concentrations remain in water. In fish farming the formed oxygen is usually a positive by-product (Powell & Scolding 2018). However, ozone reacts with different compounds at different rates, some compounds oxidize in seconds like double bonds, some may require days of exposure to ozone or don not react at all like saturated alkyls (table 1). Water pH

and alkalinity also affect ozonation efficiency and the formation products that are formed when ozone decomposes (Ershov & Morozov 2018). Ozone can also produce harmful substances into water, like for example when reacting with bromine, toxic hypobromite is formed if the process is not properly treated (Langlais 1991).

Ozone is produced on the spot by ozone generators, because as stated earlier, it is challenging to store safely needed quantities of it. The technique in ozone generators is based on the same phenomenon as the formation of ozone during lightning thunderstorms, ie ozone is produced from oxygen by electric current or in some cases with UV radiation (particularly in small generators). Simplified, the reaction proceeds according to reaction Equations 1 and 2 (Yagi & Tanaka 1979)

$$O_2 + e^- \rightarrow 2\,O + e^- \qquad (1)$$

$$2\,O + 2\,O_2 \rightarrow 2\,O_{3.} \qquad (2)$$

Generators generally tend to treat only oxygen gas because, for example, air alone contains other gases, such as nitrogen, that can compete with an oxygen molecule to reduce the efficiency of the oxygen atom and form unwanted by-products like nitrogen oxides (Yagi & Tanaka 1979).

The solubility of ozone in water is approximately 1 g / l (0 ° C). Ozone is usually added to the water by means of bubbles, which results in a large surface area and efficient dissolution. However, since ozone is not highly soluble and once dissolved into water, it begins to decompose immediately, it can be difficult to concentrate it in amounts large enough. The highest concentrations of ozone that can be obtained in pure ionized water are in practice between 20 and 40 mg / l (Roth & Sullivan 1981).

2.3.2 Ozone chemistry

The ozone decomposition in water is a complex process. Reactions generate a variety of radicals and molecules that can inhibit or catalyse ozone decomposition

reactions and affect the end products. Different compounds in water, pH and alkalinity not only contribute to the decomposition of ozone but also to which compounds ozone affects and how effectively (Langlais 1991). Figure 8 depicts ozone's direct and indirect reactions in water. Each of the reactions can occur simultaneously in water, but usually one of them is predominant.

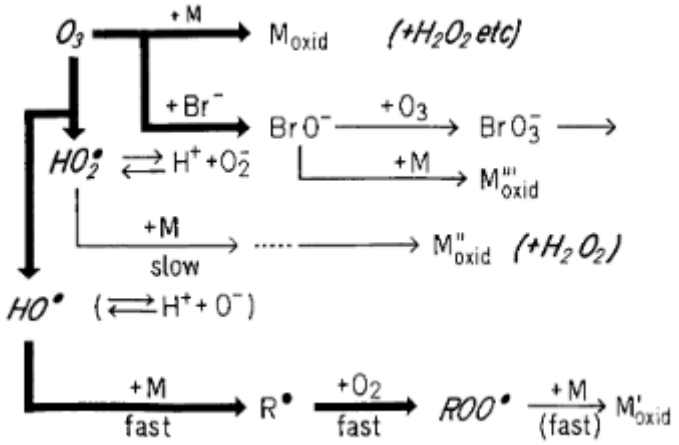

Figure 8. Different reactions of ozone when dissolved in water. M represents the solute in the figure, R represents a functional group and Br^- is bromide (Hoigne 1988).

The reactions can be subdivided into the two most important routes: its direct reaction with the compound and the reaction of the OH• - radical resulting from ozone decomposition (Beltran 2004).

When viewed from a thermodynamic point of view, ozone is a very powerful oxidant. However, its reactions are so slow that they are controlled by their kinetics rather than thermodynamics. Direct reactions of ozone with compounds can be written as first-order reactions as shown in Equations 4 and 5, when the reaction of ozone with compound M is written according to reaction Equation 3 (Hoigne 1988).

$$O_3 + M \overset{k_M}{\rightarrow} M_{oxid} \tag{3}$$

$$-\frac{d[M]}{dt} = k_M[M][O_3], \tag{4}$$

Where t is time and k_M is the rate constant of the reaction. The integral of Equation 4 gives Equation 5 which is the reduction of M.

$$-ln\frac{[M]}{[M_0]} = k_M[M]t, \quad (5)$$

where M_0 is the initial concentration of M. Thus, the logarithmic normalized concentration ratio of compound M decreases linearly if the level of ozone remains constant, i.e. ozonation is continuous.

A large number of reaction rate constants for various compounds when reacting with ozone are found from the literature. The reaction rate constants of the various compounds vary widely, and particularly selective oxidation occurs when the molecule has conjugated double bonds, or reduced sulphur compounds. Table 1 shows the reactions of various organic compounds with ozone and their reaction times. The reactions will, of course, accelerate as the concentration of ozone in solution increases. The reaction rates in Tables 1 and 2 are set at 0.5 mg / l for ozone, but if doubled, the reaction time will also be reduced by half. For example, the reaction of bromide at a concentration of 0.5 mg / l ozone occurs in about 1000 seconds, but if the amount of ozone is doubled to 1.0 mg / l the reaction time is reduced to 500 seconds (Beltran 2004).

When ozone reacts with alkenes the reaction pathway is called the Criegee-mechanism. Ozone attacks to the double bond with 1-3 dipolar cycloaddition and forms primary ozonide. This intermediate is highly unstable and decomposes fast to carbonyl oxide and carbonyl compound. From there products go through similar reaction and the end product depends about the reaction environment: if reductive the reaction gives alcohols and carbonyl compounds and if oxidative the end products are carboxylic acid and ketons. Ozone creates a oxidative environment, which leads to formation of carboxylic acids and this decreases the pH of the water. (Organic Chemistry Portal 2006)

Table 1. Reaction rates of various compounds with ozone (Hoigne 1988).

Compound	Reaction time
Saturated alkyls	No reaction
Alkenes	Seconds, except if the water contains chlorine, then the compound will chlorinate and will no longer react with ozone
Benzenes	Days
Polyaromatic hydrocarbons	Seconds
Phenols	Seconds, depending on pH.
Glyoxyl-, maleic-, oxalate-, acetate-, or formate ions	End products of oxidation, not reactive except formate ion, which may still slightly oxidize
Iodides	Immediately
Sulphides	Immediately
Nitrates	Immediately
Bromides	Minutes
The ammonia / ammonium ion	Hours
Cobolt	Days
Chlorides	No reaction

Reaction of organic compounds with ozone generally makes them more polar and water-soluble, whereby their toxicity is generally reduced (Walker et al. 2012). On the other hand, ozonation degrades the compounds, which may increase their acidity (Hoigne 1988). With inorganic compounds such as sulphides and nitrates, ozone generally reacts very quickly, while chloride and ammonia react more slowly. This is demonstrated in Table 1 (Hoigne 1988). However, all ozone reactions are affected by the pH of the water, which, when summarized, slows down the direct reaction of the ozone with the compounds and shifts the reactions more toward the OH•- radical reactions, which in turn, are much faster and less selective. However, for example, the reactions of ammonia and chlorine with ozone accelerates with increasing pH, but their reactions are still so slow that the difference is of a little importance (Hoigne 1988).

As previously stated in Table 1, ozone can react with bromine (Br) in water to form hypobromide (BrO-) and eventually bromate anions (BrO3-). They are toxic and carcinogenic to organisms, so its formation in water can be a problem when water is ozonated. However, in lake water, bromine is generally absent or very low, which means there is no problem, unlike seawater, where it is good to check and determine the amount of bromine before starting ozonation. (Hoigne 1988, Spiliotopoulou 2018).

Other ozone reactions are reactions of its degradation products. Ozone decomposition in water is a complex process that involves many different steps and can be either inhibited or catalysed by many different compounds. Certain compounds and ions also initiate ozone decomposition reactions. In water, the ion that initiates ozone decomposition is hydroxyl ion (OH-). The reactions are very rapid and can occur at the same time as ozone is rapidly degraded in water. Equation 6 is the initial step of the reaction in which the ozone reacts with the hydroxyl ion. Equations 7-13 show different reaction steps for ozone depletion (Langlais 1991).

$$O_3 + OH^- \rightarrow \cdot HO_2 + \cdot O_2^- \quad (6)$$

$$\cdot HO_2 \leftrightarrow \cdot O_2^- + H^+ \quad (7)$$

$$O_3 + \cdot O_2^- \rightarrow \cdot O_3^- + O_2 \quad (8)$$

$$\cdot O_3^- + H^+ \rightarrow \cdot HO_3 \quad (9)$$

$$\cdot HO_3 \rightarrow \cdot O_3^- + H^+ \quad (10)$$

$$\cdot HO_3 \rightarrow \cdot HO + O_2 \quad (11)$$

$$O_3 + \cdot HO \rightarrow \cdot HO_4 \quad (12)$$

$$\cdot HO_4 \rightarrow \cdot HO_2 + O_2 \quad (13)$$

The reaction is terminated if the two hydroperoxyl radicals ($HO_2\bullet$) react with each other to form an oxygen molecule (O_2) and a hydrogen peroxide molecule (H_2O_2) as shown in Reaction 14 or when an HO_4 radical reacts with another similar radical (Equation 15 or 16) (Sotelo 1987).

$$2\,HO_2 \cdot \rightarrow O_2 + H_2O_2 \cdot \quad (14)$$

$$2\,HO_4 \cdot \rightarrow 2\,O_2 + H_2O_2 \cdot \quad (15)$$

$$HO_3 \cdot + HO_4 \cdot \rightarrow O_2 + O_3 + H_2O_2 \cdot \quad (16)$$

As stated previously, the rate of ozone decomposition is greatly influenced by pH and temperature. In general, temperature increases the rate of all chemical reactions

(Powell & Scolding 2018) and in this case, pH increases the number of OH-ions in water that initiate and catalyse ozone decomposition reactions. The 2008 study by Ershov and Morozov illustrated well the linear dependence of ozone decomposition on temperature (Figure 9) and pH (Table 2).

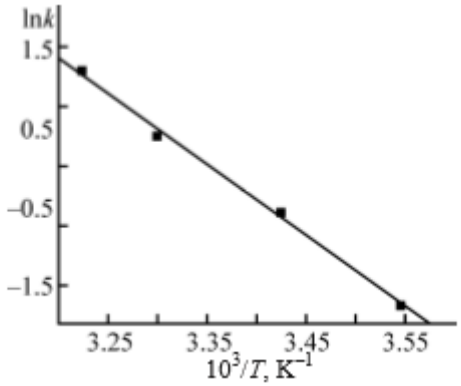

Figure 9. The linear temperature dependence of ozone decomposition is shown through the Arrhenius equation. The reaction rate constant k increases as temperature T increases. (Ershov & Morozov 2008)

Table 2. Effect of pH on ozone decomposition rate between pH 4-8. (Ershov & Morozov 2008)

pH	*k*, l mol–1 s–1
4,0	0,20
4,5	0,35
5,0	0,62
5,5	1,08
6,0	1,91
6,5	3,35
7,0	5,90
7,5	10,4
8,0	15,2

There has been a lot of research and debate on the kinetics of degradation as to whether it is a first or second order reaction. Some, based on their results, stated that the decomposition of ozone was second order, and some of the first and at different pH the order would have changed. In the end, however, it was found that the reaction is a "pseudo-first order reaction", where ozone depletion in pure water can be written as a first order reaction according to Equation 17 (Langlais 1991, Young 1996).

$$-\left(\frac{d[O_3]}{dt}\right)_{pH} = k'[O_3] \tag{17}$$

Because reaction is depended on pH, k' can be expressed to Equation 18

$$k' = k[OH^-] \tag{18}$$

By placing Eq. 18 to Eq. 17 we get the Eq. 19

$$-\left(\frac{d[O_3]}{dt}\right) = k[O_3][OH^-]. \tag{19}$$

The derived Equation 19 for the ozone's reaction rate applies only under alkaline conditions of pure water (Young 1996).

If we want to take into account the impurities in the water and the compounds that affect ozone decomposition, looking at the matter becomes much more complicated and thus empirical laboratory-scale experiments provide valuable practical information that could not be derived on a theoretical basis. Because of the

complexity of the subject, there are only a few examples of chemistry without further consideration. Equation 20 shows an equation for ozone depletion kinetics, which takes into account water impurities, pH, other chain reactions and intermediates resulting from decomposition. The equation is a first order one, but it can be used to determine the first order pseudo reaction rate constant *kc'* (Staehelin & Hoigne 1985).

$$-\left(\frac{d[O_3]}{dt}\frac{1}{[O_3]}\right)_c = k_1[OH^-] + (2k_1[OH^-] + \sum(k_{1,i}[M_i])\left(1 + \frac{\sum(k_{p,i}[M_i])}{\sum(k_{s,i}[M_i])}\right) = k_c^{'} \quad (20)$$

In equation, *Mi* presents compounds in the water that react with decomposed ozone. However, the equation does not consider the direct reaction of ozone with dissolved compounds, whereby no free radicals are formed. This means the equation has to be further expanded so, that we can calculate the reaction rate constant for the total ozone decomposition $k_{tot'}$. This is obtained by placing equation 20 to place of k_c' in Equation 21, which has the added kinetics of direct ozone reaction with M_i (Staehelin & Hoigne 1985).

$$-\left(\frac{\mathrm{d[O_3]}}{\mathrm{dt}}\frac{1}{[\mathrm{O_3}]}\right)_{\mathrm{tot}} = k_c' + \Sigma_i(\mathrm{k_{d,i}[M_i]}) = k_{tot}' \quad (21)$$

Placing Eq. 20 to Eq. 21, forms Eq. 22 that makes it possible to calculate the total ozone decomposition.

$$-\left(\frac{\mathrm{d}[O_3]}{\mathrm{dt}}\frac{1}{[O_3]}\right)_{\mathrm{tot}} = \mathrm{k}_1[OH^-] + (2\mathrm{k}_1[OH^-] + \sum(\mathrm{k}_{1,\mathrm{i}}[\mathrm{M_i}])\left(1 + \frac{\sum(\mathrm{k}_{\mathrm{p,i}}[\mathrm{M_i}])}{\sum(\mathrm{k}_{\mathrm{s,i}}[\mathrm{M_i}])}\right) + \Sigma_i(\mathrm{k}_{\mathrm{d,i}}[\mathrm{M_i}]) = k'_{tot}. \quad (22)$$

However, the practical application of the equations is difficult because the concentrations, reaction rate constants of different compounds, and reaction rate constants of ozone decomposition at a given pH should be known. Organic humic compounds in lake water are poorly known and virtually impossible to determine due to their great diversity. For this reason, it is recommended that the determination of required ozone dose, needs always be done experimentally for its intended use (Staehelin & Hoigne 1985).

The hydroxyl radicals (OH•) produced by ozone decomposition are the most powerful of the organic oxidants and their reactions are very rapid and non-selective. Reaction rates here are referred to as microseconds and reaction rate constants are generally in the range of 10^9-10^{10} (Westerhoff 2008). Figure 10 shows the expected reaction of hydroxyl radicals with a given compound. Because hydroxyl radicals are highly reactive and react indiscriminately with various compounds contained in water, the removal efficiency of certain compounds may be poor. Possibly, only a few hydroxyl radicals remain to oxidize the actual targeted compound after first reacting with other compounds in water. The kinetics of this compound to be removed can then be examined by Equation 23 (Hoigne 1988).

$$\ln\frac{[\mathrm{M}]}{[\mathrm{M_0}]} = -\eta(\Delta O_3)\frac{\mathrm{k_m}}{\sum \mathrm{k_i}[\mathrm{S_i}]}, \quad (23)$$

where *[M]* is the concentration of the compound to be removed, *[M₀]* is the initial concentration of that compound, $\eta(\Delta O_3)$ is the amount of ozone which decomposes into hydroxyl radicals, $\sum k_i[S_i]$ is the sum of the other compounds in water multiplied by their reaction rate constants and k_M is the reaction rate constant of the compound to be removed.

It can then be seen from Equation 23 that the more there are compounds *(Si)* in water, the slower one particular compound *(M)* reacts with the radical. Also, increasing the amount of ozone, which then decomposes into hydroxyl radicals, accelerates the reaction, naturally by increasing the number of radicals.

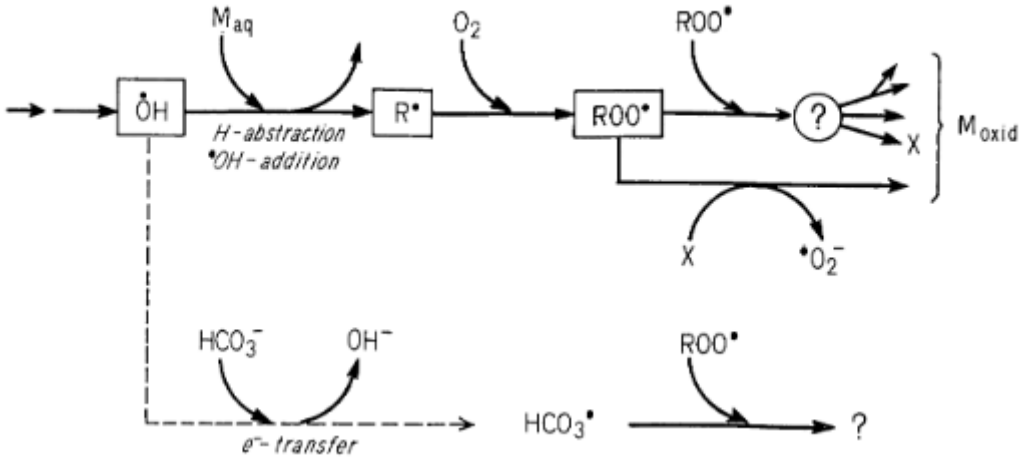

Figure 10. Reaction chain of hydroxyl radicals (Hoigne 1988).

When a hydroxyl radical reacts with compound M, it transfers an electron to it, producing a momentarily unstable new radical, which in turn reacts with the oxygen molecule to form a peroxide radical (ROO•). Peroxide radicals undergo a number of reactions which eventually lead to end product that is some kind of an oxide. An alternative route for the hydroxyl radical, is to react with the bicarbonate ion (HCO_3^-) which results in the formation of a relatively stable bicarbonate radical (HCO3•) and the hydroxyl ion (OH^-). The bicarbonate radical may possibly still react with the peroxide radical (Hoigne 1988, Powell & Scolding 2018).

The various compounds can either catalyse and initiate or inhibit ozone depletion reactions. Coarse-splitting occurs such that compounds capable of inducing the formation of the superoxide anion (O_2^-) are initiators of the reaction. Compounds that are capable of regenerating this anion from the hydroxyl radical are catalysts. On the other hand, if the compound consumes hydroxyl radicals without regenerating the superoxide anion, it inhibits ozone depletion. Table 3 lists decomposition initiators, catalysts, and scavengers (Langlais 1991, Westerhoff 2008).

Table 3. Ozone decomposing compounds and their structural formulas.

Initiators		Catalysts		Scavengers	
Compound	Formula	Compound	Formula	Compound	Formula
Hydroxyl ion	OH^-	Aryl groups	$R\text{-}C_6H_6$	Carbonates	CO_3^{2-}
Hydroperoxide ion	HO_2^-	Formic Acid	CH_2O_2	Bicarbonates	HCO_3^-
Glyoxylic acid	$C_2H_2O_3$	Primary alcohols	$HO\text{-}CH_2\text{-}R$	Alkyls	C_nH_{2n+1}
Formic acid	CH_2O_2	Phosphates	PO_4^{3-}	Tertiary alcohols	$HO\text{-}CR_3$
Humic compounds	Several	Humic compounds	Several	Humic compounds	Several

It can be seen from Table 3 that humic substances are listed to all three roles. This can make it difficult to predict ozone decomposition in waters rich in humic substances. As stated earlier, UV radiation can also initiate the ozone decomposition process. It is also noted in the table that carbonate and bicarbonate ions inhibit

ozone decomposition. Thus, ozone decomposition is slower in water with high alkalinity since alkalinity approximates the concentration of carbonate and bicarbonate ions in water (Staehelin & Hoigne 1985).

Water ozonation can be enhanced by various means to improve the water quality and speed up reactions. This can be achieved, for example, with the help of the compounds initiating the ozone depletion reactions of Table 3. Enhancement can be achieved by the addition of hydrogen peroxide (H_2O_2) which in water forms an addition of hydroperoxide-ion and this can be further enhanced with UV-light. Raising the pH also accelerates ozone depletion and the production of hydroxyl radicals by increasing the amount of hydroxyl ions in the water. The idea is to increase the formation of hydroxyl radicals, which were as oxidants much more potent than ozone, and thus accelerate the degradation and oxidation processes of the compounds. These techniques are called "advanced oxidation processes" (AOP). When the process is made more efficient, larger amounts of ozone can be used without fear of it remaining in the water as it proceeds to the next purification step or fish tank, and the size of the ozonation system becomes more compact (Hoigne 1988).

2.3.3 Ozone in fish farming

In RAS, ozonation has been found to improve water quality and significantly reduce potential fish diseases. In a study by Bullock et al. (1997), ozonation of circulating water prevented the onset of inflammation by bacteria *Flavobacterium branchiophilum*, that live in fish gills, and no chemicals or other treatments were needed to control it. The bacterial count in the system and on fish gills did not actually decrease due to ozonation, but the improved water quality and the potential deactivating effect of ozone on the bacteria may explain the disappearance of the disease. A combination of ozonation and UV irradiation has been found to effectively deactivate the system's heterotrophic bacteria to prevent disease outbreaks (Summerfelt et al. 2009). In the second part of the study (Summerfelt et al. 1997), ozonation was found to reduce solids by 35%, COD by 36%, DOC by 17%, colour by 82% and nitrite by 82%. It also increased the removal of solids by 33%,

which lead to less frequent washing of filters and reduced sludge accumulation. However, it did not affect the water turbidity on average.

Additionally, the effect of ozonation to geosmin and 2-methylisoborneol have been of interest that are known to cause bad odor and taste to fish meat. These compounds are end products of the microbial metabolism and occur naturally in surface waters, especially during summers, but tend to accumulate to RAS which causes problems with the product quality (Schrader et al. 2010, Lindholm-Lehto & Vielma 2018). However, ozonation alone has not been found to significantly reduce the amount of these compounds, even though they should react in seconds with molecular ozone (Westerhoff et al. 2006), but when combined with the addition of UV-light or hydrogen peroxide, the removal efficiency is increased (AOP). Unfortunately, other compounds in the water interfere and inhibit the reaction of geosmin and methyl isoborneol with molecular ozone (these compounds react specifically well with undecomposed ozone), and in order to effectively remove these substances, water should be pre-treated (Klausen & Grønborg 2010).

There are risks involved in ozonation with recirculating water. As stated earlier, ozone is toxic by inhalation, so it must be monitored in plant air to ensure that concentration does not become too high and dangerous for employees. On the other hand, to prevent fish or biofilter from developing oxidative stress due to ozone residues, companies recommend installing an ozone depleting unit (for example UV-light or activated carbon filter). There has been fairly new research on the online monitoring of ozone dose with fluorescence. Dissolved organic matter (DOM) contains many compounds that are fluorescent and react easily with ozone, even if ozone concentration is low. This means that change in water's fluorescence can be detected with high sensitivity when ozone oxidises those fluorescent compounds and system's ozone demand can be monitored and adjusted continuously (Spiliotopoulou et al. 2017). Because ozone is also very effective in reducing the amount of nitrite in water, its concentration can be so low that the bacterial strain in the biofilter can collapse. If disturbance in ozonation occurs suddenly, the nitrite concentration in the water may rapidly increase to a harmful level, as the bacterial

strain of the biofilter is stabilized to nitrate level with ozone and cannot adapt to the new concentration rapidly (Department of primary industries 2018). Figure 11 shows the industrial ozonation equipment recommended by Ozone Solutions for fish farming.

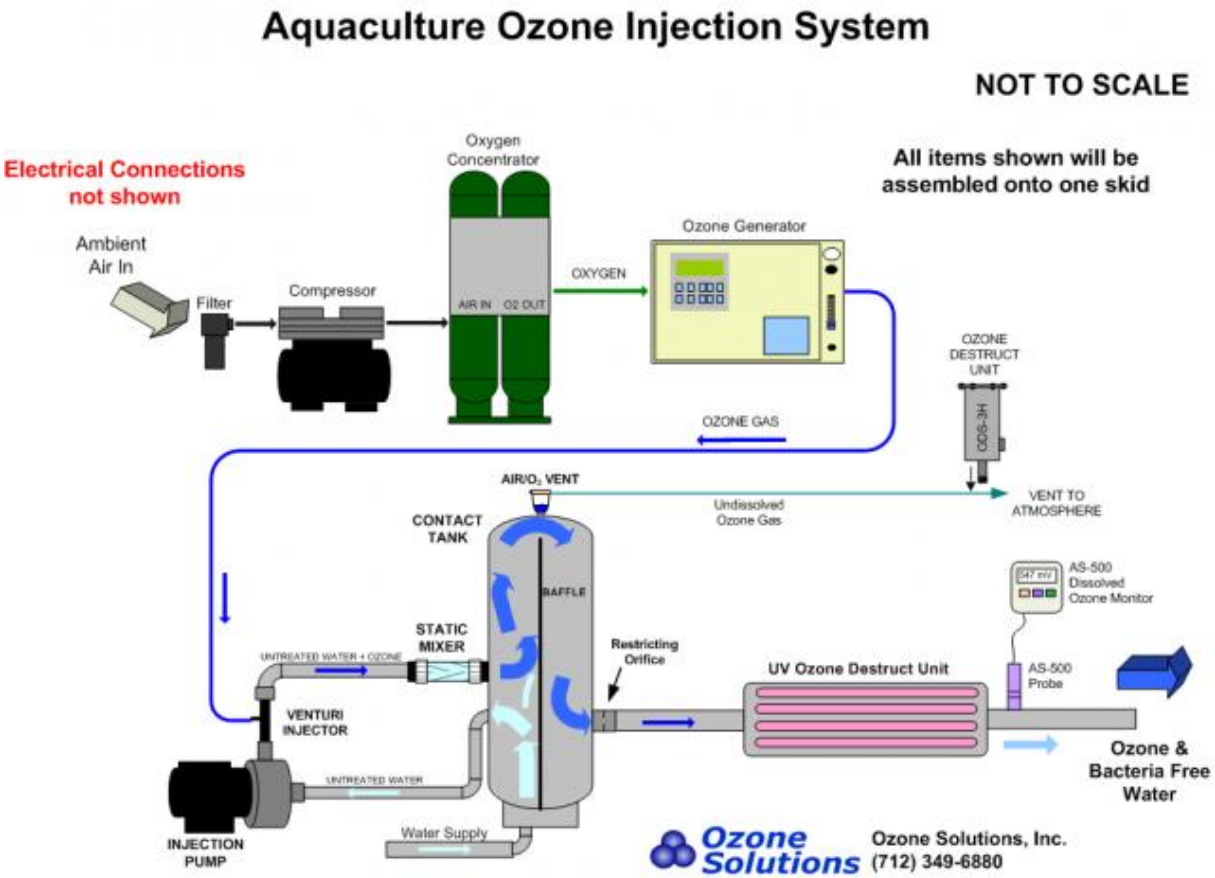

Figure 11. Potential ozonation equipment for commercial fish farming (Ozone Solutions 2014).

3 MATERIALS AND METHODS

The RAS where samples were obtained is not explained that thoroughly as the ozonation experiments were always done in lab environment and no direct ozonation of RAS took place. Initial composition of the water was always studied and results proportioned to that.

3.1 Experimental RAS platform

Experimental RAS platform is based in LUKE's fish farm in Laukaa. It consists of 10 small individual recirculating systems that were delivered and installed by ArvoTec Company. Volume of each system is 1140 l and their structures are identical, though the way they are used can be modified depending on what is desired to study. Systems consists culture tank (500 l), solids removal, biofilter, aeration, oxygen injection, pH adjustment and disinfection. System had constant monitoring that measured pH (pH::lyser, s::can, Austria), oxygen (oxi::lyser, s::can, Austria), CO_2 (Franatech Germany), Nitrogen compounds NO_2, NO_3 & NH_4^+ (spectro::lyser, p::can, Austria) and temperature. An online monitor gathered all the information from the systems individually (con::cube, p::can, Austria).

Culture tank is round bottom-drained and houses the cultured fish, that in this case was rainbow trout (*Oncorhynchus mykiss)*. Amount of fish in the tanks was first 11675 g (25 fishes, 467 g/individual) and ozone decomposition tests took place during this time. Later fishes were weighed and some were removed so the new amount of fish was 11671 g (21 fishes 555,8 g/individual). Ozone dose-tests were done during that time. Tank was covered and had constant lighting. Feeding was done with automatic feeding system that measured feed nine times per day and it was based on the undigested feed that was collected in solids removal. Used feed was Raisio circuit red (1,7 mm & 2,5 mm), that was made from vegetable oil, soy- and bean proteins, Fish meal and oil, vitamins and trace elements. Feed contained about 0,95-1,15 % of phosphorous and and 7,52-7,84 % of nitrogen (Raisioaqua 2018).

Solids removal consisted of feed collector unit, 24 cm diameter (hydraulic loading 133-531 l min−1 m-2) swirl separator (Eco-Trap Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), drum filter with 60 µm filter panels (Hydrotech HDF501, Veolia, Paris, France). More detailed explained in Pulkkinen et al. (2018). Replacement water was also added during this water purification phase (about 1-2 % of total volume).

Biofilter consisted of two 147 l serial linked tanks. A moving-bed and a fixed-bed reactor had the same kind of plastic culture mediums (Bio-Blok® 200 filter medium (EXPO-NET Danmark A/S, Hjørring,Denmark), housing about 750 m^2 m^{-3} surface area for bacteria to grow.

Aeration happened in a small aeration tower (82 cm high), to which water was added from top. Tower contained packing material to allow more efficient diffusion of carbon dioxide from water to air. After that pH was adjusted to seven using pump and NaOH. After that pure oxygen was injected using ceramic diffusers and water then disinfected with UV-light. Then water is led back to culture tank.

3.2 Materials

3.2.1 Samples

The water samples used in ozonation experiments were obtained always from the same small recirculating aquaculture system (system 2). Five litre plastic canisters were used as containers for the samples and they were washed properly before use and rinsed three times with sample water before filling them. Lake water (LW) that comes to systems from oligotrophic Lake Peurunka as a replacement water, was collected from a tube that is connected straight to the pipe that leads to systems. Tank water (TW) was collected straight from the centre of systems cultivation tank with a plastic cup, avoiding any big visible particles of solids. Samples were collected during the afternoon and stored in the fridge in about +6 °C. Experiments were always done during the next day, so the samples spent less than 24 hours in the fridge.

3.2.2 Used chemicals, solutions & equipment

Chemicals used in this study are listed in the Table 4. Solution made out them are described in more detail later in this chapter.

Table 4. Used chemicals, their chemical formulas, manufacturers and state.

Compound	Chemical formula	Manufacturer	State
Potassium iodide	KI	VWR Chemicals	Solid
Disodium phosphate	$Na_2HPO_4 \cdot 2\,H_2O$	VWR Chemicals	Solid
Sodium dihydrogen phosphate	$NaH_2PO_4 \cdot 2\,H_2O$	Merck	Solid
Sodium thiosulphate	$Na_2S_2O_3$	Merck	Solid
Sulphuric acid	H_2SO_4	Solution made in the university by lab techs	Aq. (4 M)
Starch	$(C_6H_{10}O_5)_n$	VWR Chemicals	Solid
Zinc chloride	$ZnCl_2$	VWR Chemicals	Solid
Zinc iodine	ZnI_2	VWR Chemicals	Solid
Phosphoric acid	H_3PO_4	WGK	Aq. (14,8 M)
Potassium indigo-trisulfonate	$C_{16}H_7K_3N_2O_{11}S_3$	Acros Organics	Solid
Hydrochloric acid	HCl	Solution made in the university by lab techs	Aq. (2 M)
Synthetic air	20 % O_2, 80% N_2	Linde	Gas

3.2.2.1 Solutions for the ozones iodometric determination method in gas

Buffered KI solution was prepared by dissolving 14,6 g of Na_2HPO_4, 7,0 g of NaH_2PO_4 and 40 g of KI to 2 litres of ultrapure water in 2 l measuring bottle. Salts were dissolved before KI to prevent any possible precipitation. Precision scale was used for weighing the substances and all glassware were rinsed with ultrapure water few times before use to make sure that there were no impurities. When dissolving solids to water it was always made sure that every crystal of compound was poured to bottle. Bottle was then shaken thoroughly. (IOA 1987)

Sodium thiosulphate solution was done by dissolving exactly 2,4848 g of Sodium thiosulphate to one litre of ultrapure water in measuring bottle. Solutions concentration was then 0,01 M. The instructions for this standard would use solution with the concentration of 0,1 M (IOA 1987) but it was decided that titration would be too inaccurate, so the more diluted form was used instead.

Because faculty did not have any starch indicator, it was prepared according to standards instructions (IOA 1987). 1 g of starch was dispersed to small amount of ultrapure water in the beaker. Then 5 g of $ZnCl_2$ was dissolved to 25 ml of ultrapure water in an Erlenmeyer flask (250 ml) and starch solution was added to it. Solution was stirred a bit and after that, it was boiled until the volume was reduced to about 25 ml. Finally, whole solution was poured to 250 ml measuring bottle and 0,5 g of ZnI_2 was added to it, after which solution was diluted to 250 ml and shaken to stir it completely. For storage indicator was poured to small glass vial with tight glass cap to prevent the contact with air, because it would start to react with oxygen in the air and with time be spoiled. Vial was stored in a dark place always tightly shut. Before use, indicator needed a bit of shaking because starch started to settle to bottom when left untouched.

3.2.2.2 Solutions for the colorimetric determination of residual ozone in water

First, 250 ml stock solution of indigo-trisulphonate was made. 20 µl of analytical grade H_3PO_4 was diluted to about 300 ml of ultrapure water with single channel pipette. Then 0,1541 g of indigo was weighed and dissolved to this solution in 250 ml measuring bottle and the bottle was shaken thoroughly. Stock solutions absorbance was checked in 600 nm with a spectrophotometer to check that it was over 0,16 ± 0,01 cm^{-1}. Solutions absorbance was 0,166 cm^{-1} so it was usable. Absorbance was checked every time before making new diluted solution, to be sure that stock solution was still viable, because when absorbance has dropped below 80% of starting value it must be discarded. Solution was stored in dark place all the time to prevent the absorbance loss. Stability should last one month when stored properly. (IOC 1989)

Diluted solution was made from stock solution. 1 g of NaH_2PO_4 and 0,7 ml of analytical grade H_3PO_4 were dissolved to about 80 ml of ultrapure water in 100 ml measuring bottle. Then 10 ml of stock solution was pipetted to that bottle and it was filled to the marker with ultrapure water. Bottle was then shaken thoroughly. Solutions absorbance was checked before use that it was not too dark or too bright. Solution was prepared always one day before tests to be sure that its stability would be good enough, even though solution should be usable for about one week (IOA 1989)).

3.2.2.3 Solutions for the HPSEC-analysis

For the HPSEC-analyses, a mobile phase solution was prepared. It was done by weighing 0,8900 g of Na_2HPO_4 and 0,7801 g of NaH_2PO_4 with precision scale and dissolving them to ultrapure water in a beaker. Solution was then poured to the one litre-measuring bottle and filled with ultrapure water to the mark. Solution was then suction filtered with pore size of 0,22 µm membrane filter (WhatmanTM, ⌀ = 47 mm, Germany) to ensure that no solids enter the HPSEC-machine and distort the results.

3.2.2.4 Used equipment

During the study the used equipment were kept the same all the time if it was just possible. They are listed in the Table 5.

Table 5. Used equipment, model, manufacturer and manufacturing country.

Device	Model	Manufacturer	Country of manufacture
pH-meter	PHM220 Lab pH meter	Radiometer analytical	France
Precision scale	AG204	Mettler Toledo	Switzerland
Spectrophotometer	U-1500 Spectrophotometer	Hitachi	Japan
Single Channel pipettes	Finnpipette	Labsystems Oy	Finland
Ozone generator	Ozonizer S 500	Sander	Germany
Ozone meter	Model 1180	Dasibi environmental corp.	United States
DOC-analyser	TOC-L	Shimadzu	Japan
HPSEC	C196-E061W prominence	Shimadzu	Japan

3.3 Methods

3.3.1 Ozonation setup

The ozonation setup was kept very simple and is illustrated in Figure 12.

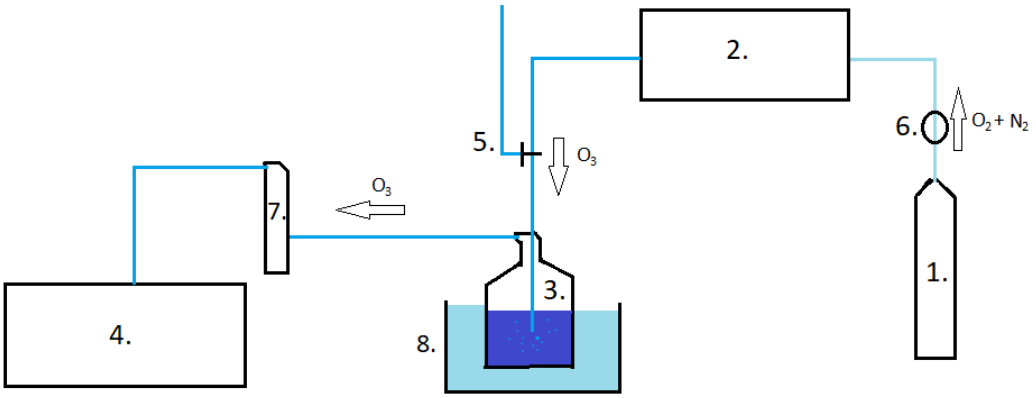

Figure 12. The ozonation setup that consists 1. Synthetic air bottle 2. Ozone generator 3. One litre ozone reactor containing the sample 4. Ozone analyser 5. Two way-valve, one way leading to reactor, another to ventilation 6. Gas flow meter 7. Smaller empty reactor and 8. Water bath.

Ozone was produced from synthetic air (80% nitrogen and 20 % oxygen) rather than pure oxygen. Custom-made flow meter was used to control the airflow and it was kept approximately in one litre per minute for all the ozonations. Air was directed straight to the ozone generator that was kept at the full power for the ozonations. Ozone was then directed through the tubes to the two way-valve. One end of the valve led to the fume hoods ventilation and the other one to the ozone reactor. Reactor was a sealed glass container with about 1 litre volume and the sample water was ozonated in it. Glass pipe bubbled the ozone straight to sample (underwater) and the non-consumed excess ozone that did not dissolve to sample was then led through tubes to the smaller also sealed empty reactor. Ozone analyser then soaked up the gaseous ozone and calculated the ozone concentration in it. Stopwatch was used to time the ozonations. In some tests, it was necessary to warm or cool samples to specific temperature and for that, a water bath was used. Temperatures were then

measured from the samples with electronic thermometer. Even though samples and the ozone reactor was kept in specific temperature gas' temperature was left untouched. This means that it was around room temperature (21-22 °C) and most likely warmed the sample when it was ozonated.

Because the ozone meter was old, its ozone concentration calculations could not be trusted completely so its current was measured with DAQ-tracer during ozonations. This current was later then used to calculate the real ozone concentration after the calibration was done for the ozone generator and the meter.

3.3.2 Ozone meter and generator calibration

Because the ozone generator did not have any real scale that could tell how much ozone it produced, evenly spaced artificial markings were done to its efficiency control knob from one to seven, one being lowest and seven being the maximum amount of ozone it can produce. Calibration was then done using the potassium iodine (KI) standard method (IOA 1987) to determine the concentration of ozone in gas with different generator efficiency levels (2, 4, 6 & 7). In this method, 0,1 l of buffered KI with concentration of 0,01 mol/l were ozonated for a duration of a one minute. Then 5 ml of 4 M H_2SO_4 and about 1 ml of starch indictor was added and solution titrated with 0,01 M sodium thiosulphate using the automated burette until the solution was clear. From the amount of consumed sodium thiosulphate, produced ozone concentration in one litre of gas could be calculated using Equation 24:

$$[O_3]\frac{mg}{l} = \frac{\left(\frac{V_{Na_2S_2O_3} \cdot c_{Na_2S_2O_3}}{2}\right)(M_{O_3})}{V_{Gas}} \cdot 1000, \quad (24)$$

Where $[O_3]$ is ozone concentration in the gas, $V_{Na_2S_2O_3}$ is volume of used sodium thiosulphate, $c_{Na_2S_2O_3}$ is concentration of used sodium thiosulphate solution (0,01 mol/l), M_{O_3} is the atomic weight of ozone (48 g/mol) and V_{Gas} is the volume of gas that was used in the ozonation (1 l).

Airflow was kept at the one litre per minute and temperature was 21 °C. Ozone generator was always warmed up properly before starting the calibration meaning that the produced ozone concentration was stable. This usually took about 30 minutes or more. With every level, three replicas were done, and mean ozone concentration calculated from them. Method is accurate with ozone concentrations of 0,1 mg/l and higher with error of ±1 %. Before every measurement Ozone analyser current was recorded with DAQ-Tracer to gain the value that corresponded the produced ozone in that level. DAQ recorded the current from the meter every second and after the ozone production was stable, few hundred measurements were chosen, and average calculated from them. Measured and calculated values are shown in the appendix 1. Calibration curve was drawn based on the results to determine the dependence between the meters current and the ozone concentration that was derived with potassium iodine-method (Figure 13).

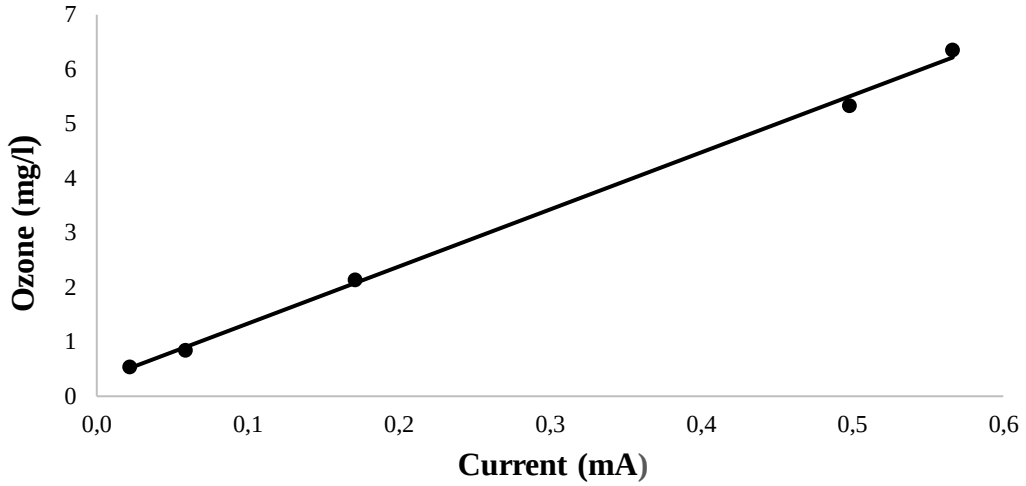

Figure 13. The calibration curve for the ozone meter. Meters current corresponding the ozones concentration in one litre of gas.

Trendlines equation from Figure 13 was then derived using excel (Eq. 25)

$$y = 10{,}464x + 0{,}2873, \tag{25}$$

where y is ozone concentration measured in one second and x is meters measured current. R^2 value was 0,9981.

With this equation, it was possible to determine the real ozone concentrations from the measured currents of the analyser. With maximal power the ozone production was about 0,4 g of ozone per hour with airflow of 1 l/min.

3.3.3 Determination of residual ozone in the water

To determine residual ozone in the water, colorimetric indigo-trisulphonate-method was used (IOA 1989). The procedure to prepare the solutions for the method is described in the materials-section so it will not be described here.

A new diluted reagent was always prepared for the test day before and stored in dark place. Before the tests its absorbance was tested to see that solution wasn't too dark of a colour. 10 ml of reagent was then pipetted using single channel pipettes to small glass vials with about 25 ml of volume. 5 ml of sample water was then taken right after the ozonation with single channel pipette and it was introduced beneath the reagents surface to prevent any loss of ozone. Vial was then sealed with a cap and shaken a bit, after that solution was poured to cuvette (cell length 1 cm), and its absorbance was measured with a spectrophotometer in wavelength of 600 nm. From each ozonated water sample, a blank sample was created. Procedure was same as with a normal sample, but ozone was removed from it by injecting air to it for about ten minutes, to make sure that all ozone had disappeared and after that sample water was introduced to reagent. Small air pump, rubber tube and glass pipette tips were used for this. Glass pipette tips were changed for every sample to prevent any contamination in samples. By comparing the blank and samples absorbance, one can calculate the ozone concentration in water by using Equation 26

$$c_{O_3}\,\frac{mg}{l} = \frac{V_{total}\cdot\Delta_{absorbance}}{l_{cell}\cdot V_{sample}}\cdot 1000, \tag{26}$$

where c_{O_3} is the concentration of residual ozone in water, V_{total} is the combined volume of sample and reagent, $\Delta_{absorbance}$ is the difference between samples and blanks absorbance, l_{cell} is the cell length of the cuvette (1 cm) and V_{sample} is the volume of the injected sample.

Used spectrophotometer was allowed to warm up for about 20 minutes and then calibrated according to its instructions, before use.

3.3.4 Ozone decomposition tests

The ozonation setup was warmed up and stabilized before the test. Airflow was kept at 1 litre per minute and ozone was directed to an ozone meter to monitor the production. Sample canister was shaken before the water was measured to 1 litre measuring bottles. Bottles were rinsed with ultrapure water a few times before samples were introduced to them. Measuring bottles were then put to water bath to reach the desired temperature (temperature measured with an electronic thermometer) and after that one bottle at the time was ozonated for 1 hour. Ozone reactor was too in water bath to keep the desired temperature. Before ozonation, water was poured from the measuring bottle to ozone reactor that was rinsed with ultrapure water. Ozone was then directed through the two way-valve, first to fume hoods air conditioning and when time started valves lever was twisted and ozone would be directed to sample. Immediately after ozonation, residual ozone was determined and the reactor was then closed and put back to water bath. Residual ozone samples were then taken in certain times as fast as possible to minimize disturbances for the decomposition. After the last residual ozone sample was taken, samples for the DOC, NT, HPSEC and pH were obtained to small plastic sample tubes. Possible ozone that was still present in the water, was removed from these samples with air in the same way as it was removed from the blank sample for the residual ozone.

Before ozonation, initial samples were taken from the water for DOC, NT, HPSEC and pH analyses. DOC, NT and HPSEC samples were stored in the fridge in about -18 °C and pH was analysed immediately after tests. Studied temperatures were 6 and 15 °C for LW, 15 °C for TW and 15 °C for ultrapure water. At least three replicates were done for each and in some cases more if there was a lot of variation.

The reaction rate constants were calculated using Equation 27 that was derived from Equation 17.

$$k = ln\left(\frac{[O_3]_0}{[O_3]}\right) \div \Delta t, \quad (27)$$

where k is the rate constant, Δt is the elapsed time in seconds, $[O_3]_0$ is the ozone concentration in the beginning and $[O_3]$ is the ozone concentration in the end.

Half-lives ($t_{\frac{1}{2}}$) for the treatmens were calculated using Eq. 28 that is the half-life equation for the first-order reactions (Chemistry Libretexts 2019).

$$t_{\frac{1}{2}} = \frac{ln2}{k}, \quad (28)$$

where k is the reaction rate constant of the reaction.

3.3.5 Ozone dose tests

Preparations and methods for ozone dose tests were similar as for ozone decomposition tests. Initial samples were taken from canister for HPSEC, NT, DOC and pH. One-litre samples in measuring bottle were first put to water bath to reach the desired temperature then poured to reactor and ozonated a specific amount of time. After the ozonation, residual ozone was determined immediately and after that, remaining ozone was purged away with air and samples for HPSEC, NT, DOC and pH were taken. Between the ozonations reactor was rinsed thoroughly with ultrapure water and smaller reactor that led to ozone meter was changed to a new one to zero the meter. Ozonation times were 2, 5, 10, 20 and 30 minutes for both LW and TW so in total 6 samples for each, including the initial one. Temperature was kept in 15 °C in all tests. Three replicates were done during the three-week time period. Ozone dose is reported as milligrams of consumed O_3 per milligram of DOC and is calculated using Equation 29

$$Ozone\ dose = \frac{O_{3\,Produced} - O_{3\,untapped}}{DOC_{sample}}, \quad (29)$$

where DOC_{sample} is sample's DOC concentration in mg/l, $O_{3_{Produced}}$ is total produced ozone and $O_{3_{untapped}}$ is ozone that wasn't consumed. Numerator can be marked too as $O_{3_{consumed}}$ if consumed ozone is immediately known.

3.3.6 Water quality analyses

DOC and NT were analysed with same TOC-L machine. Before analyses, samples were melted and warmed up a bit in room air and after that, they were filtered using syringe and filter (WhatmanTM, ⌀ = 47 mm, Germany). Samples were acidified with HCl and then inserted to machine. Laboratory technicians calibrated analyser with standard solutions of 30 and 100 mg/l of C/N as it was a proper range for the samples. Ultrapure water was used as blank sample and water from Lake Jyväsjärvi acted as reference sample. Every sample was analyzed twice and if measurements were too different it measured the sample for the third time. The result was calculated by taking the average of these measurements.

All HPSEC-samples were first filtered to 1 ml glass vials, using 0,45 µm filters (VWR, USA) and syringes. Just to be sure that filters didn't release any possible particles to samples, they were rinsed few times with ultrapure water, before samples were filtered with them. Samples were placed to the sample tray so, that machine would analyse "the cleanest" sample (the most ozonated ones) first and "the dirtiest"(initial and less ozonated ones) last, to avoid the analysers column getting dirty and then contaminating the less dirty samples. Prepared mobile phase was then placed to the machine and few runs were made with ultrapure water and mobile phase to make sure that there were no traces left from earlier experiments. Samples were then ran and the HPSEC-analyser measured UV-absorbance at 254 nm and tryptophan-, tyrosine-, fulvic- and humic-like fluorescence. A column (YarraTM 3 µm SEC-3000, 300 * 7.8 mm, Phenomenex, USA) separated different sized molecules in the sample to fractions. Each sample was analyzed twice with

two different wavelengths for fluorescence. Used wavelengths are listed in the Table 6.

Table 6. Used Wavelengths in HPSEC-analyses.

Fluorescence	Excitement (nm)	Emission (nm)
tryptophan	230 & 270	355
tyrosine	220 & 270	310
humic acid	240 & 330	440 & 425
fulvic acid	270 & 390	500

Right after the experiments of the day were done pH was measured with pH-meter, so there were only few hours between ozonation and pH measurement. Ozone was first purged from the first few samples, but as it was seen that it made no visible difference to the pH, later samples were left untouched.

3.3.7 Data analyses

Data was analysed with Windows Excel 2016 and IBM SPSS statistics 24. Excel was used to count the ozone concentration from the measured currents using the Equation 1. Excel was also used to count the means and deviations and to make the most of the data figures and tables. HPSEC results were worked with Shimadzu LabSolutions LC/GC version 5.51. SPSS was used to do the statistical tests to ozone decomposition and ozone dose data. To test the effect of ozone, before and after the ozonation, T-test test was used. when testing regression between DOC and fluorescence and UV-254 linear regression model was used, but fluorescence- and UV-data was first transformed with logarithm. For each statistical test the 95 % confidence interval was used.

4 RESULTS

Results from the ozone decomposition- and dose tests are presented in separate chapters: decomposition in 4.1 and dose in 4.2. All the data is presented in figures or tables.

4.1 Ozone decomposition

The data from water quality parameters DOC, TN & pH are first presented before and after the ozonation. Then the determined ozone doses for the treatments and finally the actual ozone decomposition data with rate constants and half-lives calculated for each treatment.

Ideally, injected ozone amount was expected to be similar in all ozonation experiments to keep the treatment identical as possible, but small differences can be observed (Figure 14). Differences are so small though, that it should not make any error to the actual results.

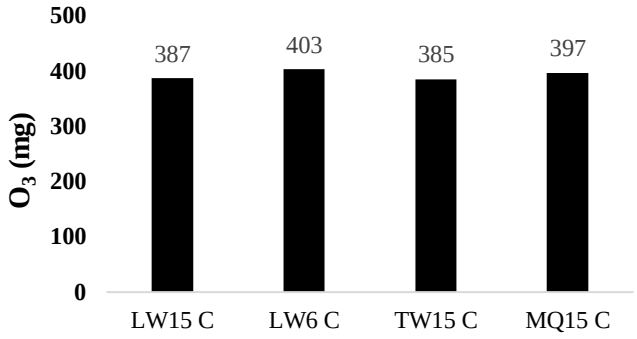

Figure 14. The amount of ozone injected to the samples (tank water TW & lake water LW) after 60 min of ozonation measured in milligrams with air flow of 1 l/min.

Observed DOC levels (Figure 15) declined slightly (t-test, t=5,339, df=15, p<0,005) after ozonation and observed DOC amounts were the highest in the tank water. Ozonation had no effect on DOC levels of MQ water. TN levels in turn, increased

slightly after ozonation (t-test, t=-15,159, df=15, p<0,005) in all treatments (Figure 16). TW contained significantly more nitrogen and DOC than other samples.

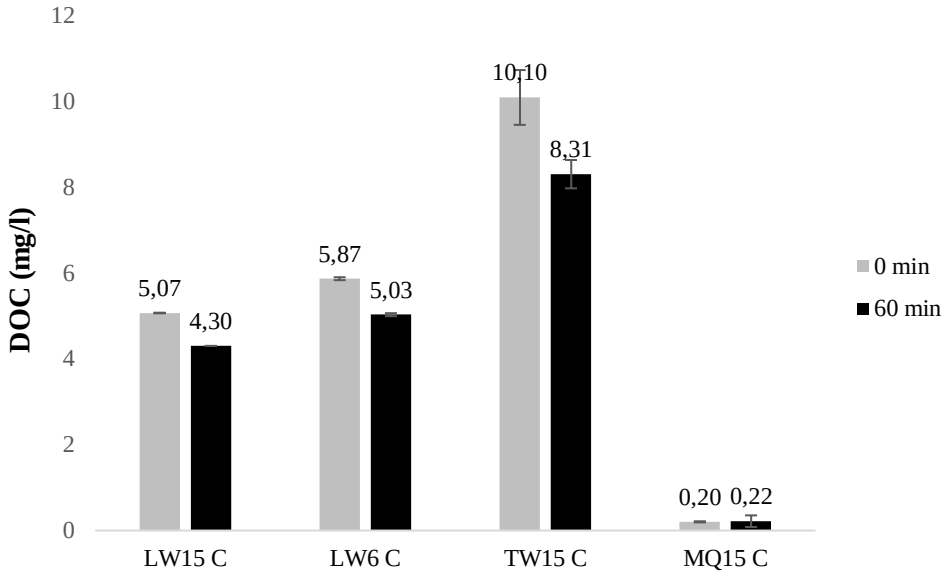

Figure 15. Measured DOC levels before (grey) and after (black) the ozonation treatment (SD, n=3).

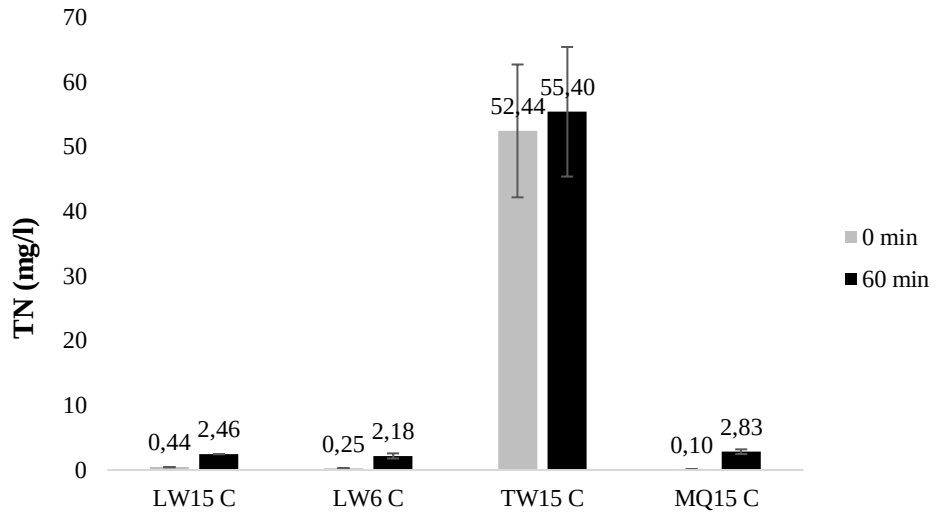

Figure 16. Measured TN levels before (grey) and after (black) the ozonation (SD, n=3)

Small change in pH was recorded as samples tend to become slightly more acidic (t-test, t=4,915, df=15, p<0,005), most notably with ultrapure MilliQ-water (Figure 17). Otherwise LW and TW were very close to neutral pH 7.

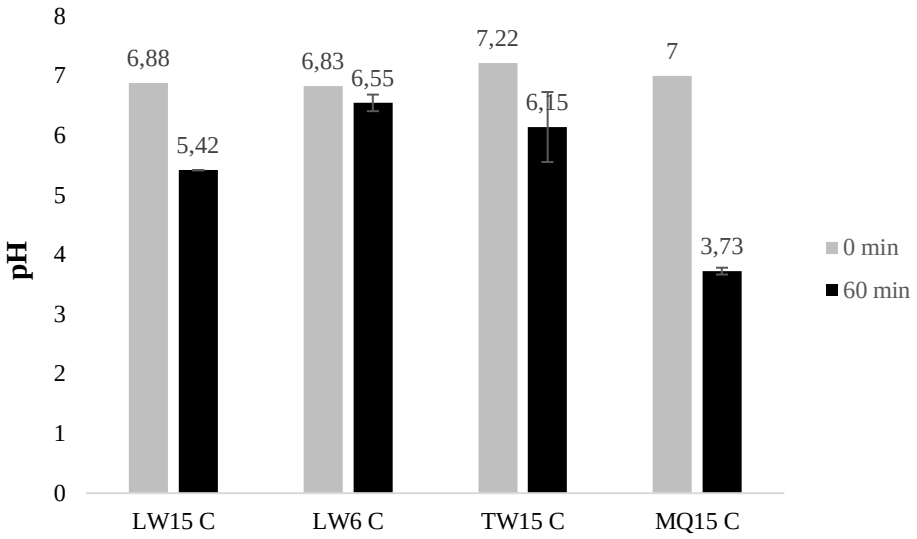

Figure 17. Recorded pH-values before (grey) and after (black) the ozonation (SD, n=3).

Derived ozone doses (Figure 18) showed large variation in their values. Largest dose was recorded with MQ-water which was to expected and lowest with lake water at 6 °C. At the same temperature, dose was higher with lake water and lower in tank water.

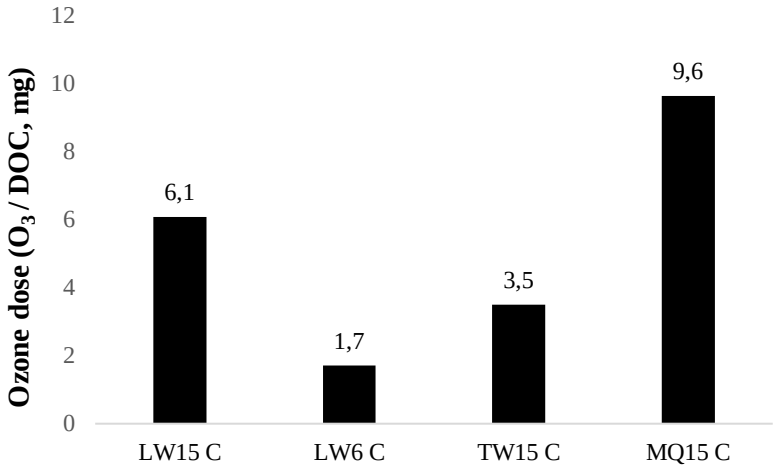

Figure 18. Calculated ozone doses after ozonation. Dose is reported as consumed ozone divided by the DOC content of the sample in milligrams.

The slowest ozone decomposition time was recorded with MilliQ-water in 15 °C its half-life being 173 min, but the highest dissolved ozone concentration was measured in LW at the 6 °C. Tank water had the fastest decomposing rate, but interestingly enough, in warmer LW ozone decomposed slower than in colder water (Figure 19). Calculated reaction rate constants and half-lives for the different treatments are listed in Table 7.

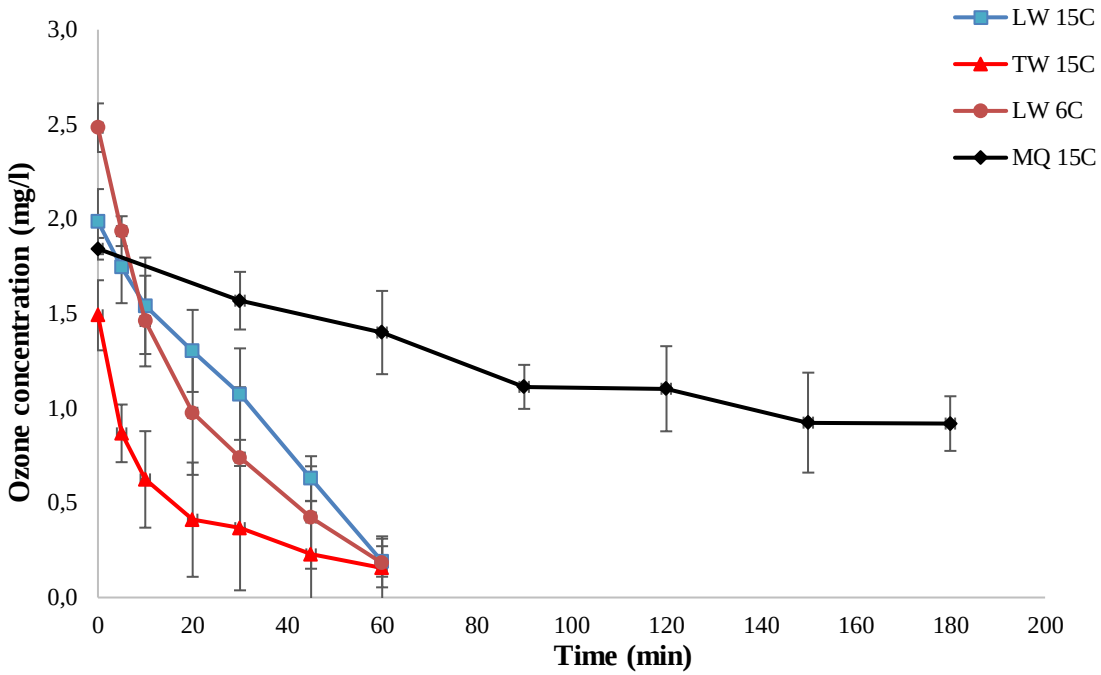

Figure 19. Ozone concentrations measured from all sample waters after ozonation at the regular intervals. (SD, n=3)

Table 7. Calculated reaction rate constants and half-lives for the ozone decomposition in different treatments.

Reaction rate constant (min^{-1})	Half-lives (min)	Treatment
$6{,}51\times10^{-4}$	18	LW 15 C
$7{,}25\times10^{-4}$	16	LW 6 C
$9{,}51\times10^{-4}$	12	TW 15 C
$6{,}67\times10^{-5}$	173	MQ 15 C

4.2 Ozone dose for LW and TW

Here water quality parameters DOC, NT, pH and ozone data are presented in first section and fluorescence data in the second. Most of the data is presented as the function for ozone dose (mg O_3/ mg DOC).

4.2.1 Water quality parameters and ozone data

Injected ozone amounts were very close to the same with every replica (n=3) as standard deviation is very low. This can be seen from Figure 20.

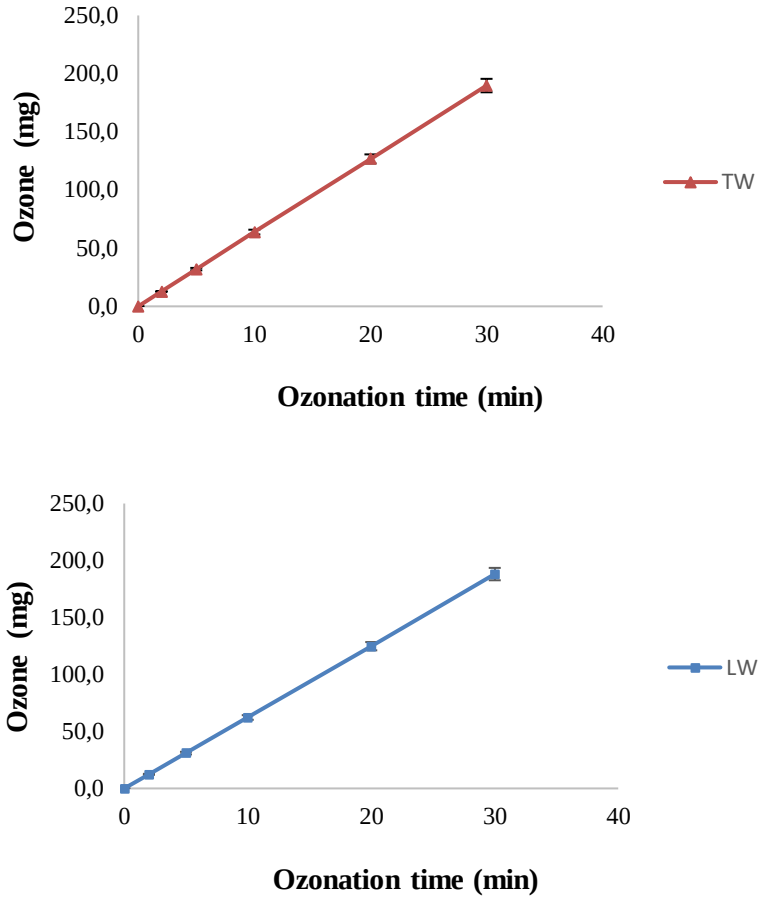

Figure 20. Mean of the injected ozone amounts from the three replicas of TW (upper) and LW (lower) ozone dose tests (SD, n=3).

Calculated ozone doses were lower for TW than LW and increase in dose seemed to be more linear with LW than with TW (Figure 21). LW's highest dose was 2,88 O_3/DOC and TW's 1,91 O_3/DOC when injected ozone amount was about 190 mg.

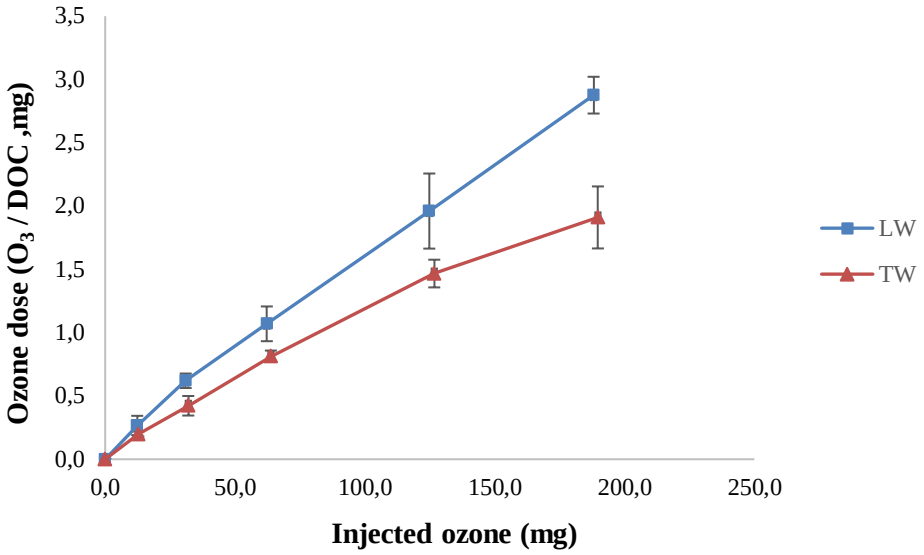

Figure 21. Amounts of injected ozone and achieved ozone doses for TW and LW (SD, n=3).

DOC and NT concentrations changed very little after ozonation. Small decreasing trend can be seen with DOC as ozone dose increased. There was not any significant difference between LW and TW, except that TW contained over 3 mg more DOC than LW in default (Figure 22). With TN though, TW contained it many times more than LW. With bigger ozone doses, minor increase in TN was observed, but with TW change is negligible as its initial nitrogen concentration is so high. TN increase in LW is more significant as the initial concentration of NT is very low and even small change is noticeable (Figure 23).

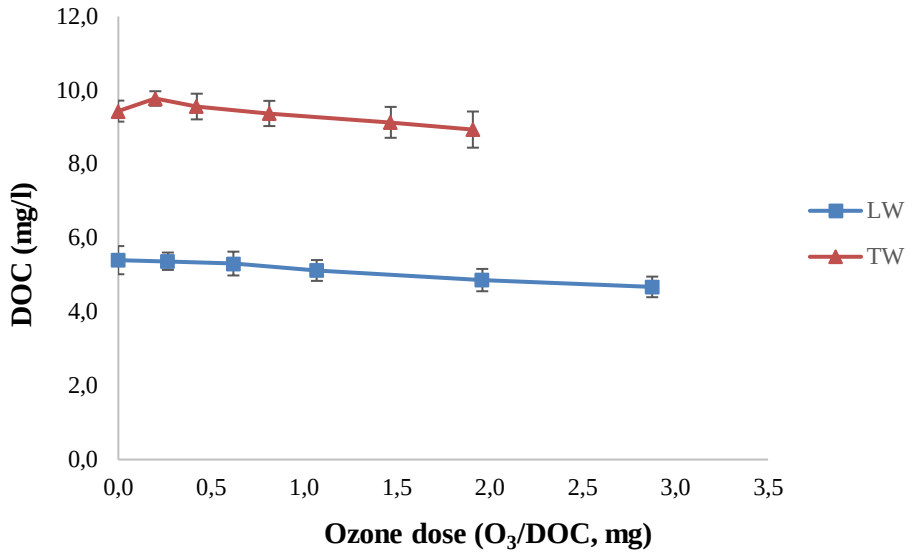

Figure 22. DOC values for LW and TW with different ozone doses (SD, n=3).

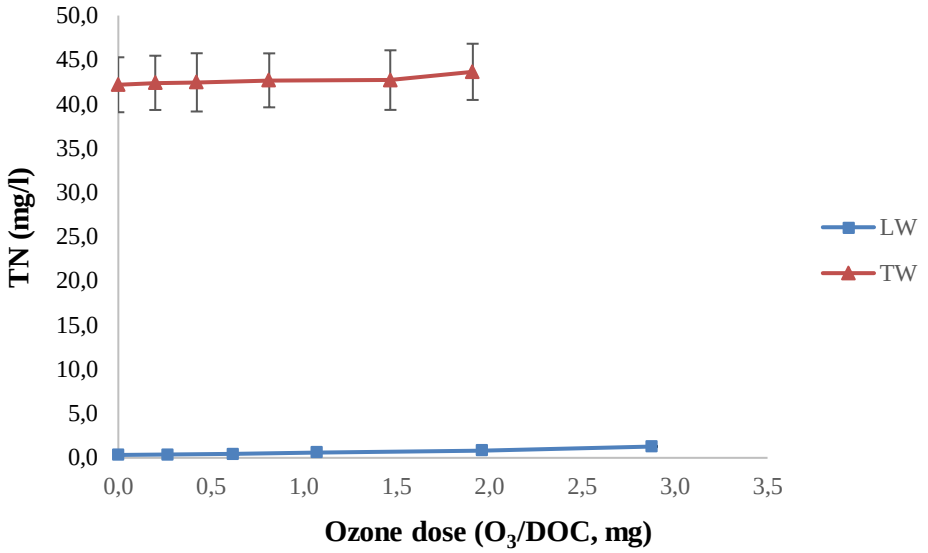

Figure 23. NT values for LW and TW with different ozone doses (SD, n=3).

Initially pH was about 0,2 higher in TW than in LW. With the highest ozone dose of 2 mg O_3/mg DOC for TW, pH dropped to 6,85 and with dose of about 3 O_3/DOC pH dropped to 6,80 in LW. As the ozone dose increased the pH decreased, exception being smallest given ozone dose when pH actually rose from its initial value (Figure 24). Deviation in TW was much greater than in LW.

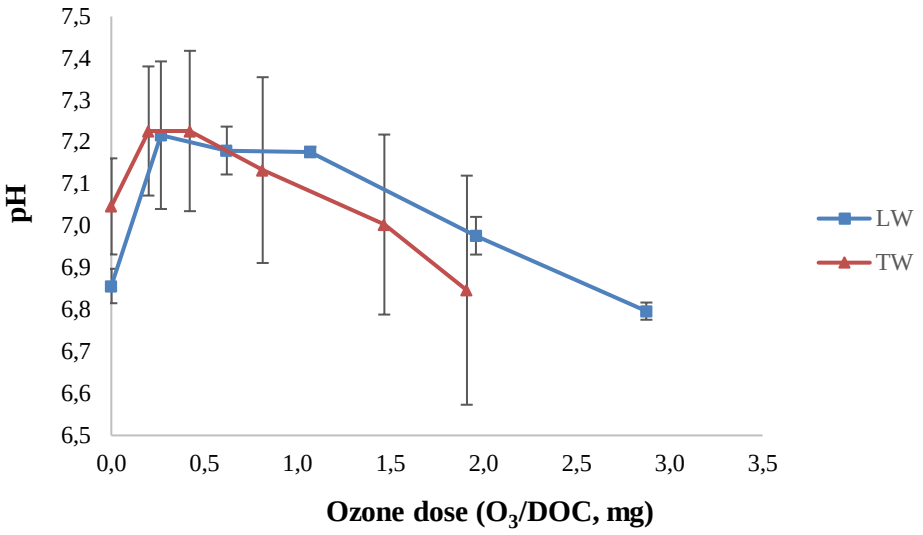

Figure 24. pH values for TW and LW with different ozone doses (SD, n=3).

As ozone dose increased the dissolved ozone in the water increased. Ozone concentration were low with first low doses and started rise when dose was around 0,5-0,7 O3/DOC. Increase in LW was first almost exponential and levelled off later when increase with TW was slower and less steep. With the highest ozone doses, dissolved ozone concentrations were 1,38 mg/l for LW and 0,91 mg/l for TW (Figure 25).

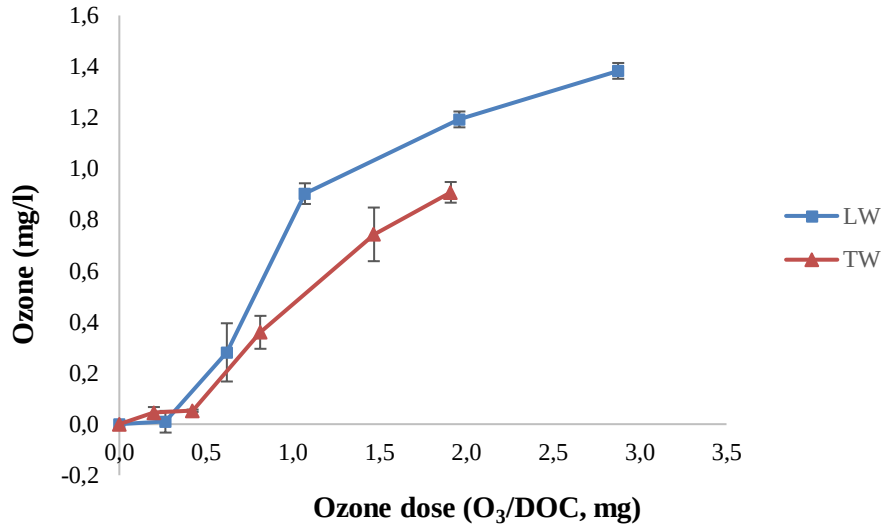

Figure 25. Dissolved ozone (mg/l) per ozone dose in TW and LW (SD, n=3).

4.2.2 Spectroscopy results

Total fluorescence (sum of peak areas) was much higher in TW than in LW. UV254-values being two times, tyrosine and tryptophan three times and fulvic and humic two and half times higher in untreated TW-samples. The most intensive fluorescence in both samples was fulvic, its values being about three times higher than tryptophan- and humic-like fluorescence that came next. Values were roughly equal in both samples when relative to total fluorescence. UV-254 absorbance and tyrosine-like fluorescence had the lowest proportional values out of all measured. (Figure 26.)

Average total fluorescence removal efficiencies were about the same magnitude in TW and LW. 80-90 % fluorescence removal was achieved with the biggest doses, but after the third dose, the efficiency fell considerably. The fourth dose was almost twice as big as the third, but only 8% additional removal was achieved in TW and no effect was observed in LW. Biggest difference between TW and LW was in the biggest doses (1,96 2,88 for LW and 1,47 & 1,91 for TW) which was about 7,8 % for lower and 6,2 % for higher. There seemed to be slightly more deviation in TW, which means that fluorescence decreased more unevenly in those samples. (Table 8.)

Table 8. Total fluorescence and UV-254 removal (%) and their standard deviations for TW and LW per ozone dose.

LW	**Ozone dose (mg O_3/ mg DOC)**				
	0,27	**0,62**	**1,07**	**1,96**	**2,88**
Fluorescence removal (%)	34,0	60,3	78,0	77,7	82,2
Std.	8,0	9,4	8,7	14,3	11,1
TW	**Ozone dose (mg O_3/ mgDOC)**				
	0,20	**0,42**	**0,81**	**1,47**	**1,91**
Fluorescence removal (%)	36,0	60,2	77,3	85,5	88,4
Std.	13,0	15,7	13,7	12,1	10,4

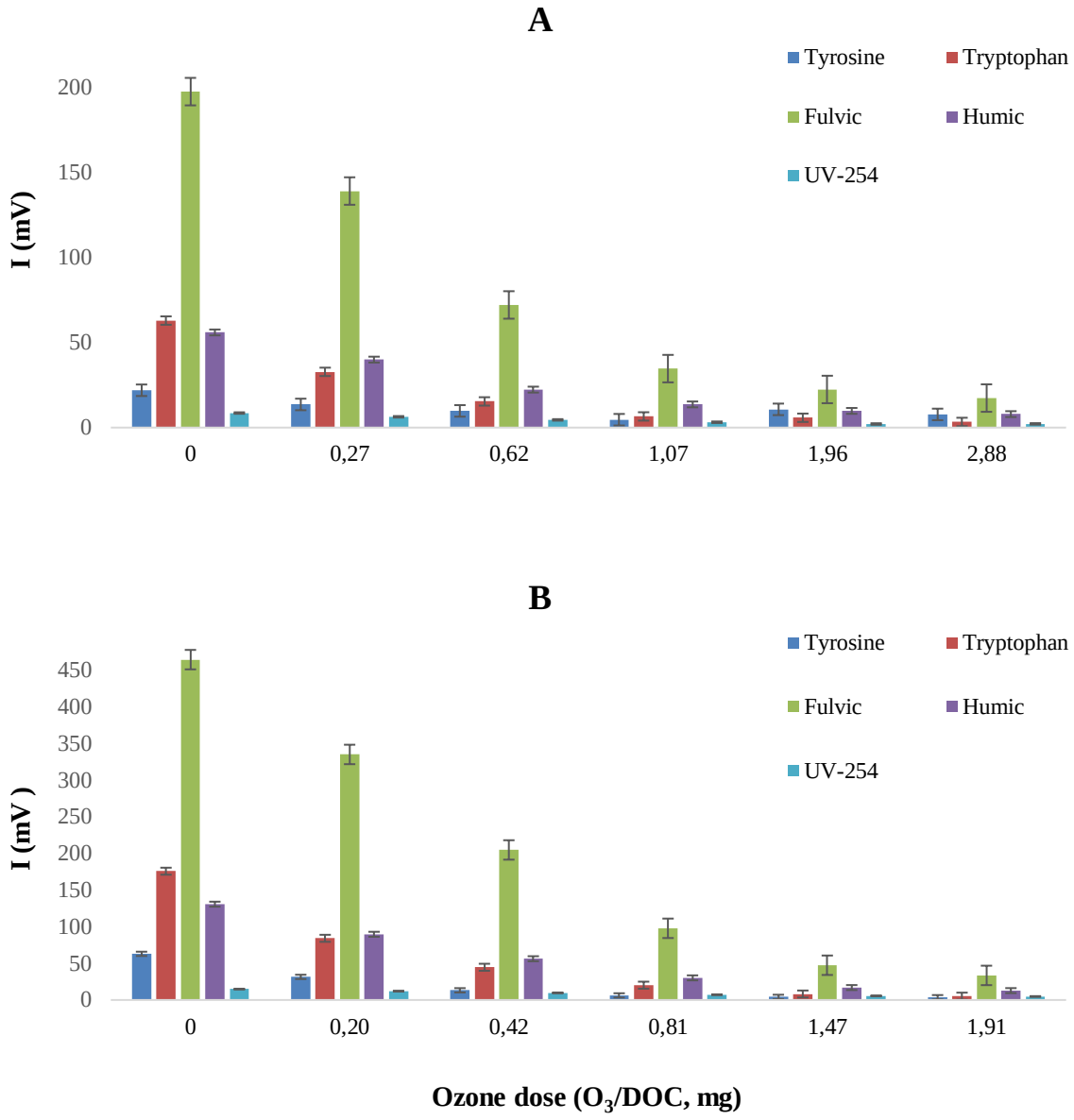

Figure 26. Total fluorescence intensities and UV-254 of LW (A) and TW (B) for initial sample (0) and all ozone doses (SD, n=3).

There wasn't big difference in removal efficiency of total fluorescence between TW and LW, but when each fluorescence was looked separately, some of them responded bit differently to ozone dose depending if water was TW or LW.

Tyrosine-like fluorescence had low intensity in comparison to others, being smaller than UV-254. In LW the removal efficiency was very poor being almost linear where in TW rate was more exponential. With the smallest dose, the tyrosine declined

almost 30 % faster in TW than in LW. The difference between samples decreased slightly as the dose increased but in the end with maximal dose in TW removal efficiency was 94 % and in LW only 67 %. (Figure 27.)

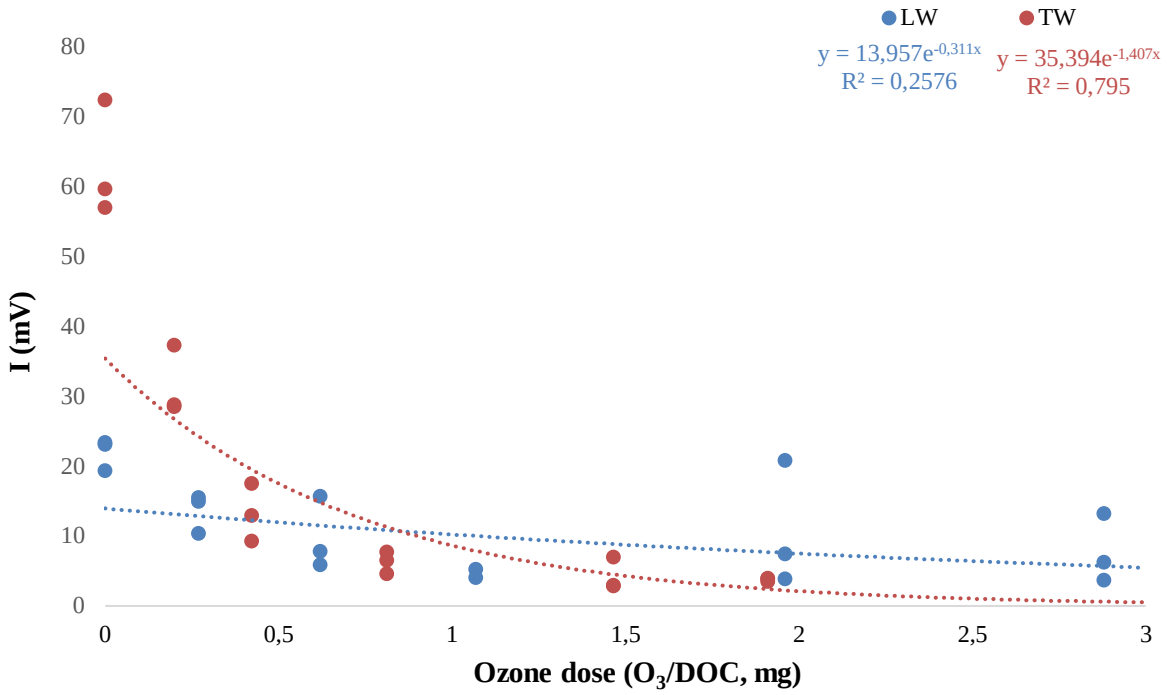

Figure 27. Intensity of tyrosine-like fluorescence for LW (blue) and TW (red) in different ozone doses. Exponential regression curves were fitted to values. Equation and R^2-values are presented in the upper right corner.

Tryptophan-like fluorescence removal efficiency was very similar in both samples. Removal rate was faster in TW and in both samples the fluorescence settled to the same values after the ozone dose of 1 mg O_3 / mg DOC and with maximal dose the fluorescence was almost completely removed (95-97 %). (Figure 28.)

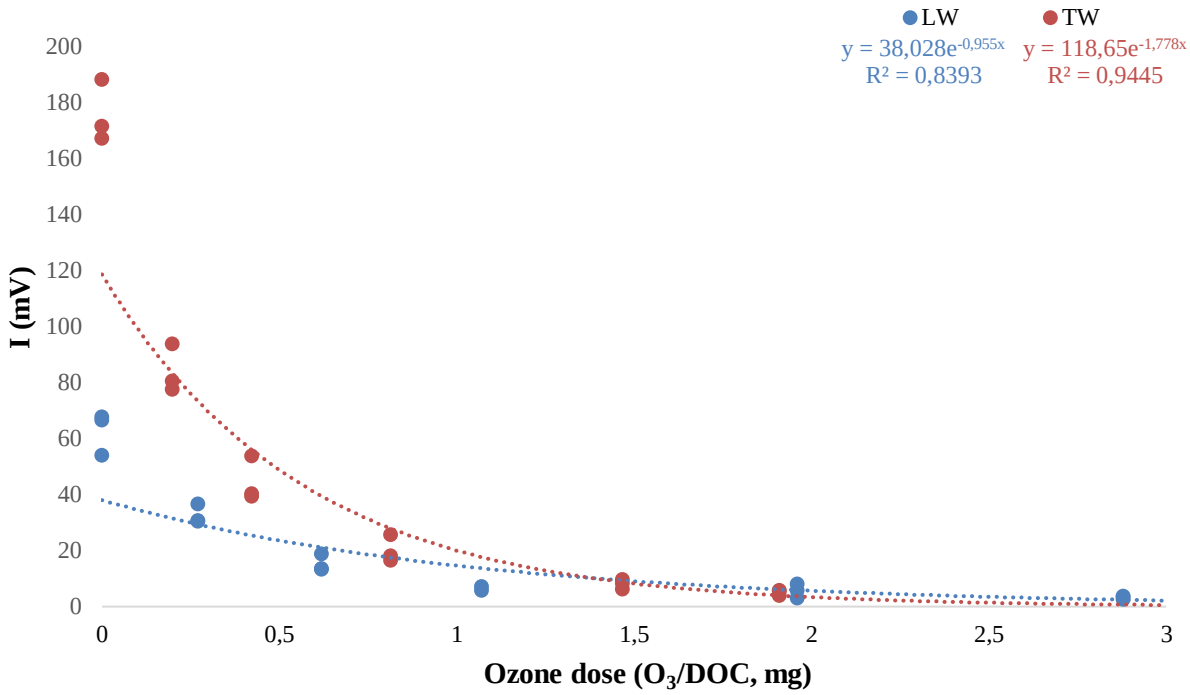

Figure 28. Intensity of tryptophan-like fluorescence for LW (blue) and TW (red) in different ozone doses. Exponential regression curves were fitted to values. Equation and R^2-values are presented in the upper right corner.

Humic- and fulvic-like fluorescences acted very similarly, noticeable difference being the intensity of fulvic-like fluorescence as it was the biggest one in both LW and TW. Intensities reached the same values when ozone dose was around 1,5 O3 mg / DOC mg, removal rate in TW being higher. With maximal dose the removal efficiency was around 86-93 %, fulvic being a bit higher. (Figure 29 & 30)

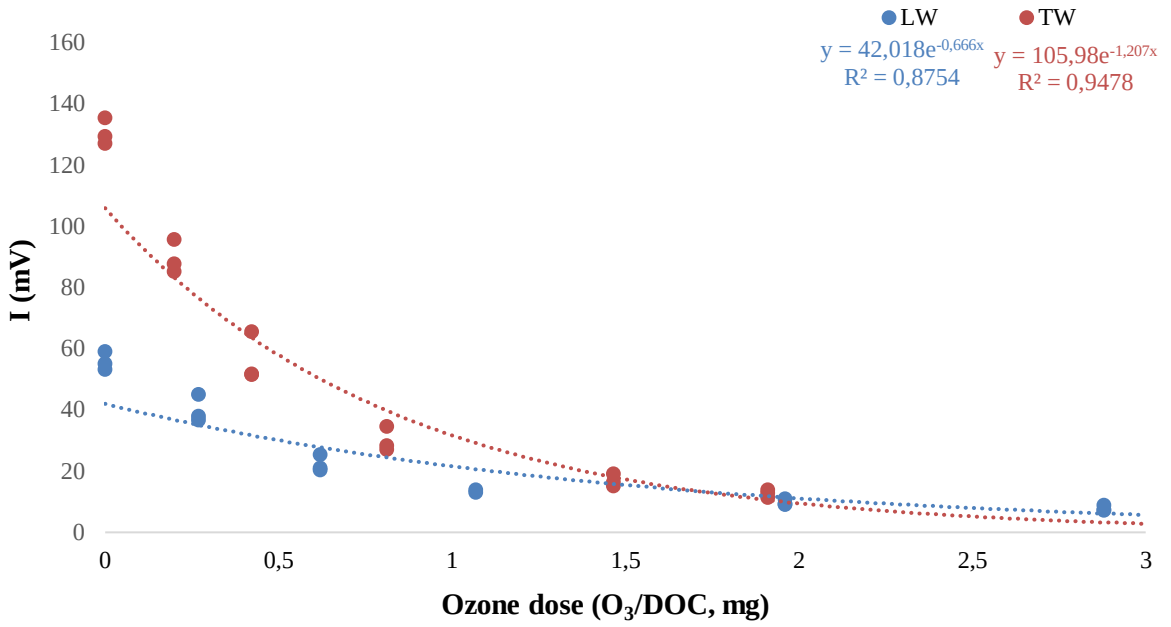

Figure 29. Intensity of humic-like for LW (blue) and TW (red) in different ozone doses. Exponential regression curves were fitted to values. Equation and R^2-values are presented in the upper right corner.

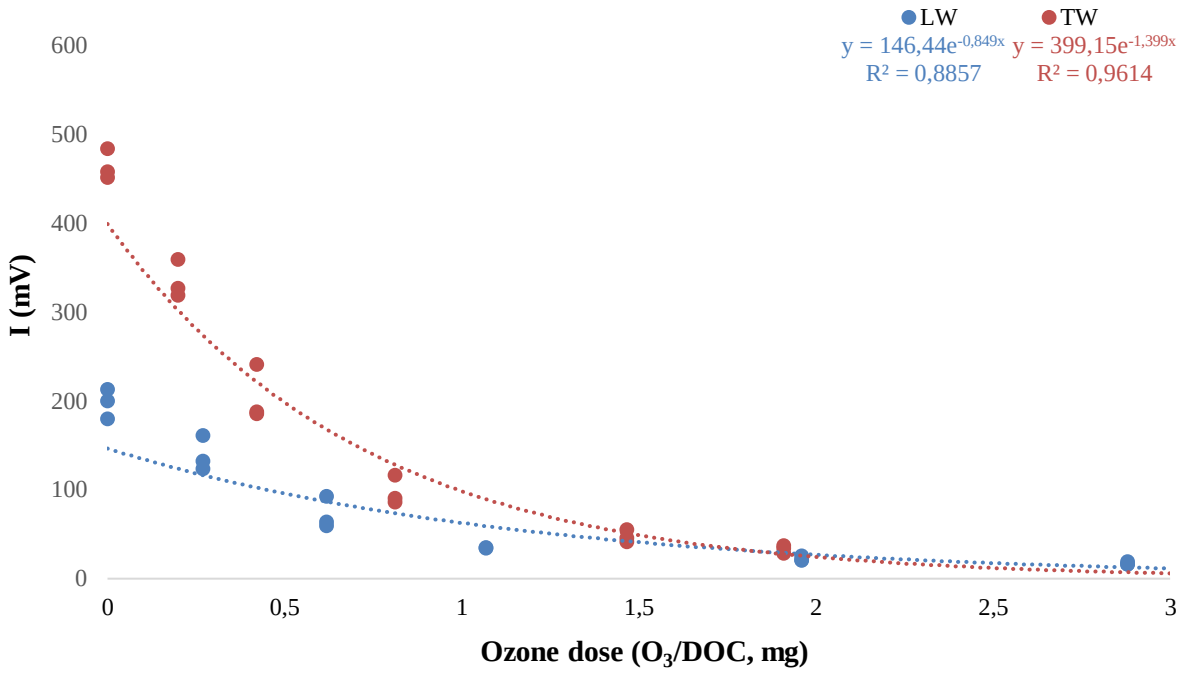

Figure 30. Intensity of fulvic-like fluorescence for LW (blue) and TW (red) in different ozone doses. Exponential regression curves were fitted to values. Equation and R^2-values are presented in the upper right corner.

UV-254 absorbance was set apart from fluorescence not because it had the lowest values, but the removal efficiency was poor and removal rates in TW and LW had less difference between them. Absorbance was initially higher in TW and it did not reach the values of LW. With maximum dose the removal efficiency in TW was 69,2 % and in LW 71,4 %. (Figure 31.)

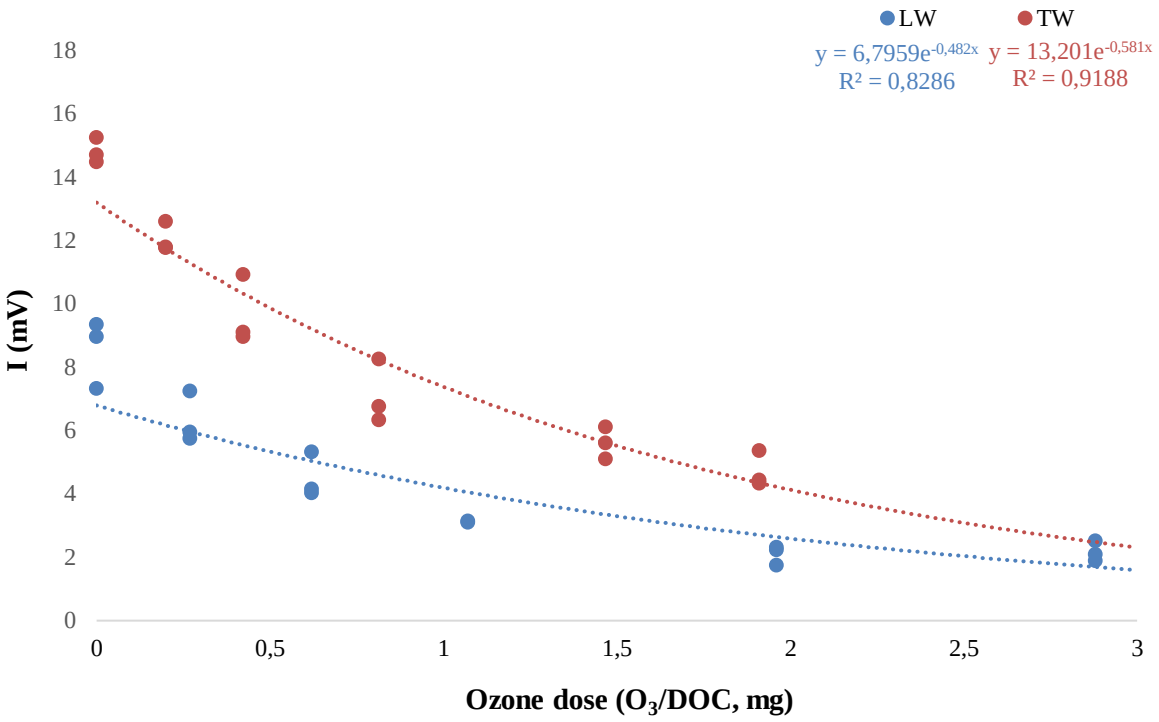

Figure 31. Intensity of UV-254 absorbance for LW (blue) and TW (red) in different ozone doses. Exponential regression curves were fitted to values. Equation and R^2-values are presented in the upper right corner.

4.2.2.1 Apparent molecular weight / size fractions

The fluorescence data was based on six different size fractions for each fluorescence and UV-254 (Size-exclusion for each measured wavelength). In the Figures 32 & 33, the size fractions were compared and their individual removal of intensity seen. The chromatograms that contain unintegrated fluorescence data can be found in appendices section. (Appendix 2 & 3)

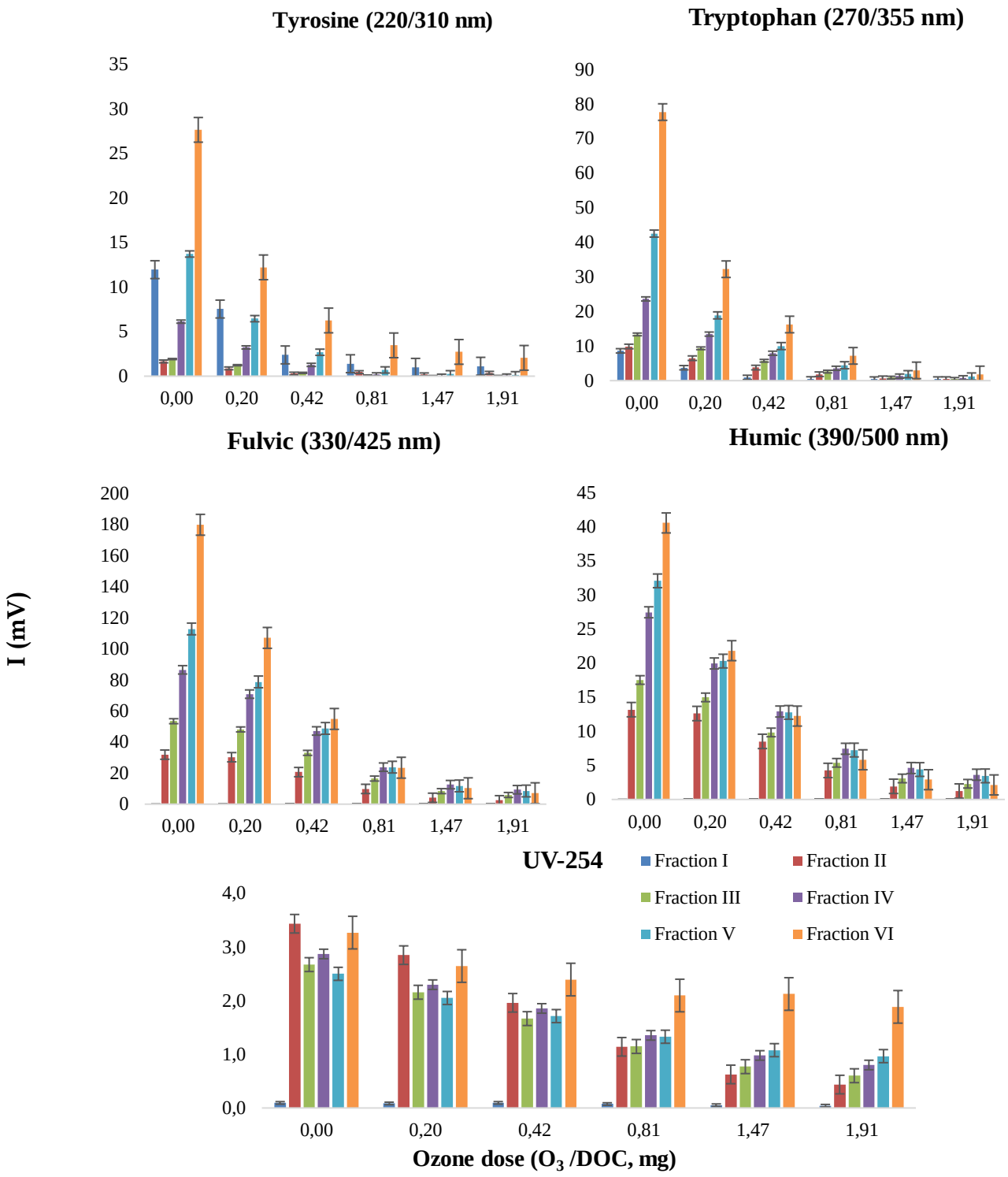

Figure 32. Intensity of each size-fraction (fraction 1 largest and fraction 6 smallest) for every fluorescence and UV-254 in TW. Fraction colours are explained in the bottom right corner. (SD, n=3)

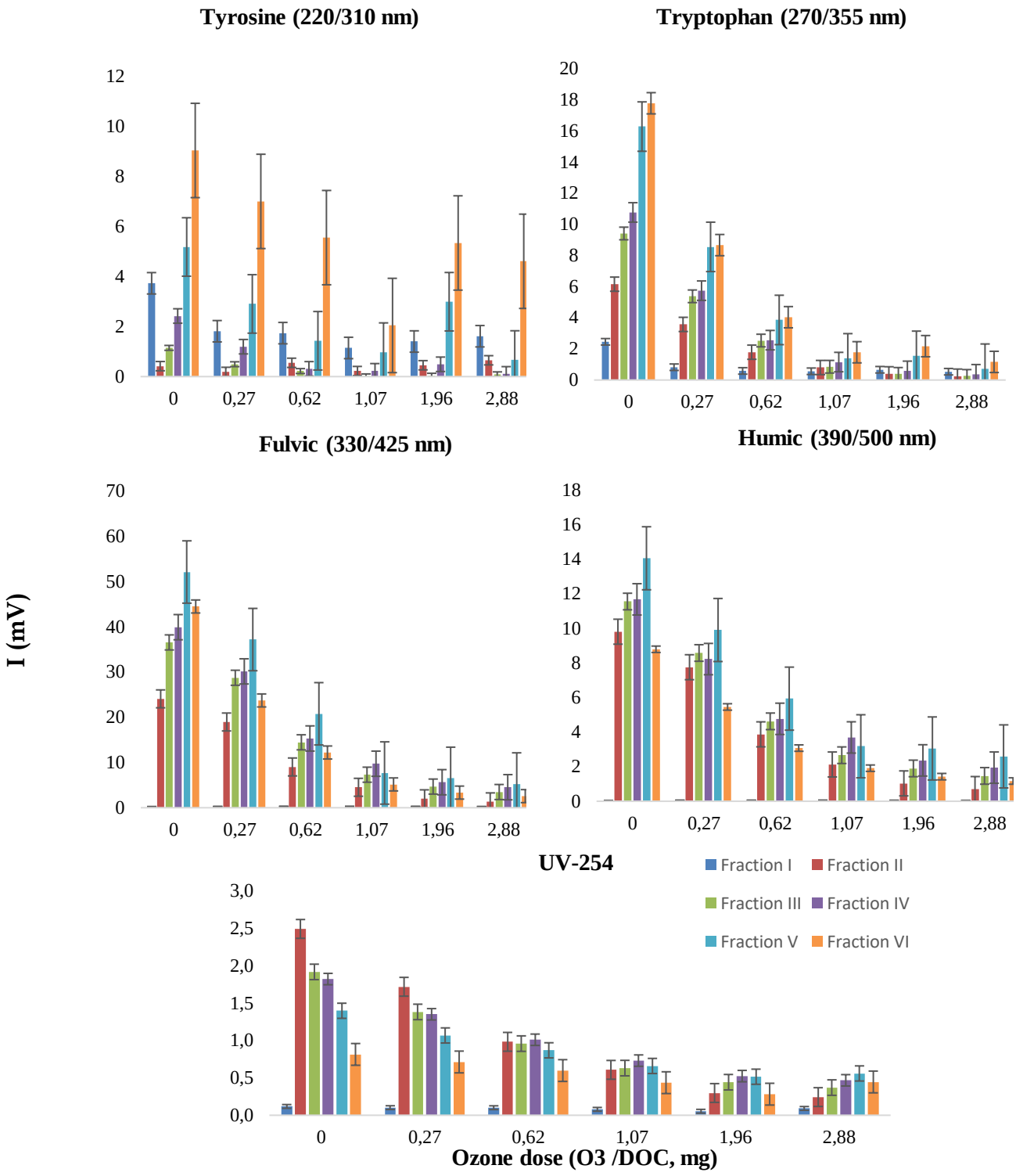

Figure 33. Intensity of each size-fraction (fraction 1 largest and fraction 6 smallest) for every fluorescence and UV-254 in LW. Fraction colours are explained in the bottom right corner. (SD, n=3)

Fraction 1, which represents the largest molecules, has the lowest intensity in all cases except in tyrosine-like fluorescence. Intensities were always higher in TW but the relations seemed to be similar when comparing to LW. The ozone dose had very little effect to this fraction.

The smallest fraction 6 had the highest intensity in TW, especially being much higher in tryptophan- and tyrosine-like fluorescence. In LW this was not the case and fractions 4 & 5 were at the same level or higher than fraction 6. Notably fraction 6 was much smaller in UV-254 where fraction 2 had the highest peak. Ozone dose did not remove intensity of fraction 6 in UV-254 and small relative increase was measured. In TW fraction 2 too had the highest peak in UV-254, but its values were very similar to fraction 6 and ozone had some impact to it. Overall, LW contained more of fractions 2, 3 & 4 in relation to TW, which means molecules were slightly bigger. In general, in tyrosine-like fluorescence smallest and biggest molecules were represented most, in tryptophan-like smallest were dominant, UV-254 had large and medium-sized ones and in fulvic- and humic-like fluorescence medium- and small-molecules seemed to have the highest intensities.

4.2.2.2 DOC-fluorescence/UV-254 relation

An exponential relation between DOC and fluorescences was observed, as fluorescence decreased exponentially when compared to linear decrease of DOC. R^2-values were overall higher in LW (R^2>0,8) than in TW (R^2>0,6) but big difference can be seen in tyrosine: in LW R^2-value being only 0,234 and in TW 0,522. Fulvic-like fluorescence had the highest R^2-values in both cases. (Fig. 34) Linear regression curves were fitted to data where fluorescence values were logarithmic and significance was found from LW in tryptophan- (df=1, F=11,336, p=0,004), fulvic- (df=1, F=16,854, p=0,001) and humic-fluorescence and in UV-254 (df=1, F=15,988, p=0,001). In tyrosine the regression was not significant (df=1, F=3,761, p=0,072). In TW significant regression was found in all fluorescences and in UV-254: tryptophan (df=1, F=4,672, p=0,046), tyrosine (df=1, F=7,496, p=0,015), fulvic (df=1, F=8,736, p=0,009), humic (df=1, F=8,035, p=0,012) and UV-254 (df=1, F=10,536, p=0,005).

The R^2-values would be much higher (close to 0,95) for TW if the initial (highest fluorescence/absorbance) DOC-value is discarded. There is most likely an error in measurements as in initial sample DOC-value is 9,44 and after first ozonation its value has increased to 9,78. After that values decrease steadily.

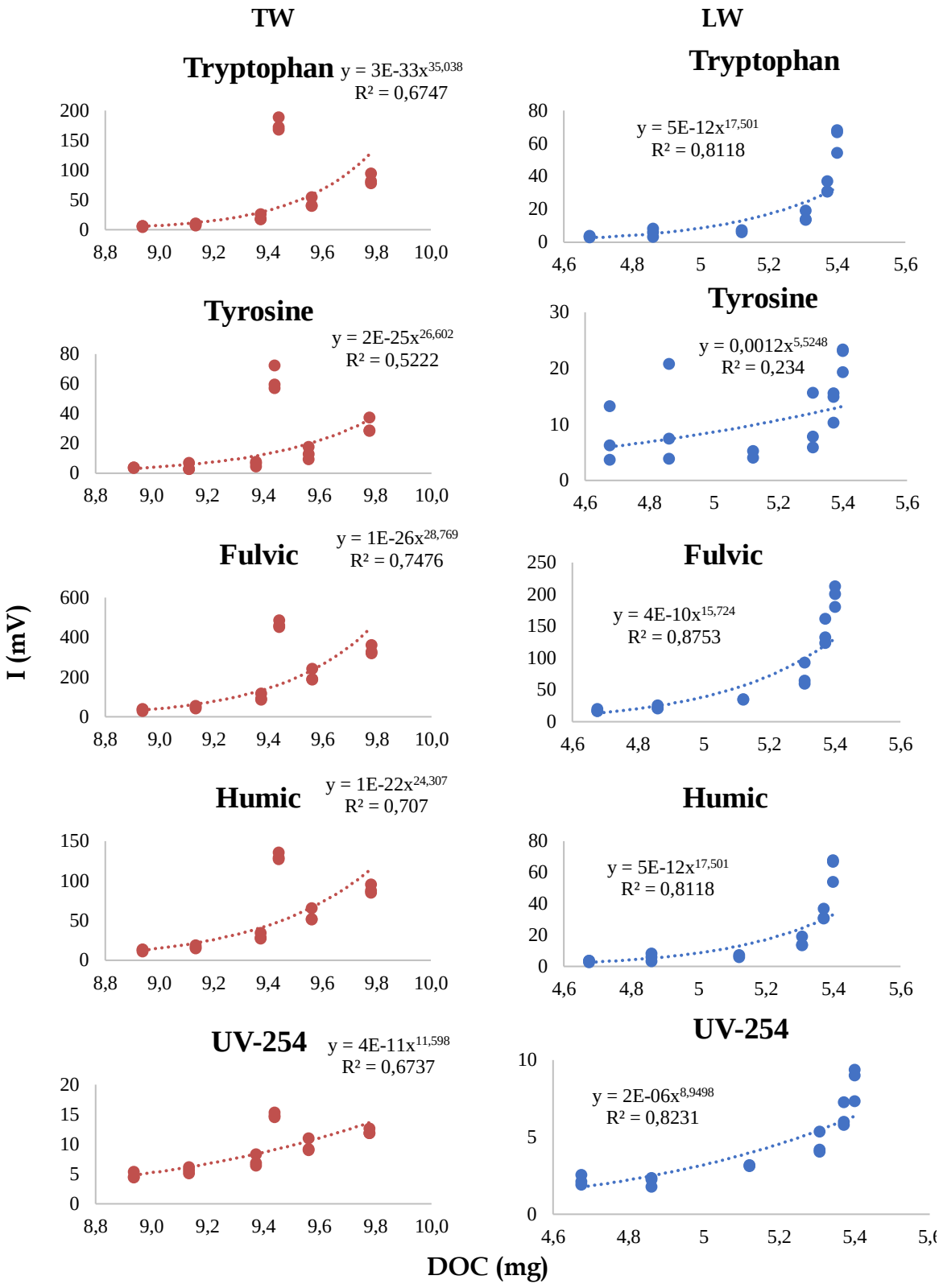

Figure 34. Relation between DOC and fluorescence and UV-absorbance. Right column's figures are for TW and left LW. Curve's equation and R^2-values are reported in the figures.

5 DISCUSSION

Decomposition and dose test results are discussed simultaneously, but the section is divided to water quality parameters, dissolved ozone concentrations and half-life of ozone and DOC-values. The organic matter components originate from three sources: the feed; the influent tap water (Lake Peuranka); and processes related to the fish and the water treatment system.

The purpose of the batch experiment was to characterise the water matrix, define the ozone demand, determine the optimal ozone dosage, which ensures improved water quality and its lifetime, and to test ozonation capacity by indicating the critical range in which ozonation can occur safely in such systems. Several ozone dosage amounts, ranging from 0 to 2,88 mg O_3/mg DOC for LW and 1,91 O_3/mg DOC for TW.

Current RAS monitoring methods typically include the analysis of bulk organic matter in the water, including particulate matter, and inorganic nitrogen species such as ammonia, nitrate and nitrite. Additionally, gross indicators such as dissolved organic carbon (DOC) chemical oxygen demand (COD) and biological oxygen demand (BOD) can be applied.

The organic matter within RASs is derived from a range of sources, each varying relative importance with time. The bulk organic matter measurements alone (e.g. DOC or absorbance at 254 nm) may not provide an adequate information of the concentration and size distribution of the extent to which organic matter character may be fluctuating and potentially influencing RAS performance.

5.1 Water quality parameters

In both decomposition and dose tests it was evident that DOC & TN values were much higher in TW than in LW. TW contained about two times the DOC and many hundred times the TN when compared to LW. This was expected as water in RAS is many times more concentrated with solids and nitrogen compounds than any

natural water. Especially accumulation of nitrogen compounds can be seen in high TN-value. Initial values were a bit lower in dose tests than in decomposition tests, even though sampling and water remained the same in both experiments and had few weeks. The water quality can naturally fluctuate in some degree as seasonal currents in lake can mix up the water and runoff, especially during spring when snow melts, from the land that contains agriculture and vegetation carry dissolved and particle matter to watercourses, which then effects the water quality that goes to the RAS. The water that came to the system was taken from two separate depths from lake Peurunka, which can reduce that water quality fluctuation to some degree, if only another input is affected. Changes in feeding can too have effect on water quality, though during this test feed amounts stayed relatively same. The DOC-N analyser has wide error range which can be seen in Figure 15 as it registered 0,22 mg/l of DOC in ultrapure MilliQ-water that should not have any measurable amount dissolved carbon in it.

Ozonation seemed to have a small decreasing effect on DOC in both TW and LW. This is more noticeable with bigger ozone amounts, like in Fig. 15, but slightly decreasing trend can be observed too in Fig 22. Values seemed to decrease similarly in TW and LW. This decreasing effect is probably due to the mineralization of organic compounds to CO_2 (Rice et al. 1981).To effectively remove DOC by this method, very intensive ozonation is required and it would most certainly be unrealistic and too costly to try achieve in any RAS as removal efficiency was around 10-15 % for 1 litre of water with 0,4 g of ozone. Same kind of removal rate was seen in a study by Park et al. 2011, but with much higher ozone amounts, which would indicate that prominent DOC removal is hard to achieve using only ozone.

Unlike DOC, TN values increased small amount in both TW and LW due to the ozonation. This can be best seen in Fig. 16 with MQ-water and in Fig. 23 with TW. In this thesis synthetic air was used to produce ozone, not pure oxygen. In air there is nitrogen that in ozone generator produces small amount of nitrogen oxides as by-product. Nitrogen oxides are then dissolved to the water with ozone and then increase the TN value (Kogelschatz et al. 1988). As TW already contains large

amounts of TN the increase from ozone should not matter, but in LW the increase is many times the amount of initial TN. This problem though can be easily avoided by replacing air with pure oxygen.

In general, pH of water decreased in all samples. This happens due to the ozone decomposing large organic molecules that form smaller slightly acidic molecules in process. Particularly, when ozone reacts with double bonds and aromatic rings with Criegee-mechanism carboxylic acids are formed, which then again indicates decrease in UV-absorbance (Organic chemistry Portal 2006). These formed smaller organic molecules are much more biodegradable and bacteria and other microbes then can use them in their metabolism (Calvosa et al. 1991). When looking at the Fig. 17, the biggest decrease was seen in MQ-water. That is because it does not contain any buffering compounds and so, nitrogen oxides that were formed as by-product in ozonation, form nitric acid in water and decrease the pH (Kogelschatz et al. 1988). At the same temperature, pH in LW seemed to decrease more than in TW. Lake waters in Finland naturally have low buffering capabilities. Additionally, buffer is often added into a RAS water to adjust pH and alkalinity, the LW so has lower buffering capabilities than TW. When temperature was lowered to 6 °C in LW, pH decreased least of all treatments. Temperature is known to slow down the chemical processes which could explain this. As the LW 6 C had the lowest ozone dose out of all treatments, it would seem like that ozone reacted less in lower temperature which decreased the amount of formed acidic compounds.

In ozone dose tests the pH overall decreased slightly too, but what is in need explanation is the increase of pH with smallest ozone doses. Change is small, there is a lot of deviation in results and only three replicas were made, so the coincidence and measurement error could explain this phenomenon. Because phenomenon seems only to be happening with small ozone doses, there could be reaction products in water that have reacted only slightly with ozone, thus forming slightly alkaline by-products and intermediates, which would normally immediately react with ozone again. Another more likely explanation is that CO_2 is stripped from the water in the beginning of ozonation. As the CO_2 is part of the complex carbonate

equilibrium cycle in the water, this could very well affect the pH of the water (Butler 1982).

5.2 Dissolved ozone concentrations and half-life of ozone

Highest dissolved ozone values were measured from LW in 6 °C after injection of 400 mg of ozone to one litre of water containing 5 mg DOC/l. After that came LW in 15 °C, then MQ and last TW. This seems logical as in lower temperature ozone reacts slower with organic matter in water and is so slower decomposed, which helps to concentrate it (Ershov & Morozov 2018). In lower temperatures gases can be dissolved more easily to water too (Chemistry Libretexts 2020). TW is much more concentrated with compounds (for example nitrates) that immediately consume ozone and it takes more ozone to first oxidize the matrix.

In Fig. 25 the concentration of dissolved ozone is properly seen. With small doses, in both LW and TW, dissolved ozone concentration barely rises, but after that it starts to increase logarithmically and then starts to smoothen. As TW was the more concentrated on different organic compounds, it achieved smaller dissolved ozone concentration. This uneven accumulation of dissolved ozone happens, because first, water contains compounds like nitrites, alkenes and polyaromatic hydrocarbons that react immediately with ozone. This means almost all ozone is immediately consumed from water when it enters it. When those compounds have been oxidized, less reactive compounds are oxidized. In the end, recalcitrant compounds remain and most of the ozone is either dissolved to liquid or escapes to air (Langlais et al. 1991).

To investigate the long-term effects of ozone on water, the samples were ozonated upon ozone depletion, to simulate the residual ozone concentration. It is crucial to determine the lifetime of ozone, since it should not enter the culture tanks or the biofilters. Ozone dose was higher in TW than LW which can be explained with DOC, as dose's unit is announced as O_3, mg / DOC, mg. TW had almost twice as much DOC than LW. If this was not the case, it would have been very strange, as it

was previously stated that TW decomposed ozone faster and more efficiently and ozone dose is calculated from consumed ozone per DOC. This is further supported by the fact that TW in 6 °C had the lowest ozone dose of all. This again being result of decreased temperature and lower ozone reaction rates.

Ozone decomposition partially followed the expected path. The slowest decomposition rate was in MilliQ-water as it did not contain any compounds that could affect the ozone decomposition process. The fastest ozone decomposed in TW as it had most of compounds that can affect the ozone decomposition. It would be logical that LW in colder temperature would have slower ozone decomposition time than LW in warmer temperature, but it actually was opposite. Half-life for LW in 6 °C was 16 minutes and in 15 °C it was 18 minutes. As the dissolved ozone amount was higher in colder temperature, it could be that dissolved ozone is escaping from the water rather than decomposing. Another explanation is that, because in colder temperatures less organic matter was oxidized, it means that more ozone decomposing ozone are abundant in water, which could lead to faster decomposing after the ozonation was ended, even though the temperature is lower. Otherwise half-lives seem to be close to other half-lives in literature, for example Hoigne & Bader (1994) achieved about 18 min half-life for ozone in water from mesotrophic Lake Zurich (DOC 3,7 mg/l) in 23 °C. Half-lives can vary greatly as stated earlier it is heavily depended on water quality.

Summerfelt et al. 2008 reported values of 2-3 mg/l of dissolved ozone to maintain a concentration of 0,2 mg/L residual after 10 minutes of time. When comparing this to results in Figure 19 it can be seen that in Summerfelts study the ozone decomposed much faster as even TW had had about 0,6 mg/l of ozone left after ten minutes in 15 °C with smaller initial ozone concentration. Temperatures were around the same values, but pH was higher in Summerfelts study and it could affect the decomposition. The ozonated water was too moving through pipes where in this thesis ozonated water was tried to be held as stable as possible. It is too possible that heir water contained more molecules that decompose ozone faster, but overall, their DOC values in water were much lower than in TW.

Samples received very close the same amount of injected ozone, which should decrease the probability of error in results. Of course, water quality fluctuated during the experiment and sometimes sampling after ozonation took longer and created possible disturbances to ozone decomposition. Three replicas were always made in default, but more replicas were made if results seemed to vary too much, which should have increased the reliability of results. This was the case with TW in ozone decomposition experiments, as ozone decomposed with very different rates in first three replicas and in the end six replicas were done in total to decrease the deviation and to produce reliable average.

5.3 DOC concentrations

From all fluorescence fulvic-like fluorescence made about 55-57% of it in LW and TW, being clearly the biggest one. Next came tyrosine with about 17-20 %and humic with 15-17 %, smallest being the UV-254 with 6-7 %. This goes well with previous thesis on the same RAS that reported same kind of values (Jäntti 2020). Same thing with intensities of fluorescence that were in the same magnitude with Jäntti's thesis.

It is logical that TW has much higher fluorescence as it was already established that TW contained much more organic material dissolved in it. DOC and intensity of fluorescence has been reported to have relation where increase in DOC would be noticed as an increase in fluorescence (Ignatev & Tuhkanen 2018, Jäntti 2020). Results back this by the fact that TW contained about twice the DOC of LW and had 2-3 times of the fluorescence of LW too. If fluorescence has decreased after ozonation to fraction of its initial value (about 90 % reduction), but DOC had been reduced only by 10-15 %. This indicates that DOC represents only partially total DOM and that DOM is composed mainly from other compounds. Other studies too suggest that DOC and fluorescence would have stronger linear relation and they suggest that fluorescence could be viable way to monitor DOC continuously (Ignatev & Tuhkanen 2018, Lee et al. 2015). This might be true for untreated natural waters, and waters that do not experience oxidation-like treatment. If the treatment is continuous then new relation could be determined and used, but this is something

yet to be investigated more thoroughly, because relation seems, at least in TW, to be more logarithmic-like rather than linear.

Reasons for this might be that in TW there are a lot of nitrogen containing organic protein like material that increase the amount of fluorescence and are oxidized very easily. This is then seen as a large reduction in fluorescence. Ozonation do not necessarily remove organic matter from water but cuts down bigger molecules to smaller ones and converts them to more biodegradable form excluding the small amount of mineralization to CO_2 (Volk et al. 1993). Though no direct accumulation of smaller molecules were observed from the size fraction results and it seemed that all fractions seemed to decrease at relatively same rate, exception being UV-254 values in TW. Linear DOC-fluorescence relation could be more correct when it is used to monitor untreated waters or waters that come from the systems where water quality stays relatively constant.

In a previous study on fluorescence, a way to control and monitor the ozone dosing to the different kind of RAS and results would indicate it to be very suitable. Indeed, this thesis' results would indicate the same as even the smallest doses effect could be seen in fluorescence change. When comparing results, in both the fluorescence seems to decrease with same kind of slope as ozone dose increases. (Spiliotopoulou et al. 2017 & 2018)

All size fractions overall decreased rather steadily and at same rate. The smallest fraction 6 had much smoother decrease in TW than LW which would indicate that it contained/consisted of mostly easily oxidized nitrogen compounds in TW. The fraction 1 was small in both TW and LW which would indicate that molecules of this size class are rather rare and protein-like as tyrosine- and tryptophan-like fluorescence had the highest intensity of them. It also seemed that molecule size in LW was bigger than in TW. This would partly explain the uneven decrease of fraction 6 in LW as bigger molecules are being oxidized and broken down to smaller ones that then belong to the next smaller size fraction. This could be seen in LW at humic- & fulvic-like fluorescence and in UV-254 absorption. There fraction 4 that is the medium size tends to stay at same point when relation to others, but decrease

can be seen in bigger and smaller fractions, bigger usually decreasing a bit faster. In TW, biofilter and microbes may have some effect to the fractions as they can use DOM in their metabolism.

When looking at the overall fluorescence removal efficiency, there seems to be a clear point, which after the efficiency decreases greatly. From the Table 8. it could said that the most efficient ozone doses to would be for LW 0, 81 O_3, mg / DOC, mg and for TW 1,07 O_3, mg / DOC, mg removal being 76-79 %, as after that the ozone dose is almost doubled but additional removal is below 10 %. When looking to Fig. 27-31, absolutely the best dose removal vice would be around 1,5 O_3, mg / DOC, mg for both TW and LW as after that in both cases the increase in dose would not improve water quality much. UV-254 removal is the slowest and hardest to achieve and in tyrosine-like fluorescence in TW seems to have edge in removal efficiency. LW might contain larger amounts of smaller molecules that are already oxidized or hard to oxidize which would explain the slow removal rate.

6 CONCLUSIONS

Organic matter, assessed as DOC and fluorescence, decreased by 17 % and 90 % respectively.

In conclusion, HPSEC and fluorescence indeed seem to be viable ways to monitor the effect of ozone to water quality due to its high sensitivity to changes that occur in water induced by the oxidation-reactions. Fluorescence as a for DOC would seemingly work for waters that haven not been treated with ozone as ozone treatment changed the relation more to a slope, but this needs more research in future to be confirmed.

Suggestion for optimal ozone dose for the system would be 0, 81 O_3, mg / DOC, mg for LW and for TW 1,07 O_3, mg / DOC, mg as it removed most of the fluorescence rather efficiently. Removal rates with thoses doses were for LW fluorescence 78 %, UV-254 52 % and DOC 5 % and for TW fluorescence 77 %, UV-254 44% and DOC 3%. Dose that would fit for each water is around 1,5 O_3, mg / DOC, mg removing most of the fluorescence. If maximum cost effectiveness is sought, ozonating TW rather than LW is more efficient.

Ozone is the easiest to concentrate to cold LW and maximum achieved dissolved ozone concentration was 2,48 mg/l its half-life being 16 min. At warmer environment concentration was 1,99 mg/l (half-life 18 min) and for TW 1,49 mg/l (half-life 12 min).

Ozonation seemed to slightly decrease the DOC concentration and pH. Increase in TN is explained as by-product from usage of air rather than oxygen in ozone generation. The increase in pH with small ozone doses is yet to be explained and would need more research. Overall, these changes should not have any undesired effect on RAS functioning.

As these results were obtained in laboratory environment, they need to be tested and confirmed in a full-scale RAS. For example, water turbidity could prove to be

problem for some kind of fluorescence probe-solution that monitors water quality in the system.

Overall, this study confirms that ozone can improve RAS water quality measured as the removal of fluorescence, absorbance and DOC. It provides a better understanding of the ozone decay kinetics and mechanisms that can be used to define further safe/optimal ozone treatment dose margins. The size exclusion chromatography combined to UV and florescence detection fluorescence could be used as a monitoring tool to control ozone since it is most sensitive method to characterise the concentration and characteristics of organic matter in water recycle. This study might be used as a tool to design ozone systems for full-scale RAS by analysing water sample from the specific RAS in the laboratory. Bench-scale experiments can predict the effect of continuous ozonation in pilot-scale RAS.

Further research is needed to find the correlation of molecular size and fluorescence to the other parameters and occurrence of single harmful compounds/ micro-organisms in the make-up and tank water.

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APPENDIX 1. Ozone meter and generator calibration table

Generator Mode	Replica	t (min)	generator warming (min)	V($Na_2S_2O_3$) ml	n($Na_2S_2O_3$) mmol	n (O_3) mmol	m (O_3) mg	O_3 mg/l	O_3 average mg/l	O_3 std.	(mA)	current std.
7	1	1	40,0	26,77	0,27	0,13	6,42	6,42	6,35	0,085	0,57	0,0073
	2	1		25,95	0,26	0,13	6,23	6,23				
	3	1		26,61	0,27	0,13	6,39	6,39				
1	1	1	5,3	2,03	0,02	0,01	0,49	0,49	0,54	0,078	0,02	0,00
	2	1		2,70	0,03	0,01	0,65	0,65				
	3	1		1,99	0,02	0,01	0,48	0,48				
6	1	1	27,3	22,08	0,22	0,11	5,30	5,30	5,33	0,028	0,50	0,0025
	2	1		22,36	0,22	0,11	5,37	5,37				
	3	1		22,19	0,22	0,11	5,33	5,33				
2	1	1	28,2	3,58	0,04	0,02	0,86	0,86	0,85	0,021	0,06	0,0025
	2	1		3,65	0,04	0,02	0,88	0,88				
	3	1		3,44	0,03	0,02	0,83	0,83				
4	1	1	17,5	8,95	0,09	0,04	2,15	2,15	2,14	0,019	0,17	0,0024
	2	1		8,82	0,09	0,04	2,12	2,12				
	3	1		9,01	0,09	0,05	2,16	2,16				

APPENDIX 2. HPSEC Chromatograms for LW

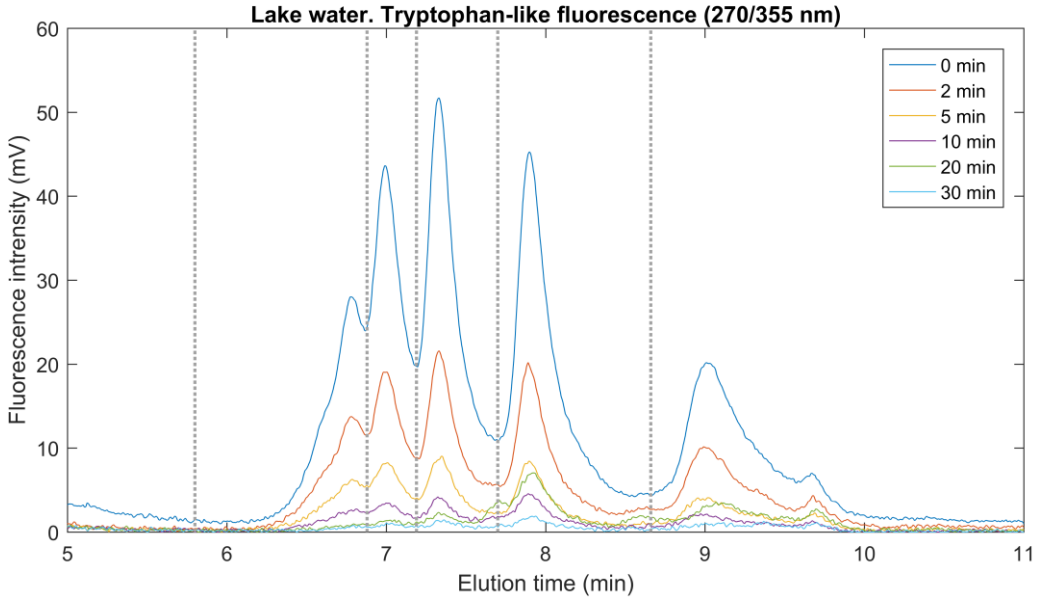

Figure 1. Tryptophan-like fluorescence chromatogram for LW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

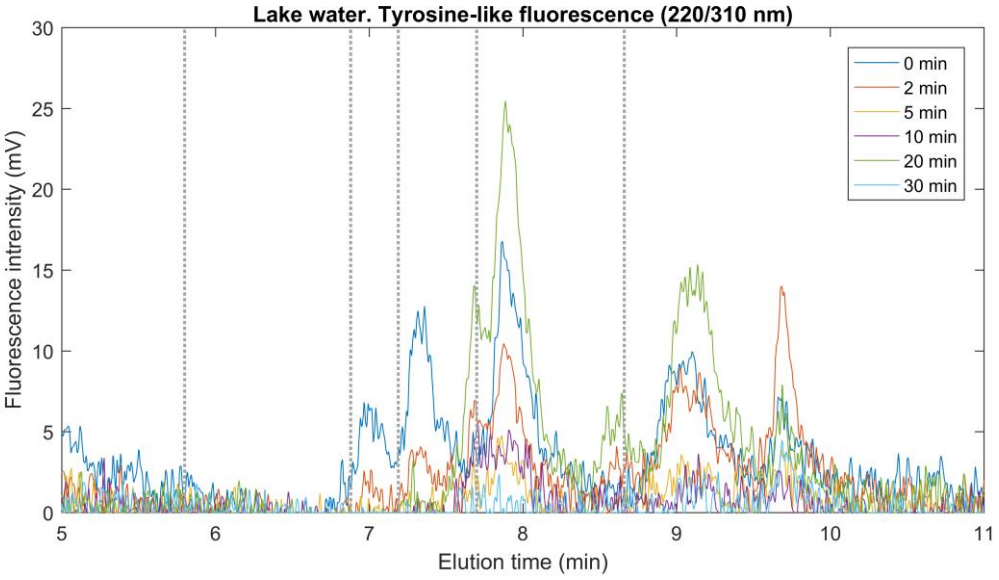

Figure 2. Tyrosine-like fluorescence chromatogram for LW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

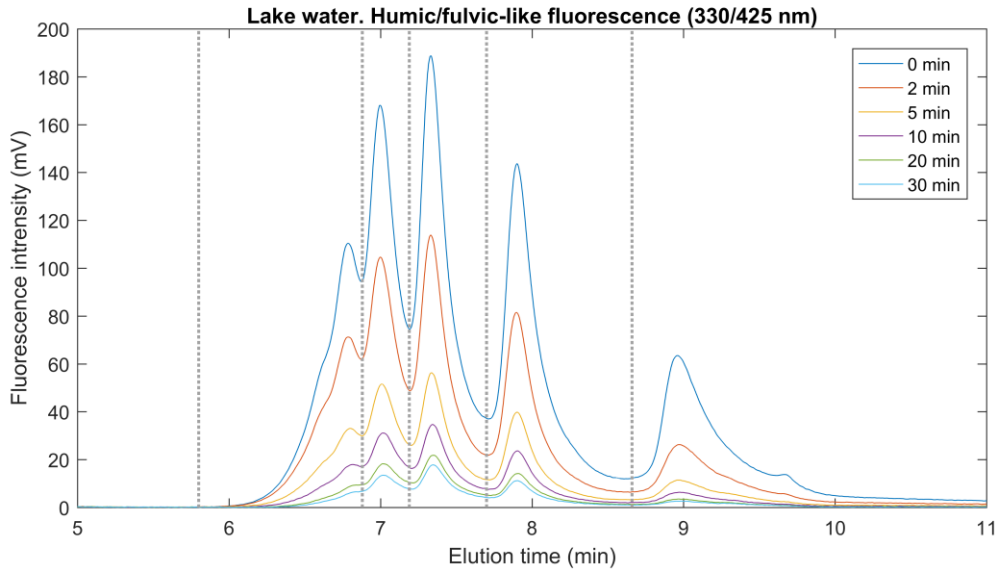

Figure 3. Fulvic-like fluorescence chromatogram for LW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

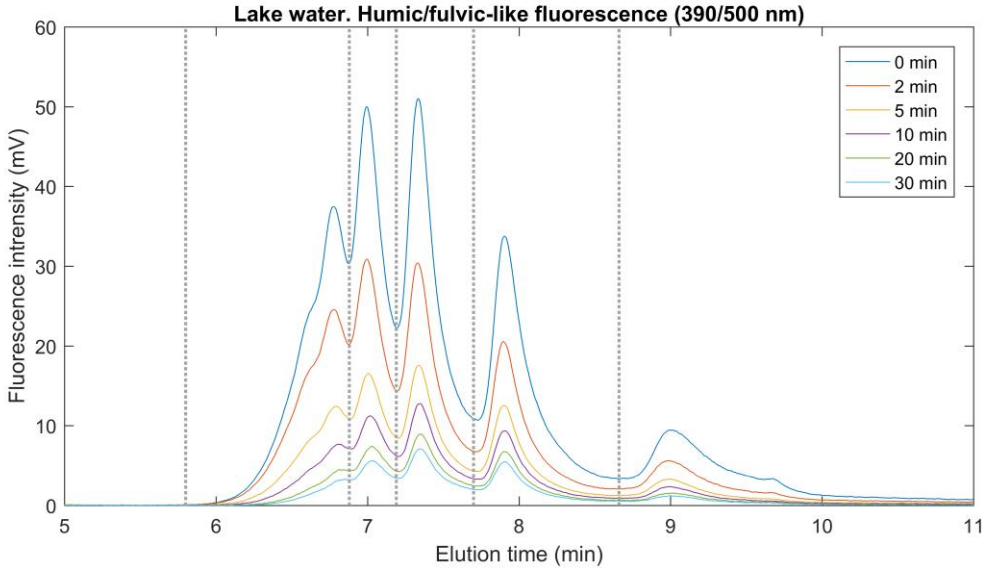

Figure 4. Humic-like fluorescence chromatogram for LW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

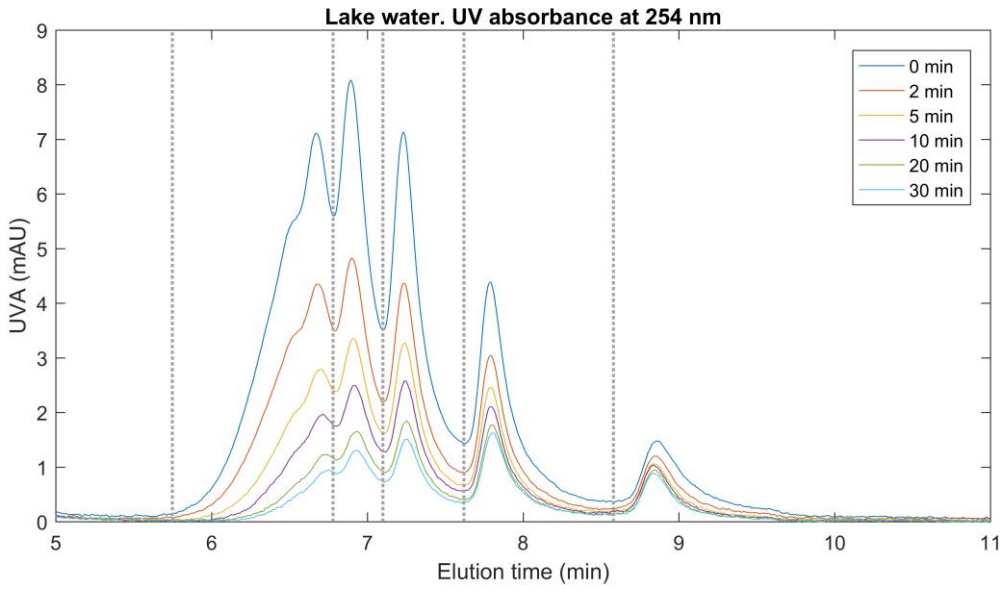

Figure 5. UV254-absorbance chromatogram for LW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

APPENDIX 3. HPSEC Chromatograms for TW

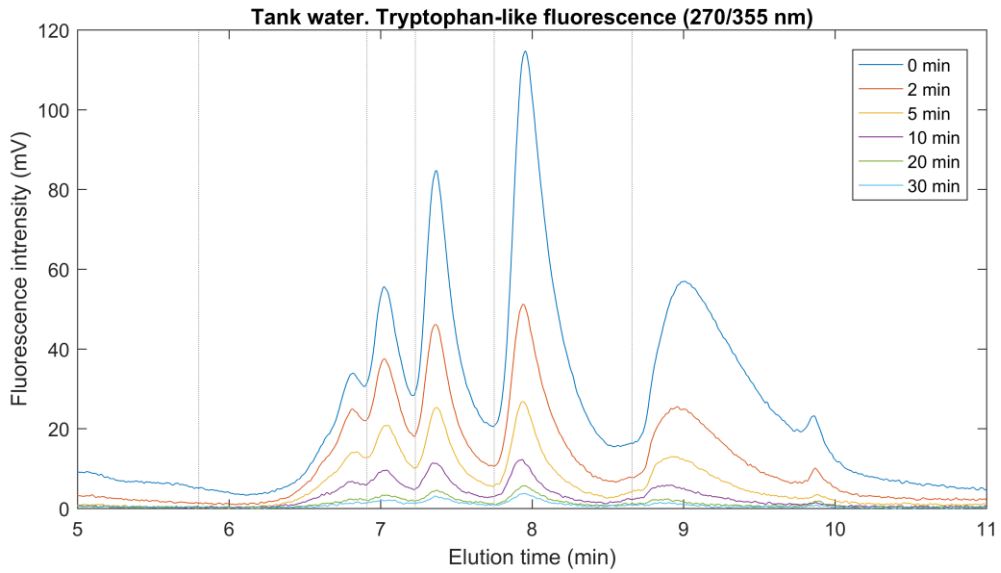

Figure 1. Tryptophan-like fluorescence chromatogram for TW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

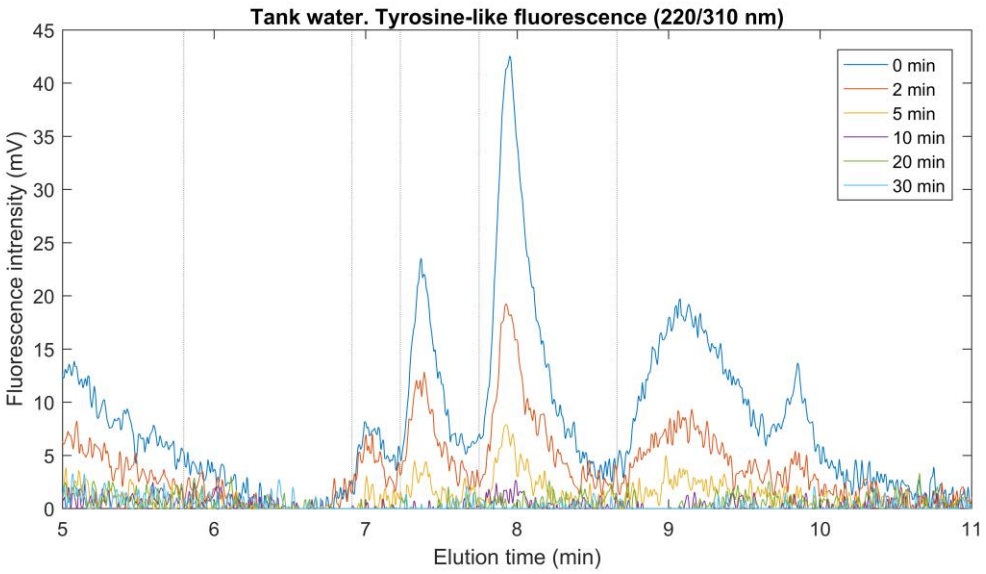

Figure 2. Tyrosine-like fluorescence chromatogram for TW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

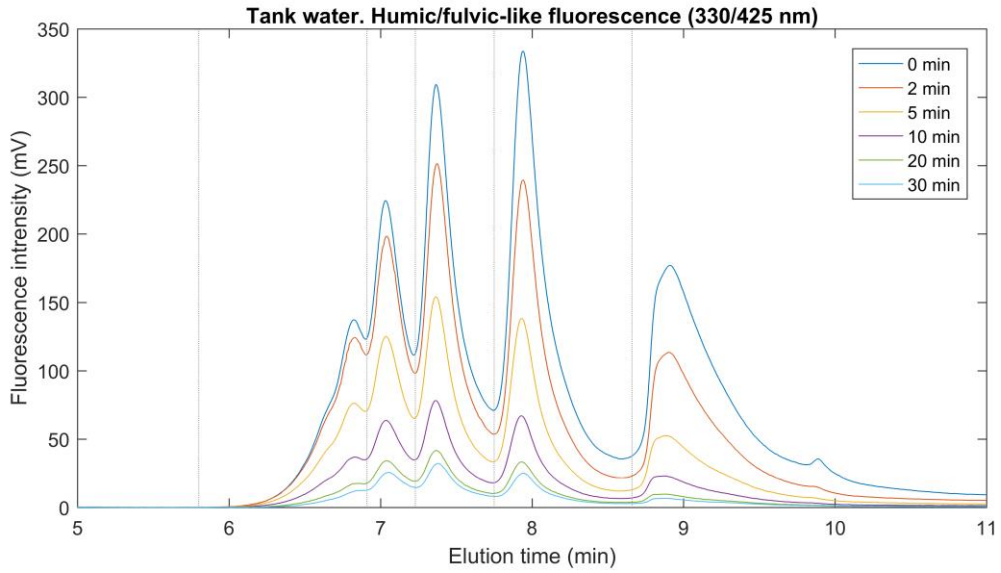

Figure 3. Fulvic-like fluorescence chromatogram for TW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

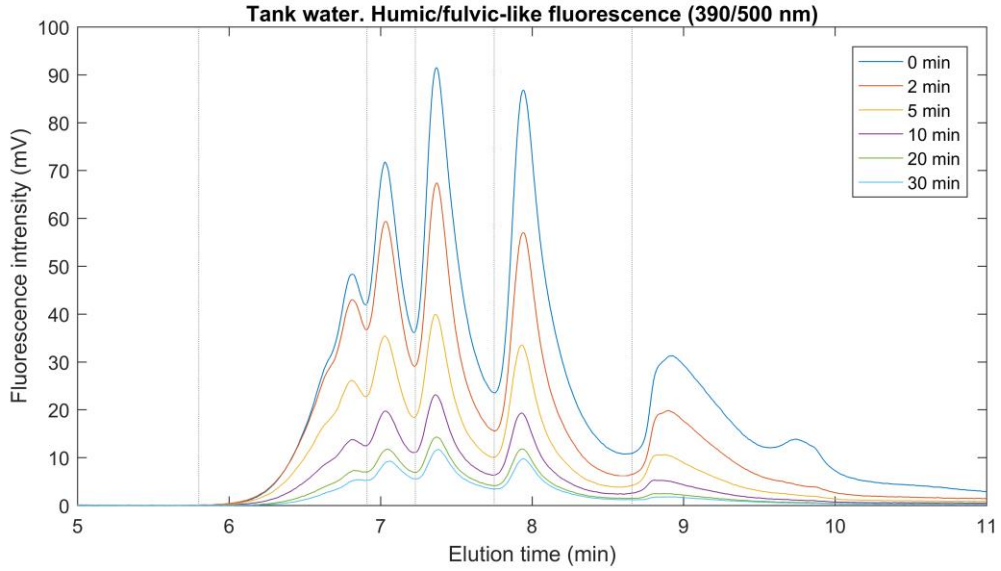

Figure 4. Humic-like fluorescence chromatogram for TW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.

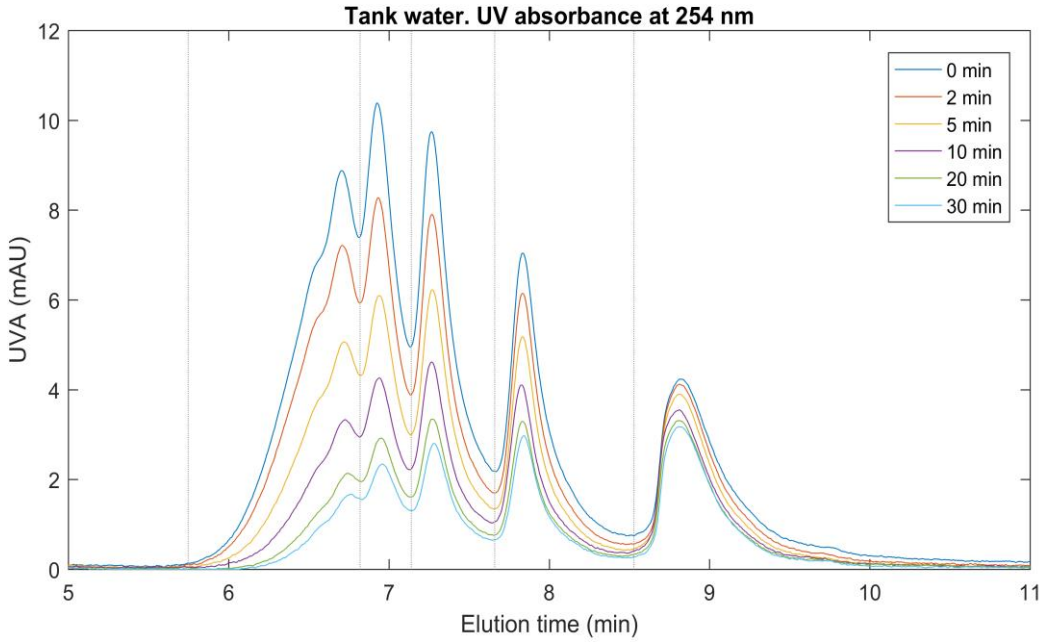

Figure 5UV254-absorbance chromatogram for TW. Vertical lines mark the size fraction areas that were integrated. Ozonation times are marked in the right corner for every curve.