Master's thesis

Organic matter characterization of circular water in recirculating aquaculture system

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TIIVISTELMÄ

Kiinnostus kiertovesilaitoksiin (RAS) on kasvamassa, koska niissä kalaa voidaan viljellä ekologisesti optimaalisissa olosuhteissa ympäri vuoden. Ratkaistavana on kuitenkin joitain teknisiä ongelmia, ennen kuin viljely on kannattavaa Suomessa. Ongelmat liittyvät vedenlaadun ylläpitämiseen hyvänä, sillä liuenneet orgaaniset aineet (DOM) kertyvät kiertoveteen heikentäen veden laatua. Tämä tutkimus painottui DOM:n karakterisointiin korkean suorituskyvyn nestekromatografilla (HPLC-SEC) kokoekskluusiota, sekä samanaikaisesti UV254 -absorbanssi ja fluoresenssi detektiota käyttäen. Tutkimusympäristönä toimi Luken Laukaalla sijaitseva kiertovesilaitos, josta kolmen RAS-yksikön viikoittaisista vesinäytteitä analysoitiin kyseisellä menetelmällä orgaanisen aineen kertymistä. RAS-yksiköt 10, 8 ja 9 toimivat 250, 500 ja 750 L kg rehua^{-1} d^{-1} korvausveden määrillä kokeen kestäessä 105 päivää. Tutkimuksen tavoitteena oli tutkia siikojen kasvua, DOM -komponenttien kertymistä ja veden laatua näissä erillisissä yksiköissä. Tulokset osoittivat, ettei siikojen kasvussa ollut merkittävää eroa yksiköiden välillä. Typpiyhdisteiden: NH_4-N^+, NO_2-N ja NO_3-N, sekä liuenneen orgaanisen hiilen (DOC) ja kokonaistypen (TN) konsentraatiot ja kaikkien tutkittujen DOM-komponenttien signaalit olivat kokeen lopussa suurimmat 10-yksikössä, jossa veden vaihtuvuus oli pienin. 8 ja 9 -yksiköiden välillä erot olivat huomattavan pieniä, joissain tapauksissa tilastollisesti merkitseviä. Yksiköiden vesinäytteiden fluoresoivista yhdisteistä suurin osa oli fulvohappomaisia, joka oli peräisin korvausvedestä. Tulokset viittaavat siihen, että korvausveden suhteella 500 - 750 L kg rehua^{-1} d^{-1} toimivien yksiköiden puhdistussysteemi pystyi tehokkaasti puhdistamaan kiertovettä. Yksikössä 10, johon korvausvettä lisättiin 250 L kg rehua^{-1} d^{-1}, DOM- ja typpiyhdisteitä kertyi kiertoveteen, mutta kokeen puolivälissä kyseisen yksikön puhdistusteho parani. DOM-komponenttien kertyminen saattaa lisätä riskiä patogeenien aiheuttamiin infektioihin ja siten lisätä kalakuolemia. On mahdollista, että täysimittaisella kasvatuskaudella intensiivinen kasvatus pienellä korvausveden määrällä heikentää kalojen kasvua ja terveyttä. Mahdollisten makuvirheiden syntyminen intensiivisessä RAS -yksikössä tutkitaan tähän pro gradu -työhön liittyvässä tutkimuksessa.

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ABSTRACT

The recirculating aquaculture system (RAS) is a prominence ecological method to farm fish in optimal circumstances annually. Interest among this aquaculture form has increased recently, however, some technical problems must be solved before RAS could be taken to commercial fish production in Finland. These problems are related to water quality, dissolved organic matter (DOM) is known to accumulate to the system and weaken the water quality by increasing microbial activity and chemical and biological oxygen demand. The focus on this study was on characterizing DOM with size-exclusion high-performance liquid chromatography (HPLC-SEC) with fluorescence and absorbance detection from the weekly samples, of Laukaa's experimental RAS facility operated by Luke. The DOM-components studied were UVA254, tyrosine-, tryptophan-, humic- and fulvic acid-like fluorescence compounds, which were separated further to seven fractions with size exclusion -column. The experiment was ongoing for 105 days. The aim was to study European whitefish (*Coregonus lavaretus*) growth, DOM accumulation and different water quality parameters of the data of the experiment in three different RAS, 8, 9 and 10, where renewal water rates (RWR) were relatively 500, 750 and 250 L kg^{-1} feed . The results showed that there were no significant differences between fish growth studied by feed conversion rate (FCR) nor by specific growth rate (SGR) between the three RAS. Concentration of nitrogen compounds NH_4-N+, NO_2-N and NO_3-N, and dissolved organic carbon (DOC), total nitrogen (TN) and signals of all DOM-components were significantly higher in RAS 10 at the end of the experiment, where the renewal water rate (RWR) was the smallest. DOM-components did not accumulate in RAS 8 and 9, but in RAS 10 accumulation was observed in all DOM-components. Tank water samples fluorescence compounds were formed in average of 55.1 ± 0.6 % of fulvic acid-like, 21.5 ± 0.3 % of tryptophan-like, 16 ± 0.2 % of humic acid-like and 7.4 ± 0.7 % of tyrosine-like compounds. This study suggests that there is no difference in whitefish growth between RWR of 250 - 750 L kg^{-1} feed in 105 days long period. When RWR is as low as 250 L kg^{-1} feed, accumulation of DOM, DOC, TN and nitrogen compounds occurs in RAS. That may increase risk of infection, or cause odor problems in fish meat. During a complete growing season, fish growth may decrease, due the worse water quality with intensive RAS system.

SHORTHANDS

BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
FCR	Feed conversion rate
GSM	Geosmin
HPSEC	High-performance size exclusion chromatography
Luke	Natural Resources Institute Finland
MIB	2-Methyleisoborneol
PARAFAC	Parallel factor analysis
RAS	Recirculating aquaculture system
RWR	Relative water renewal rate
SGR	Specific growth rate
TAN	Total ammonia nitrogen
TN	Total nitrogen
(T)SS	(Total) suspended solids

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1 INTRODUCTION

Finland is facing a tricky situation with domestic fish farming. Even though fish demand is increasing by consumers, in 2017 82 % of the fish was imported from abroad. Fish farming has significantly reduced in Finland at the turn of the millennium (Official Statistics of Finland, 2017). The main reason why domestic fish isn't finding its way on the Finnish food plates is fierce competition with imported fish. Fish quality, freshness and price are the three main factors affecting consumers purchase decision (Pro Kala, 2017). A high price of domestic fish is the cause of reduced fish production, which in turn is the result of environmental law that has become even stricter (Luke, 2019). Instead of traditional flow-through farming, other farming methods have come to prominence, one of which is a recirculating aquaculture system (RAS) (A Guide to Recirculation Aquaculture, 2015). A couple of this type of farms has been founded in Finland in recent years. In the year 2017 Finland produced 600 000 kg of fish in this type of fish farms (Official Statistics of Finland, 2017).

RAS is a modern and ecological way to grow fish in a fully controlled environment, in which water is circulated between the purification system and rearing tanks by water pumps. Fish can be grown faster and in higher densities compared with conventional flow-through systems, and the water consumption can be kept minimal because the same water is recirculated multiple times in the system (Lee et al. 2013). The make-up water accounts commonly 5 - 10 % of the total volume of the water in the system per day (Masser et al. 1999), whilst it can be in the minimum for only between 1 - 2 % (Luke, 2019). Also, the nitrogen and phosphorus load can be significantly reduced in RAS, because the effluent is compacted to a smaller volume than in traditional flow-through farm and it can be treated before discarding to the water system (A Guide to Recirculation Aquaculture, 2015).

RAS farming is based on the biofiltration, in which nitrification bacteria break toxic nitrogen compounds, to less toxic nitrate. Without these essential bacteria,

ammonium and nitrite concentrations would increase via accumulation quickly too high in the system (Timmons et al. 2002). Concentration over threshold value 1 mg L^{-1} of NO_2-N and NH_4-N^+ is harmful to salmonids (Lawson, 1995; Pillay & Kutty, 2005). Water quality is crucial for RAS, if a certain water parameter, for example, ammonium reaches a critical limit that may affect fish growth, or it might even kill all the fish in the unit in small concentrations. That for monitoring RAS water quality is very important all the time (Timmons et al. 2002).

When farming salmonids in RAS, fish growth must be high enough to cut the high electric expenses needed to pump and heat or cool the recirculating water (Timmons et al. 2002). To make fish farming profitable, some technical problems have to be solved as well, including a proper way to process unused fish feed and feces, and ensure that the biofilters operate continually with good performance (Badiola et al. 2012). Studying how to optimize RAS farming is, therefore, an important subject to renew Finnish fish farming by commercializing RAS type of fish farming (Luke, 2019).

A relative water renewal rate (RWR) can be used to tell how big portion of the system total volume is renewed by day in RAS. Furthermore, a make-up water addition is used to report how many liters per kilogram of feed it has been added. A low make-up water addition can directly affect the water quality negatively (Yamin et al. 2017). The higher the RWR is, the cleaner the recirculating water will be, but at the same time electric consumption increases, because higher volumes of water need to be pumped in the system (Timmons et al. 2002). On top of that greater volume of make-up water is needed to heat or cool down and purify. It has been studied, that lower the RWR is the more dissolved organic matter (DOM) accumulates into RAS water (Yamin et al. 2017; Pulkkinen et al. 2018). RAS purification system is capable of purifying DOM on a certain level, but after that DOM can accumulate to the system. DOM is difficult to remove from water and that for it accumulates into a system over time (Yamin et al. 2017). It is important to sort out the right RWR for a certain system to optimize fish farming (A Guide to

Recirculation Aquaculture, 2015).

There is still many technical problems RAS face, so developing new techniques are important to make it more efficient (Velichkova & Sirakov, 2013). DOM poses problems in recirculating water by reducing fish growth and impairing water quality by correlating positively with the concentration of toxic nitrogen compounds - ammonium and nitrite (Baker & Inverarity, 2004), biological oxygen demand (Hambly et al. 2015; Ignatev & Tuhkanen, 2019) and microbial activity (Blankheton et al. 2013). The Accumulation of organic matter in different renewal rates in a recirculating system requires further research. DOM and its component have been widely studied with high-performance liquid chromatography using size-exclusion chromatography (HPSEC) from drinking and wastewater (Hudson et al. 2007; Coble, 2007; Sillanpää et al. 2015; Goffin et al. 2018, Ignatev & Tuhkanen, 2019). The Method characterizes waters organic composition detecting simultaneously fluorescence and UV254-absorption of different organic compounds. It separates different DOM-components by their apparent molecular weight, depending on different components elution time in the column. DOM-components have been separated according to previous studies to tyrosine and tryptophan-like compounds (protein-like compounds) and fulvic acid- and humic acid-like compounds (fulvic acid-like compounds) (Hudson et al. 2007; Hambly et al. 2015; Nimptsch et al. 2015; Yamin et al. 2017; Ignatev & Tuhkanen, 2019).

The focus of this master thesis is to study how three differently managed RAS systems by their RWR effect on the accumulation and character of dissolved organic matter in those systems in Laukaa's experimental RAS facility operated by Luke during the three-month experiment. Fish species used in this study were European whitefish (*Coregonus laveratus*). Water samples were taken from rearing tank water and biofilter water once a week and every third week from makeup water. The characteristic of DOM in Laukaa's RAS was analyzed with HPSEC-fluorescence analysis with UVA254 and with two different fluorescence detection range for protein- and fulvic acid-like compounds. DOM characteristic was used with

measured water parameters to study the relations between three different RWR to water quality, and fish growth.

This study aimed to investigate in which unit the water quality was the best and in which the worse. The growth of the whitefish was compared with each rearing tank during the experiment. The focus of this study, however, was to find out, that do DOM-components accumulate to recirculating water, and if so, in which unit that occurs. The first hypothesis of this study was that RWR correlates with the water quality, so that more make-up water is added, the better the water quality is in RAS. The second hypothesis was that fish grow the most in the unit which has the best water quality. The third hypothesis was that organic matter is accumulating into all three systems. This study provides information about the appropriate RWR for the salmonid growth in the recirculating aquaculture systems of Finland and the characterization of DOM in recirculating water during the shortish farming season.

2 THEORETICAL BACKGROUND

2.1. Recirculating aquaculture system operation

Water must be treated constantly in a recirculating aquaculture system (RAS) to ensure high water quality. Waste products produced by fish and excess feed must be collected from the rearing tanks, otherwise, these solids will settle on the bottom of the tanks and cause problems with water quality. Water must be aerated to provide enough oxygen for the fish and the nitrification bacteria. (A Guide to Recirculation Aquaculture, 2015)

At the bottom of the rearing tanks, there is usually an excess feed and fecal matter collector. At the beginning of the purification system, the tank water is cleaned in mechanical suspended solids removal step, where larger suspended solids are being removed usually by a settling basin via gravitation. Smaller suspended solids, which can't be settled, are removed with a sand, drum or particle filter. Drum filters are quite widely used in RAS in a mechanical post-solid removal. (Timmons et al. 2002)

Drum filters solid removal efficiency increases in relation with an increase of total suspended solids (TTS) concentration in water, because small solids that would otherwise past the drum filter mesh, attaches to the surface of the bigger solid particles that block gaps of the mesh (Summerfelt et al. 2001).

After mechanical filtration, water goes to biological filtration, in which nitrogen compounds are degraded in a nitrification process. In this reaction, ammonium is first degraded to nitrite in the presence of oxygen and it is then being oxidized to nitrate (see Equation 1 & 2.). (Timmons et al. 2002)

$NH_4\text{-}N^+ + 1.5\ O_2 \rightarrow NO_2 + 2H^+ + H_2O$

$NO_2 + 0.5\ O_2 \rightarrow NO_3\text{-}N^-$ (Equation 1. & 2. Timmons et al. 2002)

There are many types of bioreactors, for example, fixed-bed and moving-bed reactors, which consist of small plastic media that provides a substrate for nitrification bacteria (Timmons et al. 2002). Besides, other materials can be used to fill bioreactors, for example, sand or wood chips (Sosa-Hernández et al. 2016).

After biofiltration, water is being aerated and stripped of CO_2 in a trickling filter. Then water pH is adjusted, and external oxygen is be added. Before water is pumped back to rearing tanks, it can be disinfected with UV-light or ozone gas. (Timmons et al. 2002)

Ozonation destroys some of the particulate and dissolved organic matter compounds from RAS water as well (Sharrer & Summerfelt, 2007). Ozone gas is lethal to fish even in small concentrations, so the residual ozone must be eliminated. Usually, before disinfection, some make-up water can be added (Timmons et al. 2002).

2.2. Salmonid farming in RAS and water quality

Traditionally valuable fish in the market, such as the European sturgeon, arctic char (*Salvelinus alpinus*) and pike-perch (*sander lucioperca*) have been farmed in RAS in Finland, but now salmonids are being started to farm also indoors. In Finland rainbow trout (*Oncorhynchus mykiss*), European whitefish (*Coregonus lavaretus*) and arctic char have been raised in RAS so far. (Luke, 2019).

Farming these cold-water fish requires clean water, with a high concentration of dissolved oxygen. Optimal water quality parameters depend on the fish species, but to salmonids, for example, following water quality parameters such as dissolved oxygen, water temperature and pH are very similar. Nitrification bacteria also require certain water quality to operate on the optimal level to remove efficiency ammonium and nitrite and some organic compounds from the recirculating water. Rearing tank water quality is monitored constantly to ensure optimal growing conditions for the fish and to make sure that water quality is not declining and

causing risks for fish health. With an online monitoring system that alarms when a certain threshold value is crossed, many problems can be foreseen, and actions can be taken in an early stage. Such action could be, for example, the emergency oxygenation of rearing tank water. That kind of system is commonly used in RAS. (Timmons et al. 2002)

2.2.1. Fish feed

Fish requires several feeding times a day and feeding can be automatized for example, with a belt feeder. While the fish grow bigger, also the amount of given feed is being increased to supply fish growth, Thus, fed given per fish body weight usually declines during the growing season. The feed is given in different pellet sizes suitable for certain fish stage, also different species require different types of feed (See Table 1 & 2). (A Guide to Recirculation Aquaculture, 2015)

The good quality fish feed provides healthy fish growth, supplying their optimal growth by containing a sufficient amount of necessary nutrients. These ingredients are proteins, fats, carbohydrates, vitamins, and minerals. The protein content of the feed can account for half of the feed mass and it is an important ingredient supplying fish biomass growth. Salmonids and fish farmed high densities require more protein than other fish families and pond farmed fish. Proteins in the feed are usually plant-based. Proteins consist of different amino acids and fish can synthesize ten of them. Lipids are high in energy content supplying fish growth and they also serve as transporters for fat-soluble vitamins. Vitamins supply fish health and vitamins synthesized to feed are usually different types of B-vitamins, and the most important vitamin C. Minerals such as calcium, sodium, potassium, and magnesium are important for the normal body functions of fish. Carbohydrates are included in the feed to reduce its cost and to bind the feed to solid pellets. (Craig & Helfrich, 2017)

Too much feed can lead to an increase of DOM and declining of the water quality and too little feed slows fish optimal growth. The feed is a large expense for the RAS

facility, therefore, the right amount of feed must be calculated and then adjusted every week. (Timmons et al, 2002)

The feed conversion rate (FCR), describes how many kilograms of feed is used to grow one kilogram of fish. That for the lower the FCR is, the less feed is needed to grow a kilogram of fish. (A Guide to Recirculation Aquaculture, 2015)

Correct pellet sizes relative to fish sizes and feeds' protein and fat content are shown for rainbow trout in Table 1. and for whitefish in Table 2.

Table 1. Content of a rainbow trout feed (Source: BioMar, 2019)

Pellet size (mm)	Fish size (g)	Protein (%)	Fat (%)
3	40 -125	43	27
4.5	100 – 500	42	28
6.5	400 – 1200	21	29

Table 2. Content of a whitefish feed (Source: Raisio aqua, 2019)

Pellet size (mm)	Fish size (g)	Protein (%)	Fat (%)
1.7	25 – 85	49	16
2.5	75 – 210	48	17
3.5	200 – 600	45	18
5/7	> 550	45	18

2.2.2. Temperature and oxygen

Water temperature directly affects how much oxygen can be dissolved into water, in cold water, there is more dissolved oxygen available for fish than in warm water. Water temperature should be held between 10 to 16 °C for most salmonid species. Also, low water temperature slows fish growth and too high increases fish metabolism, oxygen consumption and decreases the solubility of O_2. Water temperature is adjusted by controlling the air temperature in the rearing hall with

air pumps. In addition, water temperature can be adjusted directly. Fish metabolism and a nitrification process require oxygen, so RAS units are aerated constantly to keep the oxygen level and its saturation high enough for the fish and the nitrification bacteria. The optimal concentration of dissolved oxygen for salmonid is between 6 - 8 mg L^{-1}. Enough oxygen must also be left for the nitrification bacteria in the later part of the cycle. For nitrification bacteria, dissolved oxygen concentration should be higher than 2 mg L^{-1}, so the nitrification process can occur. (Timmons et al., 2002)

Lower oxygen concentration than 2 mg L^{-1} could lead to the accumulation of NO_2-N in the recirculating water because nitrification process efficiency decreases when nitrification bacteria do not get enough oxygen (Goreau et al. 1980).

Oxygen can be added into recirculating water by a gas-to-liquid or liquid-to-gas method. The Gas-to-liquid method, also called the diffused aeration method, means that air or oxygen gas is transferred to water making it saturated (aeration) or supersaturated (oxygen addition). Liquid oxygen can also be added to water, which is the liquid-to-gas method. In this method, water is diffused into droplets, which increase the surface area for air contact. (Lekang, 2013)

Every rearing tank had to have an emergency oxygen diffuser that aerates tank water in case of power failure, otherwise, the oxygen level can decline rapidly to the lethal level for the fish (Timmons et al. 2002).

2.2.3. Nitrogen compounds

Nitrogen compounds are problematic for aquaculture and fish health. They are formed from proteins as the product of fish metabolism in the form of total ammonia nitrogen (TAN), which consists of ammonium ions (NH_4-N+) and molecular ammonia (NH_3-N). The proportion of these two compounds depends on water pH so that an increase in the pH increases NH_3-N in water, which is the most toxic form of nitrogen for the fish. (Timmons et al. 2002)

Fish cell walls are relatively protective of the NH_4-N+, but NH_3-N can easily diffuse through the fish tissue. That is why short-term exposure to NH_4-N+ is not lethal for fish, but acute exposure to NH_3-N could be lethal. In addition, the long-term exposure of a high concentration of NH_4-N+ can be harmful or even lethal for fish. (Thurston et al. 1981)

TAN concentration should be held below 3 mg L^{-1} and NH_3-N concentration below 0.025 mg L^{-1} to salmonids (Timmons et al. 2002). Other sources state that TAN is toxic at 1 mg L^{-1} and nitrite (NO_2-N), which is the oxidized product of ammonia formed in the nitrification process in bioreactors, is also toxic at the same concentration (Lawson, 1995; Pillay & Kutty, 2005). NO_2-N is oxidized furthermore to the nitrate (NO_3-N) and as the final product of the nitrification process, it is the least toxic compound and its concentration should be below 80 - 100 mg L^{-1} (Boreham et al. 2004). Breaking ammonia into NO_2-N and NO_3-N leads to the accumulation of NO_3-N in the recirculating water in RAS (Timmons et al. 2002). Accumulation of it in low RWR intensity can be as high as 500 mg L^{-1} (Honda et al. 1993).

2.2.4. CO_2 and pH

Carbon dioxide (CO_2) is formed from the respiration of fish and bacterium in RAS water and metabolism products of fish lowers the water pH as well. Waters pH determines in which form CO_2 is in water. It dissolves and accumulates to recirculating water and is harmful to salmonid if it exceeds concentration between 20 - 30 mg L^{-1} (Timmons et al. 2002). Other studies suggest even smaller concentrations as 10 mg/L for salmonids welfare and optimal growth (Wedemeyer, 1996; Fivelstad et al., 1998). In Mota et al. (2019) study they found that Atlantic salmon (*Salmo salar*) post-smolts growth decreases if water CO_2 exceeds 12 mg/L and fish skin dermis layer gets thinner when fish are exposed to 40 mg/L concentration of CO_2.

pH should be kept between 6.5 - 8 for the salmonid to ensure that the fish won't get

stressed from low or high pH, and between 7 - 8 for the nitrification bacteria, so that they could function properly (Masser et al. 1999). In Finland, make-up water which is taken from a lake, river or groundwater is usually somewhat acidic (Helminen et al. 1977).

CO_2 is removed from recirculating water in draining towers, where packed columns increase dripping surface area of water to more air to diffuse with. The drainage tower is aerated and CO_2 in water is then diffused into air, according to Henry's Law. Removing CO_2 increases water pH, but the pH is adjusted usually also with some bases, for example, sodium hydroxide (NaOH) solution or common baking soda (Na_2CO_3). (Timmons et al. 2002)

2.2.5. Microbes and their by-products

Microbial communities have important roles in RAS, they consume nutrients, break down organic matter and they can control, or on the other hand, cause diseases (Zeng et al. 2017). Microbes have several different pathways into RAS, for example, make-up water, feed and dirty equipment (Sharrer et al. 2015). RAS have usually high biosecurity against pathogens, but infections can still occur (Sharrer et al. 2015). Monitoring harmful microbes in RAS water is important to protect fish from infectious diseases because in RAS fish live in high densities and are sensitive to mass infections (Timmons et al. 2002). Also, analyzing the build-up of off-flavor compounds geosmin (GSM) and 2-methylisoborneol (MIB) in fish flesh promoted by the presence of certain bacteria is important (Blankheton et al. 2013) or in circulating water (Houle et al. 2011; Lindholm-Lehto et al. 2019), because consumers do not want to buy mud-tasting fish.

Biologically degradable organic compounds metabolizing microorganisms are known to be part of RAS general microflora (Hagopian and Riley, 1998; Sharrer and Summerfelt, 2005; Guerdat et al. 2010. Hagopian and Riley (1998) and later Cytryn et al. (2005) found out that there are fulvic acid-like compounds oxidizing bacteria that coexist on the biofilter with nitrification bacteria. The increasing number of

heterotrophic bacteria in the system can affect the nitrification bacteria community negatively. In the presence of a high amount of organic matter in the recirculating water, these bacteria can displace the nitrification bacteria from the biofilter (Hagopian and Riley, 1998). Nitrification bacteria grow about 40 times slower than heterotrophic bacteria, in RAS where DOM is accumulating in the system according to Grady and Lim (1980) research. Also, heterotrophic bacteria growth may increase oxygen consumption and weaken biofilter performance (Michaud et al. 2006). Even small changes in RAS purification management via organic load control can lead to different types of microbiota in rearing tanks (Attramadal et al. 2016).

Cyanobacteria are known to produce GSM and MIB and it is coming to RAS mainly from lake water via make-up water intake (Izaguirre & Taylor, 2004). Aktino- and proteobacteria are also known as GSM producers in RAS (Suurnäkki et al. 2015, Lindholm-Lehto et al. 2019). These harmful bacteria for aquaculture can attach to any surface in RAS for example to biofilter or drum filters mesh and extract these odor problems causing compounds to the recirculating water, which then bioconcentrate to fish fat (Howgate, 2004; Hathurusingha and Davey, 2014). Even as the small concentration of these compounds as 2 – 10 ng/L causes muddy-taste flavor in fish detected by human sensory (Lindholm-Lehto et al. 2019). GSM and MIB concentration in water should be below 15 ng/L^{-1} for GSM and MIB 18 ng/L^{-1} (Persson, 1980). In fish fat, these compounds' concentration should not exceed 1 ng/g^{-1} (Lindholm-Lehto et al. 2019). This is not harmful to fish, but off-flavor is a big issue in fish marketing. Guttman & Rjin (2008) found out that chemical sorption by the sludge in the aerobic sludge digestion treatment stage was found to account for a 93 % reduction in GSM and a 79 % reduction in MIB when sludge was washed with culture water. Gerbeth et al. (2018) get relatively 99 % MIB removal with sludge digestion in their experiment in the pilot RAS facility. Other methods of removing off-flavor compounds are using micro-filtration with absorption or ozonate recirculating water (Elhadi at al. 2004).

2.2.6. Relative water renewal rate

The purpose of adding make-up water to RAS is to dilute accumulating substances, which are otherwise difficult to remove, such as nitrite, and replace lost water in the cycle (Seginer et al. 2008; Timmons et al. 2002).

In the study by Pulkkinen et al. (2018), they had eight different RWR operating independent RAS units, four different treatments with one replicate each, relatively 270, 490, 670 and 860 L kg^{-1} feed and they were using breakpoint analysis to study RWR effect on rainbow trout growth. In the study, they found out that when RWR was lower than 514 L kg^{-1} feed, SGR started to decline, and when it was lower than 478 L kg^{-1} FCR of the fish started to increase. In Seginer et al. (2008) study, in which sea breams were farmed, they found out that the minimum RWR to purge off-flavors is 280 L kg^{-1}. It has been studied with Nile tilapia (*Oreochromis niloticus*) that when RAS is being run intensively, nitrogen starts to accumulate to the system and it becomes the limiting factor for the fish growth when reaching concentration level of 500 mg L^{-1} (Monsees et al. 2016).

2.2.7. Dissolved organic carbon

Dissolved organic carbon (DOC) is typically a small carbon matter that can be filtered through a 0.45 µm filter (ISO, 2019). Its origin in water is biological and it is closely linked to water DOM concentration (Thurman, 1985). DOC is an energy source for heterotrophic microbes, so its concentration correlates with microbial activity (Kaplan & Newbold, 2000).

DOC can be analyzed from water with a total organic carbon analyzer. Before samples are added to the analyzer, they had to be acidified with, for example, HCL solution to remove inorganic carbon. This helps bicarbonate and carbonate ions to convert to CO_2 and that way inorganic carbon can be removed. Then the sample is oxidized and finally combusted at high temperature to convert the rest of the carbon which is in organic form to CO_2. DOC concentration can then be then detected from the sample with nondispersive infrared detection. Concentration between 1.0 mg L - 1000 mg L can be analyzed directly from water samples. With the same analyzer,

it is possible to measure TN as well from the sample. (Goerlitz & Brown, 1972).

On-line fluorescence DOC-detection is used to study water DOM concentrations because not all organic compounds aromatic or fluorescent structures are visible by UV- or fluorescence detection (Her et al. 2002). DOM in water correlates with COD, BOD, and UV254 -absorption and with total fluorescence (Ignatev & Tuhkanen, 2019). Hably et al. (2015) found a linear relationship between DOC and feed input in RAS, where every 125 g feed increase was linked to a 5 mg / L increase in DOC in water.

2.2.8. Solids and DOM

Generally, the index of DOM in water can be measured in DOC (Thurman, 1985). DOM origin in RAS is mainly dissolving from excess feed and fish feces to recirculating water (Timmons et al. 2002). Fish feeds quality and nutrient content affects its dissolution in water. This type of DOM is mostly proteinous. (Nimptsch et al. 2015). Proteinous DOM consist of tyrosine- and tryptophan-like compounds which are both essential amino acids for animals. Tyrosine-like compounds have one aromatic ring, and they play a role in protein synthesis, while tryptophan-like compounds consist of benzene ring fused to a heterocyclic aromatic ring (Hudson et al. 2007).

Make-up water addition is another pathway of the DOM into the system. Humic-like organic compounds, such as fulvic- and humic acid -like compounds, are coming to the system via intake water (Kothawala et al. 2013). Humic substances comprise most of the water organic matter. These substances are variable end products of complex depredated organic compounds (Aiken et al. 1985). Some humic-like compounds are the product of the bigger DOM-components degraded by microbes in the system. This type of matter easily accumulates to recirculating water (Meinelt et al. 2010) and they are difficult to remove from it, partially because of their small molecule size (Yamin et al. 2017). According to Fernandes et al. (2015) study solids < 20 µm accounts more than 90% of total suspended solids in

recirculating water of aquaculture. Timmons et al. (2002) investigated that DOM-components smaller than 30 µm in diameter can't be removed with mechanical removal.

Because of the smaller molecular size, fulvic-like compounds are more difficult to remove than protein-like compounds from recirculating water (Timmons et al. 2002). Ignatev and Tuhkanen (2019b) observed following purification rates from two Finnish drinking water treatment plants, which were on average about 70 % of DOC, UV254 -absorbance signals and total fluorescence signal 80 - 90 %, overall DOM fractions of high molecular weight (> 1500 Da) > 95 % and molecular weight (< 600 Da) 60 – 70 % were removed. The removal of total fluorescence of protein-like compounds was about 82 % and removal of humic/fulvic acid-like compounds was higher being about 88 %.

In recirculating aquaculture Stevenson (1994) found in his study that fulvic acid-like compounds are not easily biodegradable in recirculating water. Later Yamashita & Tanoue (2003) and Borisover et al. (2011) found out that fulvic acid-like compounds protect protein-like matter against biodegradation. In hammock et al. (2003) research, they found out that fulvic acid-like substances have a protective effect in fish exposed to toxic metals and Meinelt et al. (2010), later found also protective effect on ammonia and nitrite compounds.

High DOM concentration in recirculating water impairs its quality by increasing chemical and biological oxygen demand via increased microbial activity consuming dissolved oxygen. The amount of DOM in the recirculating water correlates with the number of microbes in water because microbes can harbor on the organic matter and use it as an energy source. High DOM concentration could lead to increasing levels of harmful bacteria in RAS (Pedersen et al. 2017), which in turn can lead to the increased incidence of the infections of the fish and their mortality at the facility (Moestrup et al. 2014; Timmons et al. 2002) DOM also weakness the effectiveness of UV disinfection and biofilters nitrogen compound degradation (He et al. 2012). The harm of DOM in RAS is mostly indirect to the farmed fish. According to Fernandes

(2015) and Becke et al. (2016), there is no direct harm to rainbow trout health with suspended solids concentration up to 30 mg L^{-1} in an exposure lasting from four to six weeks. Decorated DOM, which contains proteins is problematic to aquaculture because it leads to an increase in toxic nitrogen compounds in culture water (Timmon et al. 2002). There is evidence that not all DOM is harmful to aquaculture since a relatively low amount of fulvic acid-like substances can make fish more tolerant for physical handling stress. The presence of fulvic substances can even fasten the recovery of damages caused by some pathogens (Meinelt et al. 2004).

In Finland, surface water is typically rich in humus, which is making it acidic. The humus is mainly allochthonous coming into water from a catchment area with peaty soils and bogs, which Finland has many. Therefore, Finnish surface waters are typically rich in dissolved organic substances, but in contrast, the concentration of inorganic substances in water is low (Skjelkvåle et al., 2001). In Arvolas et al. (2016) study, they found out that medium-size Finnish lakes' (lake area 10 – 100 km^2) the average colored dissolved organic matter (CDOM) concentration was: 96 ± 9.6 mg Pt l^{-1} and in larger lakes' (lake area > 100 km^2 it was: 79 ± 9.8 mg Pt l^{-1}.

DOM can be removed from water by oxidation and biosynthesis (Gray, 2004). Ozonation is a widely used efficient treatment method for wastewater organic matter removal because ozone can break down the double bonds of organic matter by oxidation and kill pathogens at the same time (Sharrer & Summerfelt, 2007). Degraded compounds can be furthermore removed by activated carbon filtration or by some other biological filtration method (Barbu et al. 2016).

There is coming an online fluorescence sensor on the market according to Hambly and Stedmon (2018) paper. The Online fluorescence sensor can be used to monitor recirculating water DOM concentration, which could save up to 30 % per year of the fish farms water and energy consumption with the water treatment and nitrogen removal (Eding et al. 2006).

2.3. Methods to characterize DOM in water

There are several methods of characterizing DOM in water, such as fractionation, spectroscopic and chromatographic methods (Michael-Kordatou et al. 2015; Sillanpää et al. 2015). These methods divide organic matter into groups by their chemical or physical properties (Michael-Kordatou et al. 2015). On top of that oxidation-based methods, the analysis of chemical and biological oxygen demand can be studied to determine the amount of the organic matter in water (Standard methods, 2020). DOM in water can be easily analyzed by liquid chromatography and fluorescence detection, because many organic components have fluorescent or aromatic structure (Her et al. 2002).

High-performance liquid chromatography (HPLC) -analyzes have been in popular use monitoring DOM from drinking and wastewater (Hudson et al. 2007; Coble, 2007; Sillanpää et al. 2015; Goffin et al. 2018; Igantev & Tuhkanen 2019a & b), because analysis itself is cheap, requires small sample volume and is fast to accomplish, since one sample can be analyzed in a half of hour and the cost per sample can be just a couple of euros. Also, with high pressure in the column, small DOM matter can be separated (Michael-Kordatou et al. 2015). One advantage of chromatography analysis is that the same sample can be analyzed multiple times and different analytes can be analyzed from the same samples in the same run (Dong, 2006). With chromatographic methods, organic compounds' molecules can be separated into fractions in a column by intermolecular interactions (Vitha 2017).

Liquid chromatography requires a mobile phase where the sample is diluted, and which then can be carried through the column. The mobile phase is selected depending on what kind of samples are being analyzed. There are many HPLC-modifications for chromatography to separate molecules, for example, normal-phase-, reversed-phase-, ion-exchange- and size-exclusion chromatography. In HPLC, usually UV254-absorption simultaneously with fluorescence is used to analyze organic compounds in water. (Dong, 2006)

2.3.1. Spectroscopic methods

In High-performance size-exclusion liquid chromatography (HPLC-SEC) sample is carried with a mobile phase through a column that separates organic compounds by their size. Smaller compounds elute to detectors after the bigger compounds, because they penetrate column pores more easily and are transported that for slower through the column. The different elution time of the different sizes of compounds helps to separate them into different fractions. HPLC-SEC provides information about apparent molecular weights. (Striegel et al. 2009)

The mobile phase, column, and detectors are selected for analysis depending on what kind of samples is being analyzed (Moldoveanu & David, 2016). For example, phosphate and acetate buffers have been used as a mobile phase when analyzed water samples DOM. The column used in HPLC-SEC can be silica- or polymer-based. The method also divides analysis requires minimal sample pretreatment and is fast to run (which makes it a very good DOM analyzing method for water samples (Her et al. 2002).

2.3.2. High-performance size exclusion chromatography

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(Her et al. 2002). To use the size exclusion column, it must be calibrated before the analysis. Commonly used calibration standards for the size-exclusion column are the polystyrene sulfonate of 210, 1600, 3200, 4800, 6400, 17000 and 32000 Da (Shon et al, 2006).

2.4. Characterization of DOM in RAS

From fish feed and feces originated fluorescent DOM consists mostly of protein-like fluorescence: tyrosine-like, and tryptophan-like compounds (Nimptsch et al. 2015). DOM of the boreal lakes is formed mostly fulvic-like compounds (Aiken & Cotsaris, 1985), which can be divided into fulvic acid and humic acid-like compounds (Sierra et al. 2005). That kind of DOM in coming mainly via make-up water intake into RAS (Stedmon et al. 2007; Walker et al. 2009; Kothawala et al. 2013). There is also another fulvic/humic acid-like compounds found in the studies the origin, which is not terrestrial, but inside the RAS. This type of compound is less commonly found on aquatic DOM and it might have a lower molecular weight, which makes its origin microbial (Fellman et al. 2010 Osburn et al. 2011). Leonard et al. (2002) observed in their study, that in RAS in which tanks water retention time is long, fulvic acid-like compounds can account for 90 % of the DOM.

Table 3. λex/λem Ranges to detect different DOM-components from aquaculture water samples with HPLC-fluorescence.

λex (nm)	λem (nm)	Character of DOM	Component	Reference
270 - 280	310 - 320	Protein-like	Tyrosine-like	Baghoth et al. (2009)
220 & 270	310			Ignatev & Tuhkanen (2019)
270 - 285	340 - 360		Tryptophan-like	Hudson et al. (2008), Baghoth et al. (2009)
230 & 270	355			Ignatev & Tuhkanen (2019)
320 - 350	400 - 450	Fulvic-like	Fulvic acid-like	Spencer et al. (2007), Baker et al. (2008)
240 & 270	440 & 500			Ignatev & Tuhkanen (2019)
320 - 390	410 - 500		Humic acid-like	Yamashita & Jaffé (2008), Baghoth et al. (2009)
330 & 390	425 & 500			Ignatev & Tuhkanen (2019)

3 MATERIALS AND METHODS

3.1. Experimental RAS platform in Laukaa

Laukaa's RAS is an experimental research and learning environment run by Natural Resources Institute Finland (Luke). Technical and biological solutions can be tested via trialing in this environment and the solutions that are the best can be taken in use in Finnish RAS fish farming. Biological limits for fish can be examined as well as the purifying efficiency of biofilters of a different kind. (Luke, 2019)

Arvo-Tec Oy set up the RAS. There are overall 10 separate RAS units in the experimental recirculating system and three of them were used in this study - units 8, 9 and 10. These three units are independent of each other, having own rearing tanks and purification systems. The total water volume of one unit is 1140 L. Process diagram of Laukaa's RAS is shown in Fig. 1. Renewal water ratios of different RAS units are shown in the middle of the diagram.

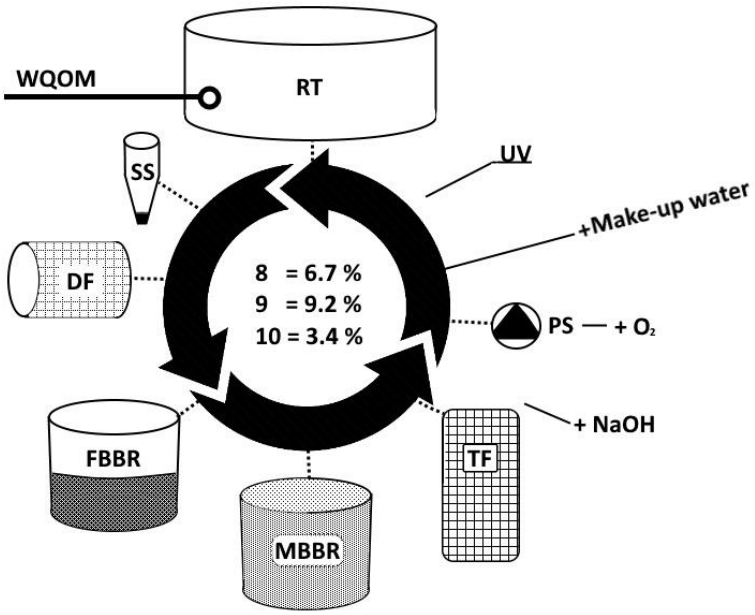

Fig. 1. Laukaa's RAS process diagram, showing water quality online monitoring (WQOM), rearing tank (RT), swirl separator (SS), drum filter (DF), fixed-bed (FBBR) and moving-bed bioreactor (MBBR), trickling tower (TF) and pump sump (PS).

3.1.1. Rearing tanks and online monitoring system

The rearing tanks were bottom-drained, round shape and 500 L in volume. In this study, each of three tanks contained 5023 ± 2 g of whitefish with an average weight of 53 ± 1 g between the different tanks at the start weighing on 7th February. There was an automatic feeder (T Drum 2000, Arvo-Tec Oy, Joroinen, Finland) on top of each tank cover and beneath the cover is constant lighting with the LED light system. Fish are feed at the beginning of the experiment with 5 mm pellets (Silver, Raisioagro, Raisio, Finland) amount relative of 1 % of fish body weight per day in 2 h cycles. Later, the amount of feed given is calculated from the size of the fish and their growth every weekday, and feeding is reduced in case of a decrease in appetite. The feeding amount is increased once a week because while the fish grows, they require more feed to sustain the growth. The feeding rate varied from 1.2 to 2.1 % of fish biomass in the tank per day, during the experiment.

The oxygen saturation of the fish tanks is manually adjusted with a constant flow regulator (Model 2851, Kytola® instruments Oy, Muurame, Finland) and kept over 80 %. The temperature of the system water is kept at 16 °C by cooling the experiment halls' air temperature with industrial fans. Each RAS unit has an online monitoring system that consists of O_2-(oxi::lyser, s::can, Vienna, Austria), pH- (pH::lyser, s::can, Vienna, Austria) and spectrometer –probes (spectro::lyser, p::can, Wien, Austria), and a CO_2 -sensor (Franatech, Lüneburg, Germany). The Spectrometer sensor is used to monitoring TSS, NO_2-N, NO_3-N and NH_4-N^+ concentration, UV254 absorbance, and water turbidity. At the bottom of every tank, there are emergency oxygenation diffusers, which can be turned on manually from a switch or automatically, in case of a power failure or if the oxygen levels drop drastically. All the data is collected at farms on-line computer (con::cube, p::can, Vienna, Austria).

3.1.2. Make-up water intake

RAS is located next to an oligotrophic Lake Peurunka (62.446°N, 25.852°E), where the make-up water is taken from two different depths, 6 m and 10 m deep, where

water is pumped of the facilities storage tank. Hypolimnetic water of the lake stays cooler around the year than water in an epilimnion, what is the one reason hypolimnetic water is used Other reason is that it has lower level of pathogens. Make-up water is added a minimum of 1 - 2 % of the recirculating water to the system (Luke, 2019). Make-up water temperature is adjusted with heat exchangers and a heat pump (30 HM-065, Carrier, Farmington, USA).

Make-up water is pumped from a 600 L storage tank with a water-dosing pump (DDI-222, Grundfos, Bjerringbro, Denmark) and it is added to the feed collector units. Make-up water alkalinity is increased if needed by adding NaHCO3 with an automated belt feeder. Three units are operated with a different make-up water intake ratio: 10 = 250 L kg^{-1}, 8 = 500 L kg^{-1}, and 9 = 750 L kg^{-1} feed, which corresponds RWR of 3.4; 6.7 and 9.2 %. The flow rate is measured with a flow meter (type 8012, Bürkert, Ingelfingen, Germany) from the tank inlet water pipe. Make-up water volume is increased while the amount of given feed increases.

3.1.3. Recirculating water purification system

3.1.3.1.Suspended solids removal

At the bottom of each tank is a water outlet from where the water is flowing through a metal screen that filters uneaten feed and fish feces. Water goes to a swirl separator (Eco-Trap Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), which removes larger suspended solids (SS) via gravitation. Then the water goes to a drum filter (Hydrotech HDF501, Veolia, Paris, France) which has 60 µm filter panels and can remove some of the smaller SS as well. SS are settled into the drain, while some of the formed sludge can be removed via swirl separators detachable container.

3.1.3.2.Biological filtration

Toxic ammonia, nitrite, and some finer organic compounds are removed from circulating water with different kinds of bioreactor: moving-bed and fixed-bed reactors, which are serial linked. Both are 147 L in volume and have plastic media

(RK Bioelements, Skive, Denmark) with the surface area for nitrification bacteria of 750 m^2 m^{-3}. In the moving-bed biofilter reactor, water column is aerated with 15 L min^{-1} airflow keeping the media in constant motion. Water is pumped first to the fixed-bed biofilter reactor and after that, it is pumped to the moving-bed biofilter reactor.

3.1.3.3.CO_2 removal and chemical adjustment

After the water is processed in biological treatment, it flow to an 82 cm high trickling tower, where water drain through its Bio-Blok®200 -packing media (EXPO-NET Danmark A/S, Hjørring, Denmark) with a surface area of 200 m^2 m^{-3}. Air is blown from the bottom of the trickling filter with a channel blower (Online CK 100 A, Onninen, Vantaa, Finland) to reduce CO_2, which is produced by fish and nitrification bacteria. Packing media adds a surface area in the water for air to diffuse with. Because the metabolism products from the fish and carbon dioxide lower water pH, it is maintained around 7.0 by adding a base solution, 20 % NaOH (aq). Before pump sump, pH can be adjusted by a single channel control unit (Dulcometer, ProMinent, Heidelberg, Germany) using pump sumps pH-probe and low-pressure metering pump (Beta b, ProMinent, Heidelberg, Germany) dosing NaOH solution to the trickling filter. Then water goes to 70 L pump sump, where it is supersaturated with pure O_2 via ceramic diffusers. At the end of the purification, process water is pumped from the pump sump with a recirculating pump (Magna 3, Grundfos, Bjerringbro, Denmark) back to the rearing tanks.

3.1.3.4.Disinfection

Make-up water is disinfected with UV light (Duv 01 A, Lit, Moscow, Russia) and also it can be filtered with a string-wound cartridge (Shelco RHS, Charlotte, USA) or a carbon block filter (5FOS, Shelco, Charlotte, USA), which are part of the system.

3.1.4. Water sampling

The technical staff of the Laukaa's fish farm took once per week water samples for

the HPLC-SEC and DOC analyses from three different points: the rearing tanks feed collector, on the surface of the fixed-bed bioreactor and the make-up water container tank. Sampling points are shown in Appendix 1. The water samples were taken with clean 0.25 to 0.50 L plastic bottles. They were taken from each three RAS units from the same points of the water cycle. Tank water samples were taken from separate receptacles that were connected to tanks and had fish feces screens on top of them (see Appendix 2.). From now on, for simplicity, three units with RWR of 6.7 %, 9.2% and 3.4 % are relatively called units 8, 9 and 10.

Fixed-bed biofilter samples were taken from that bioreactors water phase so that water samples presented water purified with a fixed-bed biofilter, not moving-bed biofilter. Make-up water samples coming from Lake Peurunka were taken from a make-up water reservoir tank every third week, to see if there were any changes in make-up water quality during the experiment. The fish were reared starting on 7th February, but the actual experiment, during which the samples were taken, was going on from 1st March to 11th June. After the samples were taken, they were immediately stored cold and transported to the University of Jyväskylä within 24h.

3.2. Fish weighing

Fish were weighed at the beginning, when they were brought to the rearing tanks on 7th February, two times in the middle of the experiment on 21st March and 25th April, and at the end on 29th May. Fish were netted from the rearing tank and weighed in a tared water-filled container. The total weights of the fish in each tank were measured by weighing all the fish together from the tank, so the individual weights of the fish were not measured.

3.3. Sample preparation

3.3.1. DOC and TN samples

DOC samples were analyzed in three separate runs during the experiment, weekly samples were kept in a freezer. For a total organic carbon analyzer (Shimadzu TOC-

L, Japan) 24 mL glass test tubes were precombusted at 600 °C for 4 hours to get rid of organic matter. 20 mL of samples, reference, and blank samples were pipetted to test tubes. Blank samples made of Milli-Q-water and references were filtered samples of Lake Jyväsjärvi. In each tube, 80 µL of 2 mol L^{-1} HCL was added to acidify a sample.

Standard solutions were prepared by the laboratory technician from KHP (C8H5KO4, M = 204.22 g/mol) for carbon and from KNO_3 (M = 101.1032 g/mol) for a nitrogen calibration curve. The concentrations in the first two runs for carbon were 2 mg C/L, 10 mg C/L and 100 mg C/L, and for nitrogen 0.15 mg N/L, 1.5 mg N/L, in the third run both standard concentrations were increased to 30, 100 mg /L, because of the high concentration of carbon and nitrogen of the samples. Samples were placed in an autosampler which temperature was adjusted to 4 °C. The autosampler injected 100 µL of each sample at least two times during the analyze making at least one replicate. In case these two injections concentrations differed greatly the autosampler took the third injection from that sample. The results were obtained from each sample injections average.

3.3.2. HPLC-SEC samples

All the samples were filtered through a 0.45 µm syringe filter (VWR, USA) that was attached to a 20 mL plastic syringe. Before the actual samples were filtered, the syringe filters were rinsed with Milli-Q-water to make sure nothing from the filter ended up in the sample vials. The first 5 mL of the sample was discarded, and then the other 5 mL were filtered to a 50 mL sterile centrifuge tube for DOC and TN analyses and after that 1 mL glass vials for the HPLC-SEC were filled with the filtered sample. The centrifuge tubes were filled up to 30 mL mark, named and moved to a freezer to -20 °C for later DOC and TN analyses. The HPLC-SEC vials were stored cold if they couldn't have been analyzed on the same day. Three blank samples were made of Milli-Q-water, to make sure there was no contamination in the chromatogram during the run.

There was also an HPLC-SEC sample of fish feed from 21st February. Feed was the same that was given to fish in the experiment. A small amount of it was diluted in Milli-Q water in a closed glass flask for a couple of days. Extract represented given feeds DOM character, thus, there was only a single sample to run in HPLC and the feed was not fully diluted in the water.

3.4. HPLC-SEC Analyses

On the same day or the day before the analyses, a mobile phase was made to 2 L long-necked glass bottle containing Milli-Q-water, by adding two sodium phosphate compounds (Na2HPO4 * 2H2O = 0.8900 g L^{-1} and NaH2PO4 * 2H2O = 0.7801 g L^{-1}). A precision scale was used weighing the right amount of the compounds with tared weighing dishes. After the compounds were added, the bottle was shaken well, so that the compounds dissolved fully to the water. Solution was filtered through a membrane filter pore size of 0.22 µm (Whatman™, ⌀ = 47 mm, Germany) by a vacuum.

The samples were analyzed within 24 hours after sampling with high-performance liquid chromatography (Shimadzu, Perkin-Elmer SL 55 spectrophotometer, DGU-20A5R, and DGU-20A3R gas exchange unit, Japan), which measures simultaneously samples' organic compounds UV254 -absorbance with a diode array detector (SPD-M20A, Shimadzu, Japan) and fluorescence with fluorescence detectors (Shimadzu SPD-M20A and Shimadzu RF-20A XS). The HPLC-SEC method modified by Iknatev and Tuhkanen (2019), were used in this study. Yarra™ 3 µm SEC-3000 (300 * 7.8 mm, Phenomenex, USA) column was used to separate different apparent molecular weights of compounds. The column oven (Shimadzu CTO-20AC) temperature was adjusted to 25 °C. The system was rinsed before the analysis with the mobile phase to clean it from the possible substances of earlier analyses of other users.

The samples were arranged to a sample table so that the presumably cleanest samples were analyzed before the dirtier samples. In the beginning, several Milli-

Q-water samples were run if the first Milli-Q sample that was analyzed was contaminated. That way the injector could have been purified with water so that there were no significant contamination peaks in the chromatogram results that were originated from earlier analyzes with different phases. After every different sample, a Milli-Q-water sample was run to indicate if there was any contamination as well. Injection volume was 30 µL for the samples and 50 µL for the Milli-Q-water samples. Each sample was injected and analyzed twice with two different fluorescence wavelengths that analyzed proteinous and fulvic acid-like compounds. Each run cycle took 30 minutes. A photodiode array (PDA) and fluorescence detectors were used simultaneously to detect absorbance and fluorescence intensities of different compounds. The PDA-detectors range was 200 - 400 nm and fluorescence-detectors excitation and emission were respective for tyrosine-like compounds Ex./Em. 220/310 nm and 270/310 nm, tryptophan-like compounds Ex./Em. 230/355 nm and 270/355 nm, humic acid-like compounds Ex./Em. 240/440 nm and 330/425 nm, and fulvic acid-like compounds Ex./Em. 270/500 nm and 390/500 nm.

3.5. Conductivity and pH

The conductivity and pH were measured from water samples first each week. These measures are temperature dependent, so the samples were kept at room temperature for 2 - 3 hours before the actual analyses to avoid interference in results due to storing them in the cold. The needed amount of a sample was poured into a 50 mL centrifuge tube, in which conductivity and pH sensors were dipped. In the conductivity analysis, a conductivity-meter (Hanna instruments, HI 9635, Italy) was used, and in the pH analysis, a pH-meter (PHM220 MeterLab™, Villeurbanne, France) was used. After each measurement, the sensors were rinsed with Milli-Q-water and dried with a tissue.

3.6. Data processing

3.6.1. Chromatogram data

After the analysis, chromatogram peaks were integrated manually in the data post-processing software (Shimadzu LabSolutions LC/GC Version 5.51), by selecting an elution range from 4.5 min to 20 min and then separating each peak from each other to seven different fractions (1 - 7). Each peak then represented different molecular weights that helped to characterize different DOM-components. Total fluorescence areas (mV min) and total absorbance areas (mAU min) of the peaks were processed in Microsoft Excel 2016. The measurements of the first fraction was removed from the final data because values were unreliable for this fraction, due exceed methanol or some other contaminant in the chromatogram's injector. The raw intensity signals from chromatogram were divided by 1000 to scale intensities down. Outlying observations have been removed and introduced in a results section.

3.7. Statistical analyses

All the statistical analyses were done in IBM SPSS Statistics 24. Meta-analysis: Skewness, Kurtosis test, and Shapiro-Wilk test were used to investigate the normality of the data, to pick the right statistical analysis. If meta-analysis supported the normality of the data, parametric tests were used. For all the statistical tests, a 95 % coefficient interval was used to detect the statistical significance, but 99% and 99.9% were also reported if tests were very significant. The data considering of the whole experiment was used to study differences between water quality between the units.

3.7.1. Water quality parameters

Instead of variance analyses, Friedman test and repeated measures ANOVA were used to compare differences in the samples' water parameters between the treatments, because each unit data points are dependent of each other due to water

quality was studied over time. The differences of the ranked values of the different treatments were then analyzed with Wilcoxon Signed-Ranks test pairwise, so that W-value was used to report the differences because the sample size was < 20. Each tank samples were compared with relative fixed-bed biofilter samples of a certain week with Wilcoxon Signed-Ranks test as well. For parametric data, repeat measures ANOVA were used to find differences between the treatments. Furthermore, dependent sample t-test was accomplished to find the differences in means.

Data of DOM-components fluorescence intensities were studied also with a curve estimation and linearity assumption was tested with a Pearson correlation. Regression was done by using components intensities total area as an independent variable, and passed time in days of the experiment, as a dependent variable. A one-way-ANOVA was used to test the significance that was each observed compound intensity values fitting the model. Finally, the coefficient was tested between how well the time in days of the experiment predicted DOM accumulation in RAS.

A correlation matrix was made for the data of every tank water quality parameter, using Spearson correlation, because the combined data were non-normal distributed. The relationships between DOC versus total UV254-absorbance and the total fluorescence of different compounds were tested with a linear regression model for the whole data (n = 42). Each tank's data was used also independently (n =14).

3.7.2. Fish growth

Because of the lack of the individual weighing of the fish, fish growth, FCR and SGR between each weighing (n = 3) were used to investigate the difference between each tank median fish growth during the experiment. Analyze for this was made with the Kruskal Wallis test.

4 RESULTS

4.1. Water quality parameters

For units 8, 9 and 10, the water average exchange ratio was respectively 6.7, 9.2 and 3.4 % of its total volume per day. Make-up waters average (n = 5) pH was 7.3 ± 0.3, conductivity: 36.1 ± 23.9 µSv, DOC: 7.0 ± 2.2 mg L and TN: 0.4 ± 0.1 mg L.

In Table 4. are introduced the results of all water quality parameters in different RAS units' tanks and fixed-bed biofilter, from three different periods of the experiment, which were measured every week during the 15 weeks long experiment. The average values of the tank and biofilter samples are displayed in each period (n = 5) with standard deviation. More detailed results are reported under the subheadings.

Table 4. The differences between different RAS tanks water quality parameters. Fixed-bed biofilters' and make-up water quality have been reported in the table as well. Non-analyzed parameters are left blank. In make-up water column one asterisk (*) means sample size was 2, and two (**) means there was just a single measure.

1.3. - 28.3.19	Tank						Make-up
Parameter	8	9	10	FB8	FB9	FB10	water
RWR (%)	6.7	9.2	3.4	6.7	9.2	3.4	
pH_{post}	7.3 ± 0.1	7.4	7.3 ± 0.1	7.1 ± 0.2	7.3 ± 0.1	7.2 ± 0.1	7.6 ± 0.3 *
pH_{online}	6.8 ± 0.2	7.1 ± 0.1	7.1 ± 0.1	-	-	-	-
Alkalinity (meq/L)	18.5 ± 5.3	36.0 ± 10.5	27.9 ± 1.0	-	-	-	-
Conductivity (µSv)	371.8 ± 92	365.8 ± 91	492.3 ± 163	356 ± 72	369 ± 73	491 ± 130	54.5 ± 21.5 *
DOC (mg L)	10.5 ± 0.7	10.2 ± 0.4	11.2 ± 1.3	10.4 ± 0.6	10.3 ± 0.4	11.2 ± 1.1	8.9 ± 2.5 *
TN (mg L)	39.8 ± 7.6	37.1 ± 5.9	48.6 ± 13.8	40.2 ± 6.7	38.1 ± 5.2	49.2 ± 12.2	0.3 *
NH_4-N^+ (mg L)	0.4 ± 0.1	0.4	0.4 ± 0.1	-	-	-	-
NO_2-N (mg L)	0.08 ± 0.02	0.07 ± 0.1	0.08	-	-	-	-
NO_3-N (mg L)	32.2 ± 10.7	30.9 ± 8.3	37.7 ± 15.9	-	-	-	-
UVA254	599 ± 34	557 ± 24	632.8 ± 60.1	595 ± 31	572 ± 19	630 ± 47	414 ± 14
Tyrosine	2049 ± 158	2550 ± 235	2286 ± 367	2106 ± 153	2615 ± 328	2194 ± 102	1827 ± 626
Tryptophan	6437 ± 546	6499 ± 336	7465 ± 1187	6409 ± 554	6606 ± 382	7523 ± 991	2956 ± 525

Fulvic	17328 ± 1370	16719 ± 1081	19897 ± 2871	17399 ± 1278	16869 ± 1036	19929 ± 2519	8196 ± 82
Humic	5123 ± 474	4946 ± 394	5857 ± 922	5097 ± 442	4964 ± 344	5848 ± 802	2242 ± 12
29.3. - 30.4.19		Tank					
Parameter	8	9	10	FB8	FB9	FB10	Make-up water
pH_{post}	7.2 ± 0.1	7.3 ± 0.1	7.2 ± 0.2	7.0 ± 0.1	7.3 ± 0.2	7 ± 0.2	7.3 ± 0.2 *
pH_{online}	6.8	7.2 ± 0.2	7.0 ± 0.1	-	-	-	-
Alkalinity (meq/L)	50.6 ± 4.1	44.8 ± 6.8	83.1 ± 40.9	-	-	-	-
Conductivity (µSv)	609.5 ± 244	673.8 ± 260	926.9 ± 278	587 ± 49	525 ± 66	1093 ± 158	17.6 ± 0.1 *
DOC (mg L)	11.4 ± 0.9	11.2 ± 1.2	13.5 ± 1.8	11.2 ± 1.0	8.3 ± 4.1	11.5 ± 5.8	5.9 ± 0.4 *
TN (mg L)	56.7 ± 23.3	62.1 ± 25.4	85.3 ± 26.7	55.8 ± 8.0	37.6 ± 18.9	80.4 ± 42.2	0.2 *
NH_4-N^+ (mg L)	0.7 ± 0.2	0.7 ± 0.1	1± 0.5	-	-	-	-
NO_2-N (mg L)	0.09 ± 0.05	0.13 ± 0.01	0.18 ± 0.12	-	-	-	-
NO_3-N (mg L)	56.1 ± 4.7	44.6 ± 2.4	94.2 ± 14.2	-	-	-	-
UVA254	588 ± 60	591 ± 68	690 ± 92	615 ± 16	555 ± 49	749 ± 25	323 **
Tyrosine	2277 ± 248	2442 ± 223	2772 ± 415	2397 ± 169	2586 ± 322	2854 ± 333	996 **
Tryptophan	7201 ± 1117	7587 ± 1197	9775 ± 1856	7256 ± 366	6782 ± 591	10489 ± 610	2250 **
Fulvic	1894 5 ± 3122	19407 ± 3435	24741 ± 4581	19083 ± 774	17168 ± 1682	26982 ± 1047	7505 **
Humic	5659 ± 719	5738 ± 835	7465 ± 1337	5669 ± 324	5099 ± 581	8010 ± 458	1984 **
1.5. – 11.6.19		Tank					
Parameter	8	9	10	FB8	FB9	FB10	Make-up water
pH_{post}	6.8 ± 0.1	6.7 ± 0.1	6.9 ± 0.1	6.7 ± 0.1	6.6 ± 0.1	6.9 ± 0.1	7.0 **
pH_{online}	6.9 ± 0.1	6.9 ± 0.3	7.1 ± 0.1	-	-	-	-
Alkalinity (meq/L)	18.8 ± 1.5	17.2 ± 1.1	38.5 ± 1.3	-	-	-	-
Conductivity (µSv)	597.5 ± 118	419 ± 67	1134 ± 261	550 ± 49	401 ± 65	1143 ± 274	-
DOC (mg L)	9.5 ± 0.6	9.4 ± 0.8	13.0 ± 0.5	9.6 ± 0.6	9.0 ± 0.7	12.9 ± 0.5	5.6 **
TN (mg L)	50.8 ± 7.5	42.2 ± 7.9	107.8 ± 16.5	52.4 ± 7.4	42.7 ± 7.3	111.1 ± 16.6	0.6 **
NH_4-N^+ (mg L)	0.61 ± 0.11	0.64 ± 0.08	1.04 ± 0.34	-	-	-	-
NO_2-N (mg L)	0.06 ± 0.01	0.07 ± 0.01	0.17 ± 0.04	-	-	-	-
NO_3-N (mg L)	55.6 ± 6.2	41.6 ± 5.3	115.1 ± 14.4	-	-	-	-
UVA254	510 ± 45	505 ± 24	699 ± 54	535 ± 32	503 ± 57	699 ± 42	361 **
Tyrosine	2588	2349	3727	2319	2490	3858	1366 **

	± 760	± 207	± 597	± 538	± 291	± 379	
Tryptophan	6154	6105	9970	5946	5796	9807	2249 **
	± 996	± 666	± 976	± 951	± 472	± 857	
Fulvic	16084	15099	24707	16028	14712	24571	13596 **
	± 1094	± 1649	± 2466	± 1155	± 1675	± 2491	
Humic	4433	4221	6694	4408	4089	6652	3224 **
	± 350	± 505	± 781	± 362	± 492	± 738	

4.1.1. Online monitoring data

4.1.1.1. Nitrogen compounds

At the end of the experiment, there were significant difference between each of the nitrogen compounds' concentrations: NO_3-N (Friedman test, Chi-square = 24.7, df = 2, $p < 0.001$), NO_2-N (Friedman test, Chi-square = 19.5, df = 2, $p < 0.001$) and NH_4-N+ (Friedman test, Chi-square = 15.4, df = 2, $p < 0.001$). Significant differences were found between tanks 8 and 10 in NO_3-N (Wilcoxon Signed-Rank test, W = 0, $p < 0.01$), between tanks 9 and 10 (Wilcoxon Signed-Rank test, W = 4, $p < 0.01$), differences was found also between tanks 8 and 9 (Wilcoxon Signed-Rank test, W = 0, $p < 0.01$). In NH_4-N+ there were significant differences between tanks 8 and 10 (Wilcoxon Signed-Rank test, W = 5 ,$p < 0.01$) and 10 and 9 (Wilcoxon Signed-Rank test, W = 29, $p < 0.01$) and barely between tanks 8 and 9 (Wilcoxon Signed-Rank test, W = 33, $p < 0.05$). Significant differences in NO_2-N between tanks 8 and 10 was found (Wilcoxon Signed-Rank test, W = 0, $p < 0.01$), 9 and 10 (Wilcoxon Signed-Rank test, W = 8, $p < 0.01$). Of all nitrogen compound concentrations, unit 10 had the highest mean values (See Fig. 2).

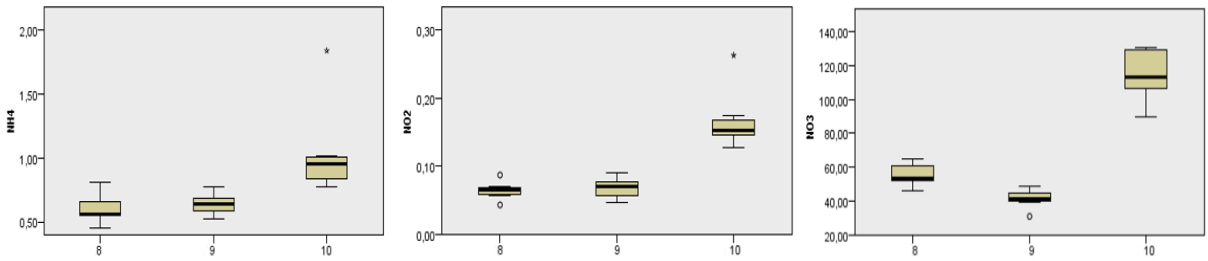

Fig. 2. Whisker boxplot chart of the mean nitrogen compounds' concentrations between tanks at the end of the experiment (n = 14).

Changes in nitrogen compounds NH_4-N+, NO_2-N and NO_3-N concentrations (mg L) in three tanks over time are shown in Fig.3. On the days 4th April and 28th May abnormally high NH_4-N+ and NO_2-N concentrations (1.79 and 1.84 mg/L, 0.36 and 0.26 mg/L) were measured from tank 10. NO_2-N showed to increase in tanks 8 and 9 a little bit until April, and after that, the concentration started to decline. Tank 10 concentration increased until the end of the experiment. The same trend was seen with NO_3-N, but in tanks 8 and 9, the concentrations stabilized on a certain level around April. On 21st March, and 25th April, feeding was paused for a day due to the weighing of the fish, which is displayed as a decrease in NH_4-N+ and especially with NO_2-N concentrations.

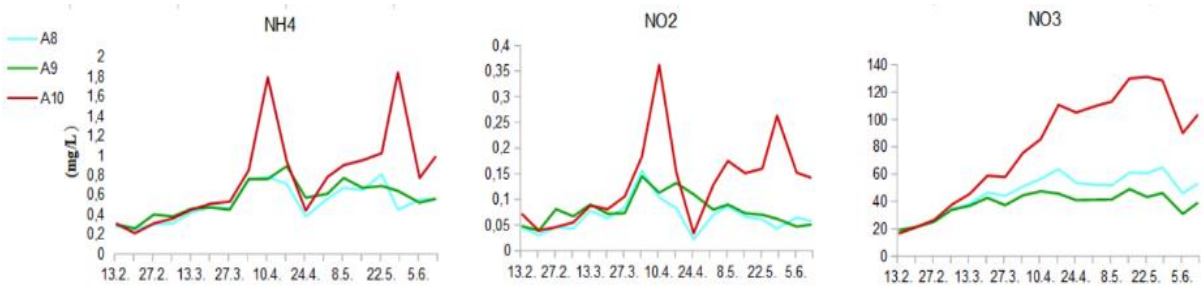

Fig. 3. Change in three nitrogen compounds in tanks over time.

4.1.1.2. Alkalinity and pH

Alkalinity values were stable in tank 8 and they were increasing a little in tank 10 towards the end of the experiment. Tank 9, which had the highest make-up water addition, alkalinity varied a lot during the experiment. pH did not vary drastically during the experiments in the tanks. Tank 8 pH was below 7.0 during the whole experiment, being on average (6.8 ± 0.1). the pH of 9 was (7.0 ± 0.2) and 10 (7.1 ± 0.1), being almost all the time just over 7.0. Tank 9 had the greatest variation.

4.1.2. Water quality post-analysis

4.1.2.1. DOC and TN

The concentration of TN and DOC matched well to the amount of feed given in all

three units (See Fig. 4. and 5.). In units, 8 and 9 there was barely any increase in these two concentrations during the experiment. DOC concentrations started to decline in all units after Mid-April. Feeding continued until June although the charts end at the end of May.

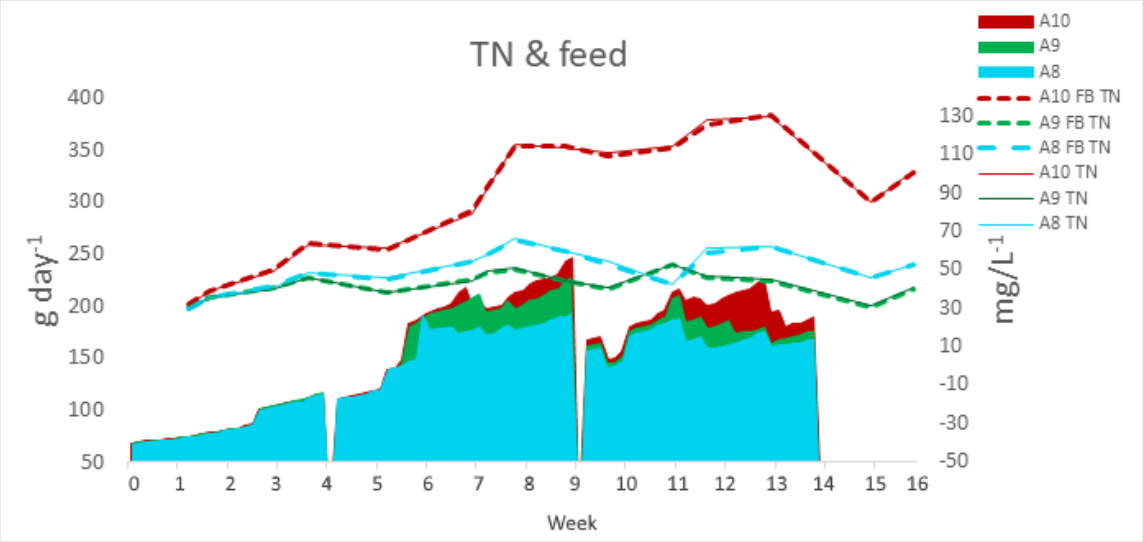

Fig. 4. Increase of TN concentrations during the experiment on the right vertical axis measured from the weekly tank (solid line) and fixed-bed samples (dotted line). The feed given to different tanks is on the left vertical axis that colored areas present.

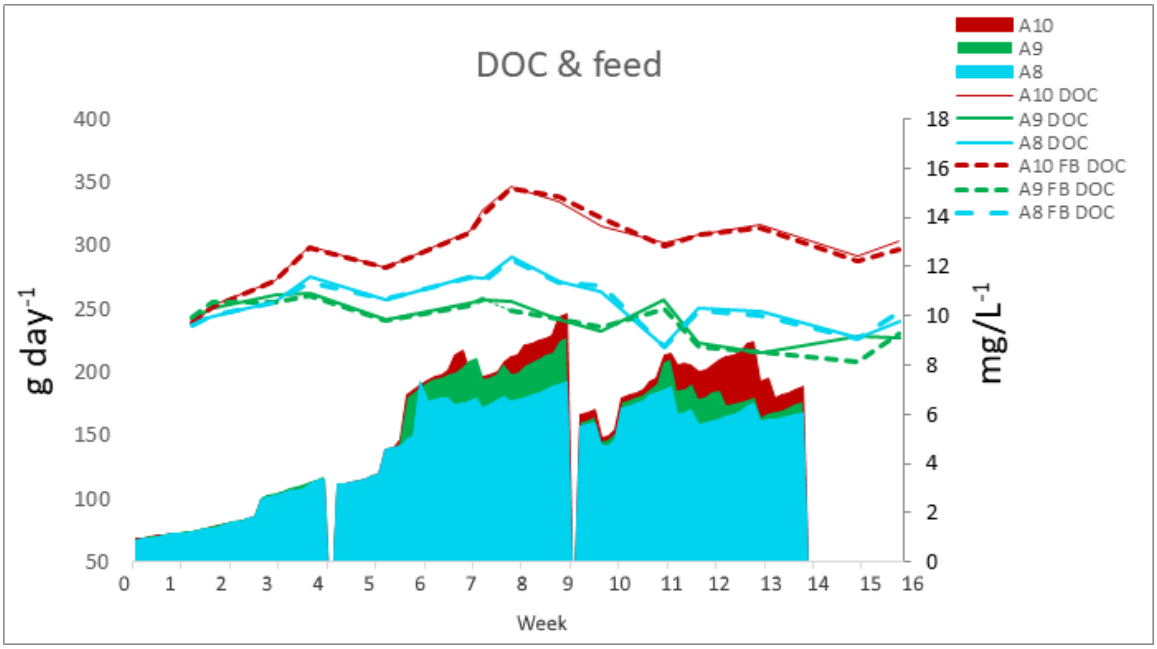

Fig. 5. Increase of DOC concentrations during the experiment on the right vertical axis measured from the weekly tank (solid line) and fixed-bed samples (dotted line). The feed given to different tanks is on the left vertical axis that colored areas present.

Unit 10 had highest concentration in TN and DOC concentrations (See Fig.6.). In TN concentrations there was a significant difference between the tanks (Friedman test, Chi-square = 24.2, df = 2, $p < 0.001$). The significance was found between tank 8 and 10 (Wilcoxon Signed-Ranks test, W = 0, $p < 0.01$), tank 9 and 10 (Wilcoxon Signed-Ranks test, W = 0, $p < 0.01$) and between 8 and 9 (Wilcoxon Signed-Ranks test, W = 8, $p < 0.01$). There was a significant difference between some of the tanks DOC (RM-ANOVA, $F(2,28) = 36.4$, $p < 0.001$). The significance was found between tank 8 and 10 (T-test, $t(13) = 6.8$, $p < 0.001$), tank 9 and 10 (T-test, $t(13) = 6.4$, $p < 0.001$). Tank 9's DOC concentration did not differ statistically from tank 8's values.

In fixed-bed biofilter samples, there were also significant differences in TN (Friedman test, Chi-square = 22.6, df = 2, $p < 0.001$) and in DOC (RM-ANOVA, $F(2,28) = 38.9$, $p < 0.001$). In TN values, the significance was found between tank 8 and 10 (Wilcoxon Signed-Ranks test, W = 0, $p < 0.01$), tank 9 and 10 (Wilcoxon Signed-Ranks test, W = 0, $p < 0.01$), and also between tanks 8 and 9 (Wilcoxon Signed-Ranks test, W = 10, $p < 0.05$). In DOC concentrations the differences were found between tanks 8 and 10 (T-test, $t(13) = 7.2$, $p < 0.001$), tanks 9 and 10 (T-test, $t(13) = 6.5$, $p < 0.001$) and tanks 8 and 9 (T-test, $t(13) = 2.9$, $p < 0.05$).

Combined data of units' tank water samples were compared with all the units' fixed-bed biofilter water samples, and no significant difference was found in DOC and nether in TN concentrations.

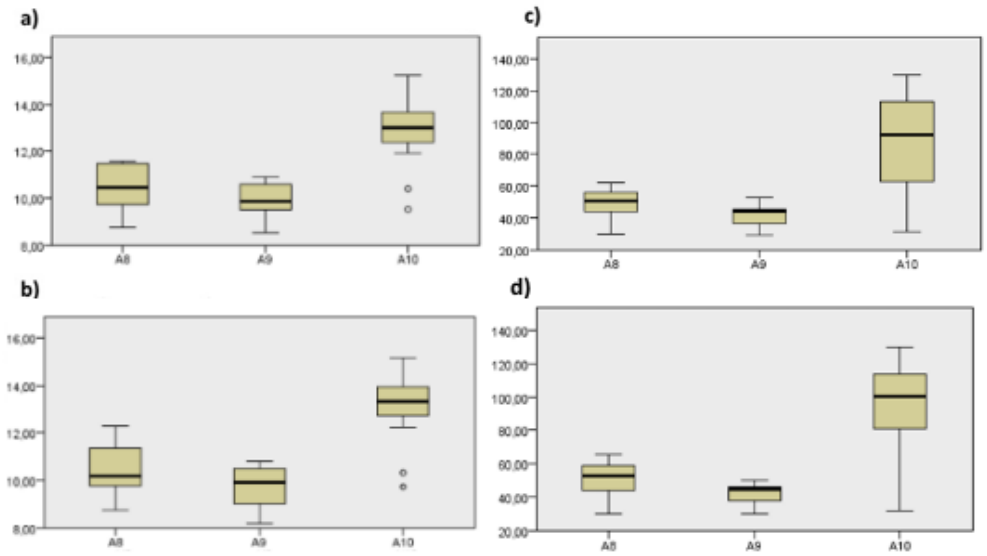

Fig. 6. Mean DOC concentration (mg/L) differences between the three units of the weekly a) tank water samples and b) fixed-bed biofilter samples and TN concentrations (mg/L) of c) tank water samples and d) fixed-bed bioreactor samples (n = 14).

4.1.3. Post measured conductivity and pH

Conductivity was increasing in all tanks during the experiment. 30th May there was a high pH value of 7.94 measured from tank 9, otherwise, the pH did not vary greatly.

4.1.4. Fluorescence chromatograms

In Fig. 7. is shown an example chromatogram of tryptophan-like fluorescence (270/355 nm) of make-up water and feed at the end of February and a fixed-bed biofilter sample of mid-April. The chromatogram was divided into seven fractions and was normalized by the intensity of the highest peak (fraction 6 of feed sample).

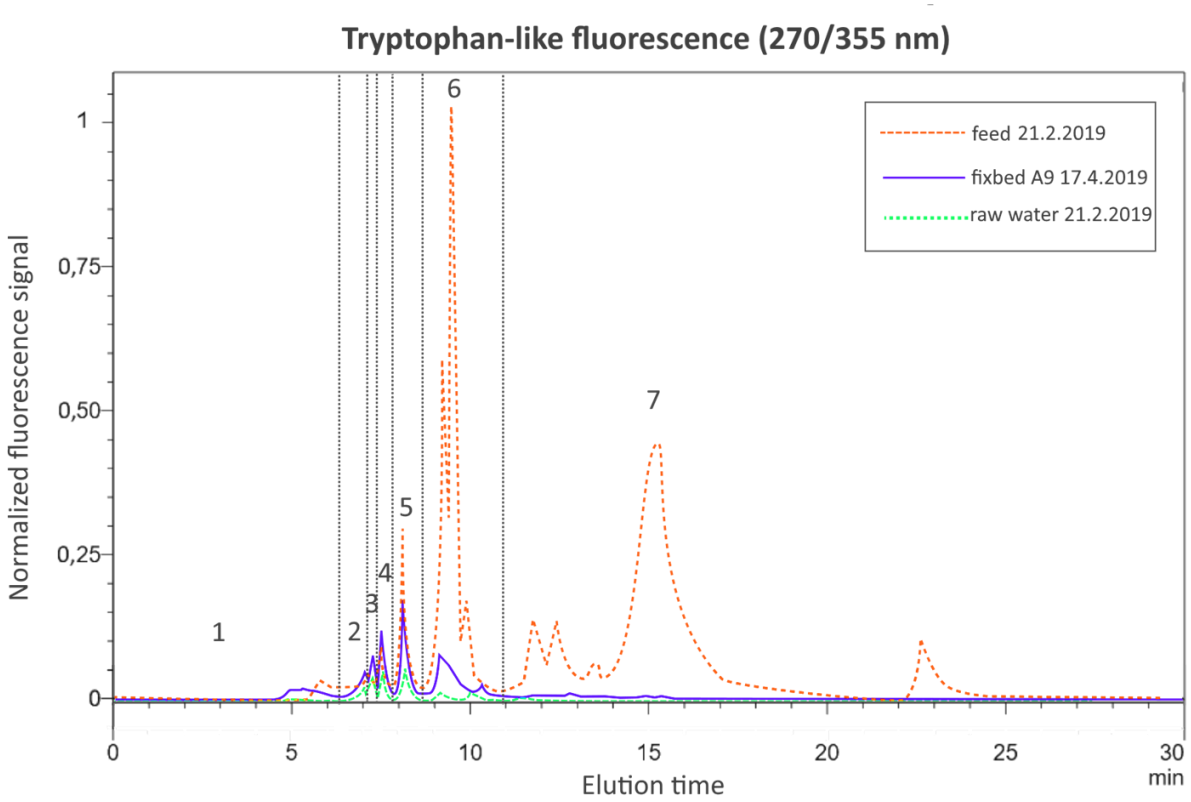

Fig. 7. The fluorescence chromatogram of tryptophan-like compounds (Ex./Em. 270/355 nm), from diluted feed sample, make-up water (raw water in the diagram) sample and fixed-bed biofilter sample from the end of February- The chromatogram has been divided into seven fractions marked as numbers over the peaks.

4.1.4.1. Accumulation of HPLC-SEC detected fluorescent DOM-components

In Fig. 8. – 12. there are changes of UV254 -absorbance intensities and fluorescence DOM-components of the intensity values of each tank and fixed-bed bioreactor samples, with the food consumption of each tank from the time of the whole experiment. HPLC-SEC analysis was ran starting from week 1 until week 16. Feeding was paused on three weighing days on weeks 4, 9 and 14. Fish were fed until the end of the experiment unlike shown in the following figures.

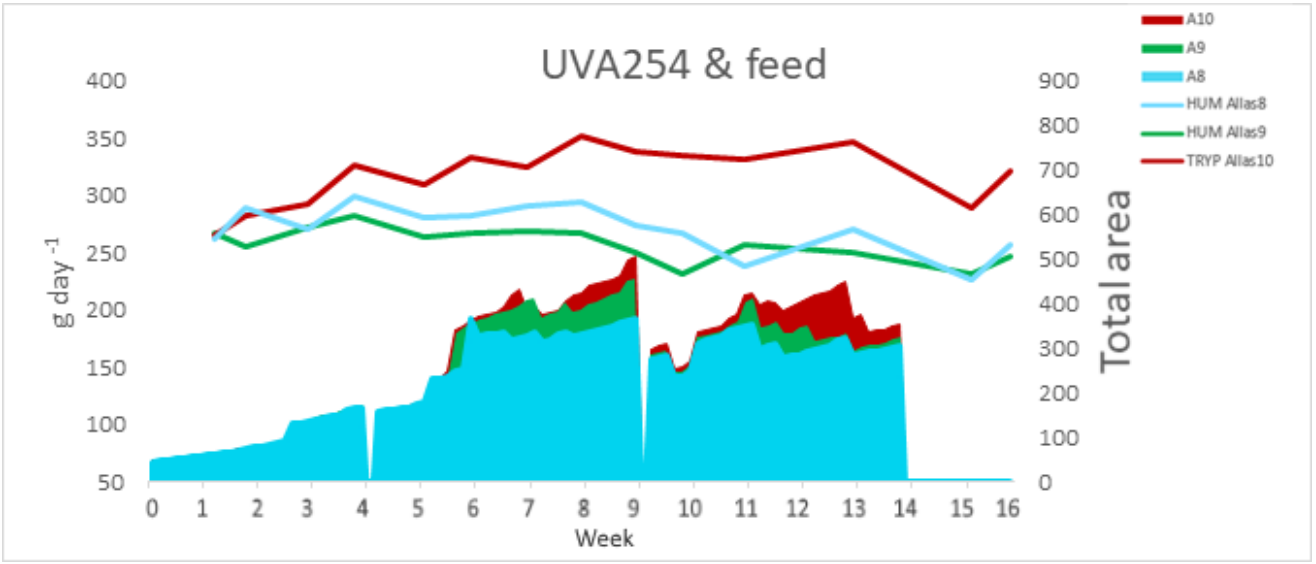

Fig. 8. Feed consumption on the left vertical axis and the corresponding total intensity value of UVA254 on the right.

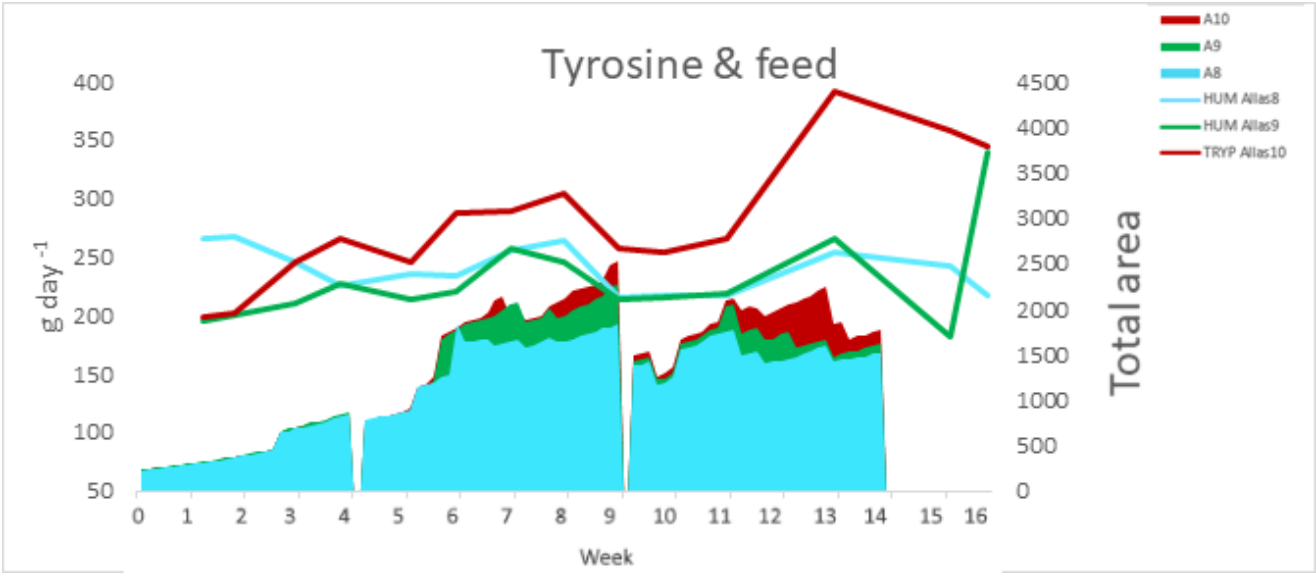

Fig. 9. Feed consumption on the left vertical axis and the corresponding total intensity value of tyrosine-like fluorescence on the right.

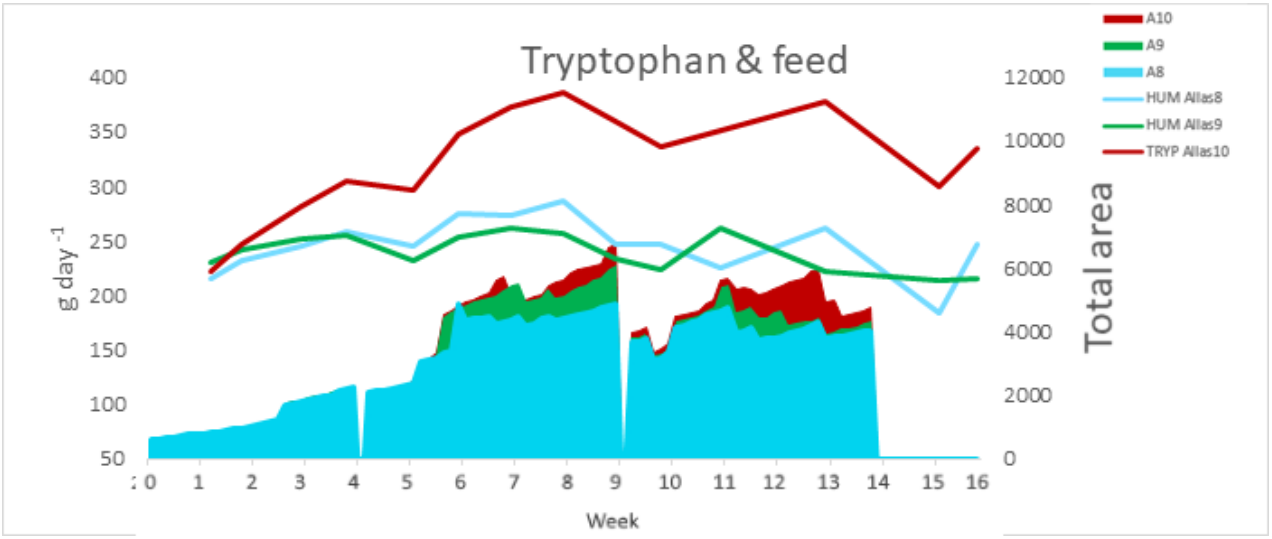

Fig. 10. Feed consumption on the left vertical axis and the corresponding total intensity value of tryptophan-like fluorescence on the right.

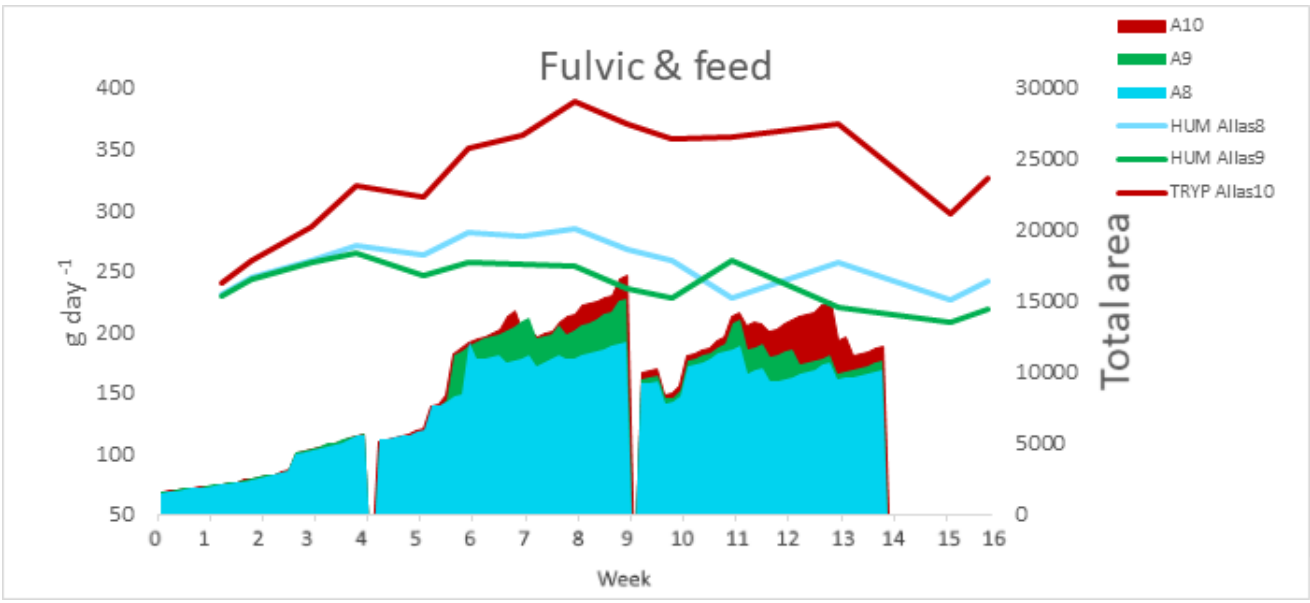

Fig. 11. Feed consumption on the left vertical axis and the corresponding total intensity value of fulvic acid-like fluorescence on the right.

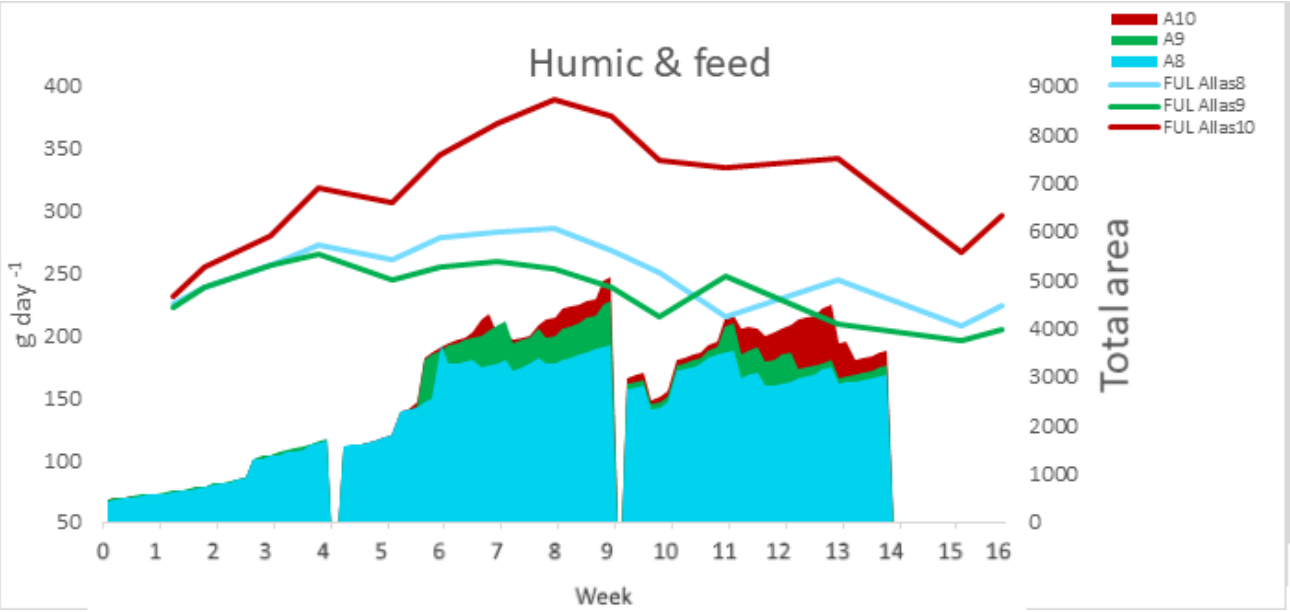

Fig. 12. Feed consumption on the left vertical axis and the corresponding total intensity value of humic acid-like fluorescence on the right.

The best fitting model for tanks 8 and 9 detected observations was a linear model and for tank 10 was a quadratic model. In UVA254 samples a significant but not that strong regression equation was found for tank 8: $F(1, 11) = 6{,}96$, $p < 0{,}05$ and for tank 9: $F(1, 11) = 9{,}06$, $p < 0.05$ with R^2 of 0.39 and 0.45 (See Fig. 13.). According to the linear model, UVA254 decreased per day in tank 8 by -0.62 $t(11) = -2.63$, $p < 0.05$ and in tank 9 by -0.67 $t(11) = -3.01$, $p < 0.05$ during the experiment. For tank 10 quadratic model showed significant regression: $F(2, 10) = 13.96$, $p < 0.01$ with high R^2 of 0.74. Tanks UVA254 increased: $b_1 = 2.93$, $t(10) = 5.19$, $p < 0.01$ until the mid-experiment and started to decline after that point: $b_2 = -2.65$, $t(10) = -4.69$, $p < 0.01$.

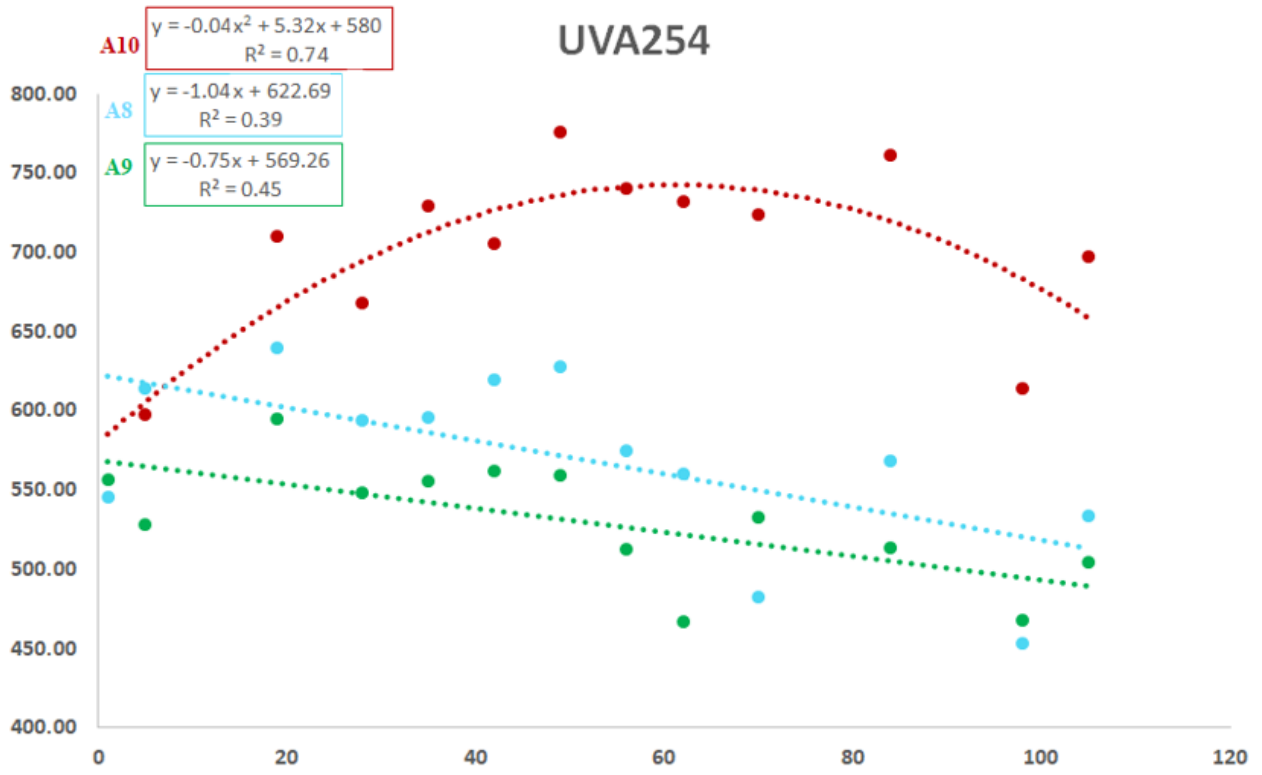

Fig. 13. Regression between UV254-absorbances divided by 1000 (y-axis) and time as the passed days of the experiment (x-axis). For tank 10 samples a polynomial regression model was used, while for tanks 8 and 9 samples a linear model was used.

Linear models in Fig. 14. of tank 8 and tank 9 did not explain the change in tyrosine-like fluorescence during the experiment, because the regression equation was not significant. Tanks 10 linear model, however, did explain increasing intensities during the experiment: $F(1, 11) = 24.43$, $p < 0.01$ with R2 of 0.69. The coefficient was 0.83: $t(11) = 4.94$, $p < 0.01$.

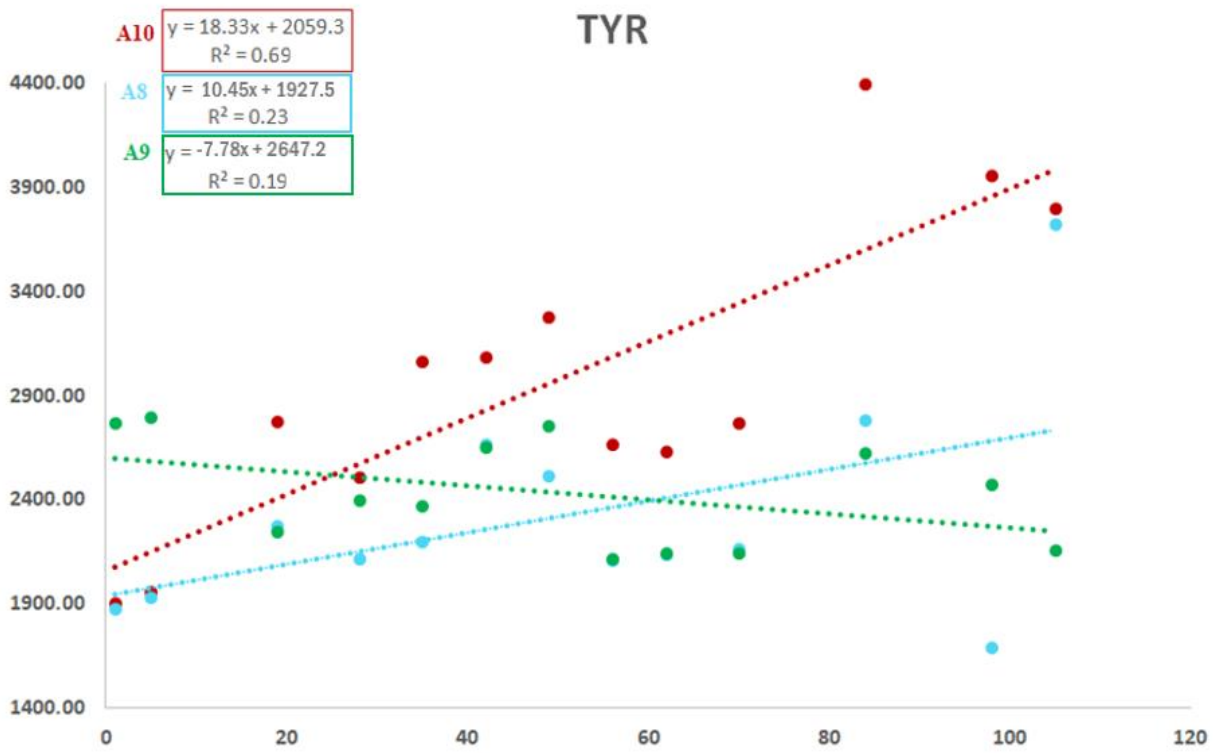

Fig. 14. Linear regression between tyrosine-like compounds fluorescence intensities divided by 1000 on the y-axis and passed days of the experiment on the x-axis.

The best fitting model for tanks tryptophan-like fluorescence was a quadratic model. In tryptophan-like fluorescence samples a significant regression equation was found for tank 9: $F(2, 10) = 5.14$, $p < 0,05$ and for tank 10: $F(2, 10) = 23.11$, $p < 0.01$ with R2 of 0.51 and 0.82 (See Fig. 15). Tank 8 regression equation was not significant: $F(2, 10) = 2.96$, $p = 0.10$. Time did not explain significantly the increase of tank 9's tryptophan-like fluorescence signal at the beginning of the experiment: $b = 1.30$, $t(10) = 1.69$, $p > 0.05$, but around mid-experiment there was significance in the decrease of the signal: $b = {}^{-1}.85$, $t(10) = -2.40$, $p < 0.05$. Tank 10 tryptophan-like fluorescence signal was increasing until around the 60th day: $b = 2.9$, $t(10) = 6.36$, $p < 0.01$ and started to decline after that: $b = -2.50$, $t(10) = -5.40$, $p < 0.01$.

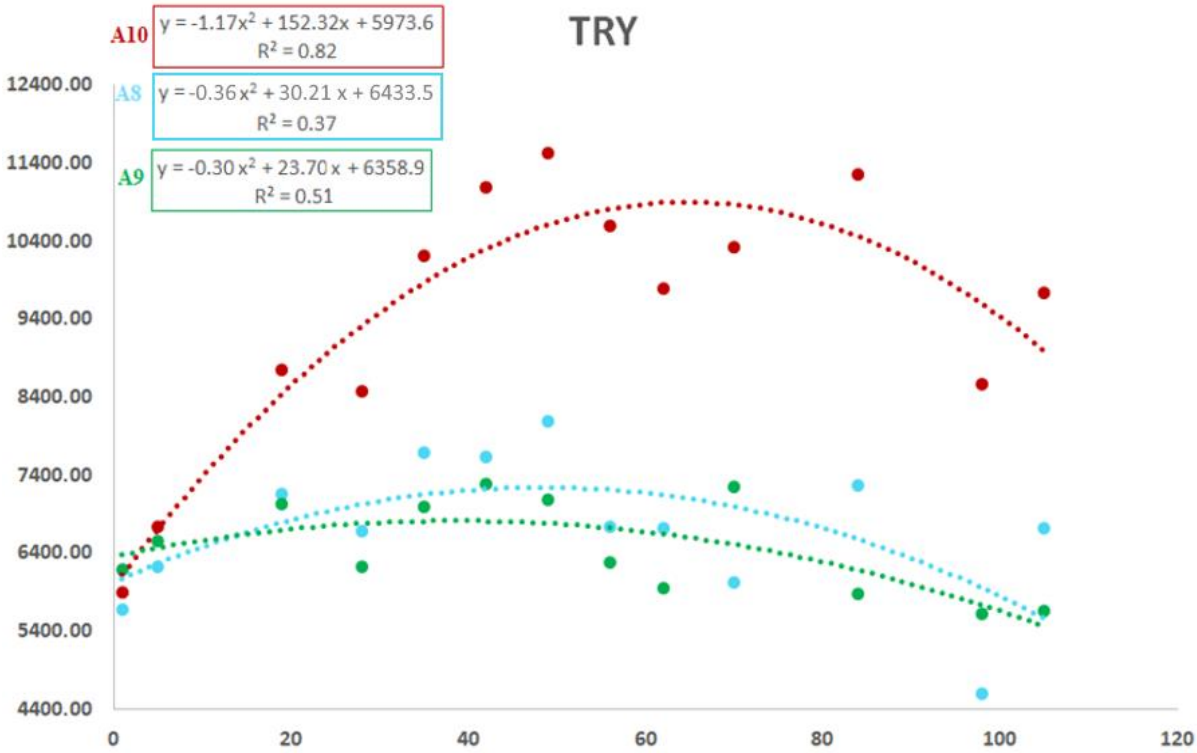

Fig. 15. Quadratic regression between tryptophan-like compounds intensities divided by 1000 on the y-axis and passed days of the experiment on the x-axis.

The best fitting model for tanks fulvic acid-like fluorescence was a quadratic model. In fulvic acid-like fluorescence samples a significant regression equation was for tank 8: $F(2, 10) = 5.35$, $p < 0.05$, for tank 9: $F(2, 10) = 7.58$, $p < 0.05$ and for tank 10: $F(2, 10) = 38.11$, $p < 0.01$ with R^2 of 0.52, 0.60 and 0.88 (See Fig. 16.). Tank 8's increasing regression equation was significant: $b = 1.96$, $t(10) = 2.57$, $p < 0.05$ and declining after the midpoint: $b = -2.32$, $t(10) = -3.04$, $p < 0.05$. Time did not explain the increase of tank 9's fulvic acid-like fluorescence signal significantly at the beginning of the experiment: $b = 1.22$, $t(10) = 1.76$, $p > 0.05$, but around until 40th day on there was significance in the decrease of the signal: $b = {}^{-1}.86$, $t(10) = -2.56$, $p < 0.05$. Tank 10 tryptophan-like fluorescence signal was increasing until around the 60th day: $b = 3.17$, $t(10) = 8.49$, $p < 0.01$ and started to decline after that: $b = -2.82$, $t(10) = -7.55$, $p < 0.01$.

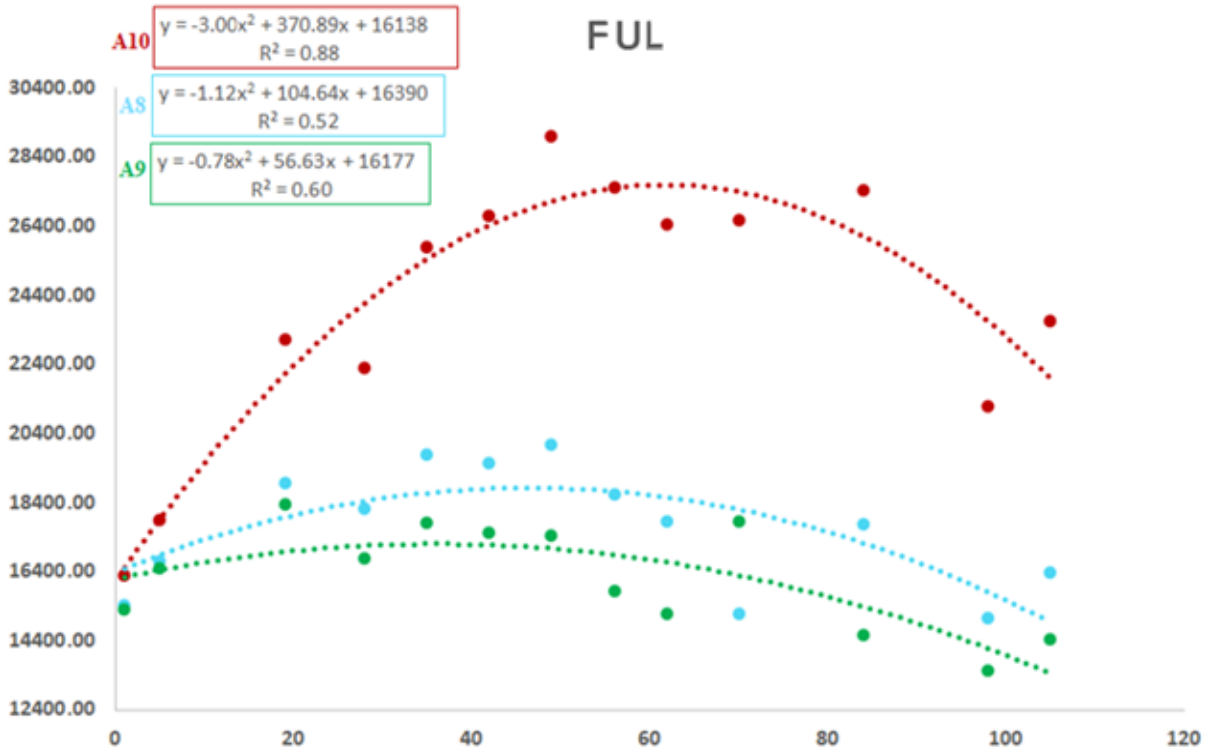

Fig. 16. Quadratic regression between humic acid-like compounds intensities divided by 1000 on the y-axis and passed days of the experiment on the x-axis.

The best fitting model for tanks humic acid-like fluorescence was also quadratic model. In humic acid-like fluorescence samples a significant regression equation was for tank 8: $F(2, 10)= 7.53$, $p = 0,01$, for tank 9: $F(2, 10)= 10.56$, $p < 0.01$ and for tank 10: $F(2, 10)= 23.15$, $p < 0.01$ with R^2 of 0.60, 0.68 and 0.84 (See Fig. 17). Tank 8 increasing regression equation was significant: $b = 1.82$, $t(10) = 2.63$, $p < 0.05$ and declining after the midpoint: $b = -2.31$, $t(10) = -3.34$, $p < 0.01$. Time did not explain quite significantly increase of tank 9 fulvic acid-like fluorescence signal at the beginning of the experiment: $b = 1.21$, $t(10) = 1.95$, $p = 0.08$, but until around 45th day on there was significance in decrease of the signal: $b = -3.06$, $t(10) = {}^{-1}.90$, $p = 0.01$. Tank 10 tryptophan-like fluorescence signal was increasing till around midpoint: $b = 3.18$, $t(10) = 7.23$, $p < 0.01$ and started to decline after that: $b = -3.07$, $t(10) = -6.98$, $p < 0.01$.

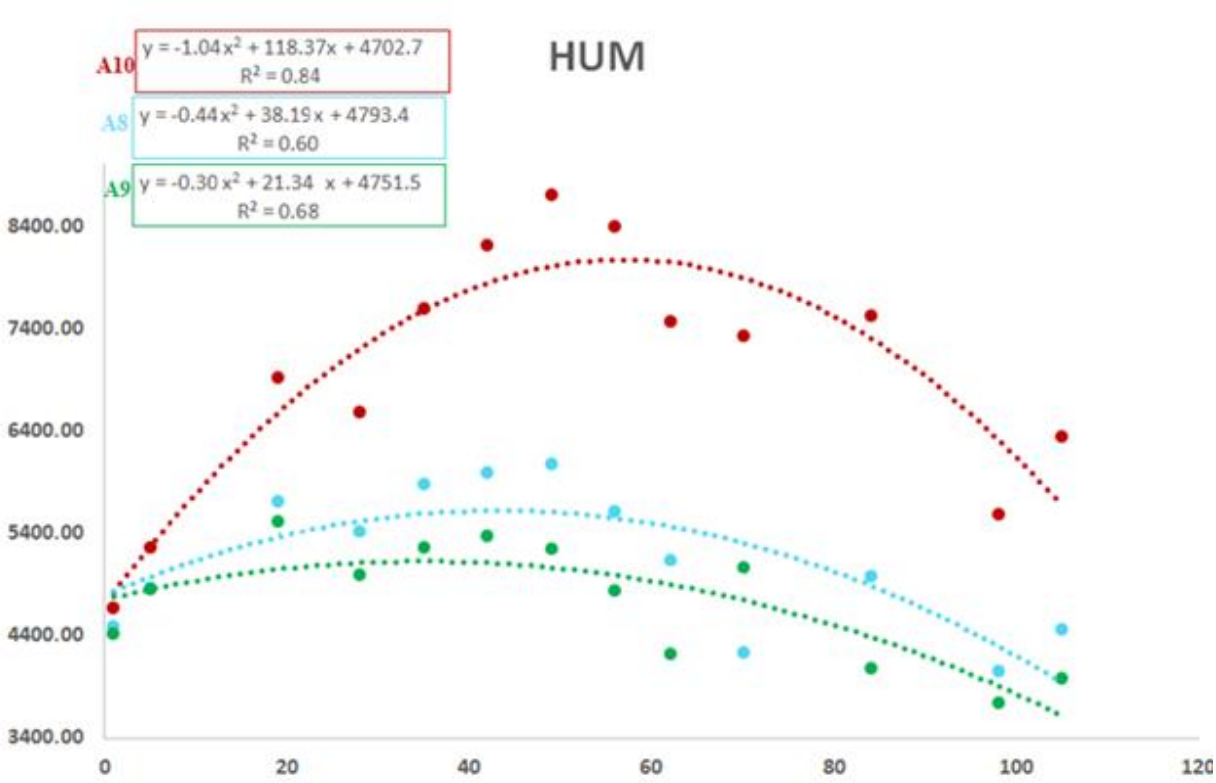

Fig. 17. Quadratic regression between humic acid-like compounds intensities divided by 1000 on the y-axis and passed days of the experiment on the x-axis.

4.1.4.2. Characteristic of DOM-components

Change of different DOM-components' six fractions (2 – 7) are shown in Appendix 4. for tank water samples and fixed-bed biofilter water samples. Some of the observations which had very high-intensity value are screened out because they were considered contaminated. There were observations from 14 weeks in total. The highest point of the most DOM-components signals occurred during the mid-experiment. Between tank and fixed-bed biofilter samples of every unit, there were no big differences in the trend of the fractions change. Thus, in tryptophan-like fluorescence compound samples of all fixed-bed biofilter units from 8th May, there was an abnormally high total area intensity value compared with other dates.

In UVA254, the absorbance of the fraction 2 decreased in all units during the experiment. In units 8 and 9 fractions 3 – 6 did not change during the experiment, except in unit 8 there was a minor increase in the signal of fraction 6. In unit 10 those

fractions increased, but the fraction 6 increased the most being two holds higher after the beginning of May compared with the beginning of March.

In tyrosine-like fluorescence, there was a lot of variance in different fractions' signals in all units. In unit 8 only small molecule fractions increased somewhat. In unit 9, all fractions seemed to decrease, except for the last fraction which increased towards the end of the experiment. In unit 10, all the fractions increased, especially fraction 4.

There was a similarity in units' fractions 2 – 4 of the tryptophan-like fluorescence signals that were the signal were low and did not change during the experiment. Fractions 6 and 7 did increase in 8 and 10, but in 9 only fraction 6 increased. Increasing of fraction 5 occurred in 10. In unit 10, all three fractions increase were noticeable.

In fulvic acid-like fluorescence fraction 2 decreased and fractions 5 – 7 increased in all units. Fraction 3 decreased somewhat in 8 and 9. The increase in fractions 5 – 7 were great in 10. Humic acid-like fluorescence signals change were very similar to fulvic acid-like fluorescence signals changes.

In all four DOM-components and UVA254, there were abnormally high total area intensities in fraction 7 in tank 8 samples taken on 13th March. The samples of that date have been removed due to that. Also, there were high intensity values in unit 8 tyrosine-like compound from the last sampling date 11th June and because of that, all unit is tyrosine-like signals have been removed from the tanks that date.

Differences occurred in UVA254 between the tanks (RM-ANOVA, $F(2,26) = 41.7$, $p < 0.001$). Tank 10 had significantly higher UVA254 compared with tank 8 (T-test, $t(13) = 6.0$, $p < 0.001$) and tank 9 (T-test, $t(13) = 7.5$, $p < 0.001$). There was also a significant difference between tanks 8 and 9 values (T-test, $t(13) = 3.3$, $p < 0.01$), tank 8 having higher signals. There were differences in tyrosine-like fluorescence compounds intensities (RM-ANOVA, $F(2,26) = 7.2$, $p < 0.01$). Tank 10 had significantly higher average intensities in comparison with tank 8 (T-test, $t(13) = 3.9$,

$p < 0.01$) and tank 9 (T-test, $t(13) = 2.4$, $p < 0.05$). Statistical differences did not occur between tanks 8 and 9. In tryptophan-like fluorescence there were differences in fluorescence intensities between tanks (RM-ANOVA, $F(2,26) = 40.6$, $p < 0.001$). Tank 10 had significantly higher tryptophan-like fluorescence total signal in comparison with tank 8 (T-test, $t(13) = 7.3$, $p < 0.001$) and 9 (T-test, $t(13) = 6.4$, $p < 0.01$). In fulvic-like fluorescence differences occurred (RM-ANOVA, $F(2,26) = 47.6$, $p < 0.001$), as well as in humic-like fluorescence (RM-ANOVA, $F(2,26) = 44.5$, $p < 0.001$). Tank 10 had significantly higher fulvic- (T-test, $t(13) = 7.1$, $p < 0.001$) and humic-like fluorescence total signals compared with unit 8 and unit 9 (T-test, $t(13) = 7.0$, $p < 0.001$). Differences occurred between tank 8 and 9 in fulvic-like fluorescence (T-test, $t(13) = 3.2$, $p < 0.01$) and humic-like fluorescence (T-test, $t(13) = 3.0$, $p < 0.05$). The statistical significances are included in bar diagrams shown in Figures 20 – 24, where one asterisk equals test significance level of $p < 0.05$, two of $p < 0.01$, three of $p < 0.001$.

The differences between intensity signals were greatest in tryptophan-, fulvic- and humic acid-like compounds, and in these compounds, fractions 5, 6 and 7 were significantly the greatest in 10 than in the other units. In tyrosine-like fluorescence, 10 had also significantly larger intensity in fraction 5. Variances between weekly samples were greatest in the smallest compounds, that fraction 6 and 7. presents. See Appendix 3. for more detailed box and whisker plot diagram of the DOM-components in different tanks, make-up water and diluted feed.

The absorbance of the second fractions UVA254 was the highest of all fractions UVA254 signals in all units. In unit 10, also the intensity of the 6th fraction was the highest while its standard error was high. Fraction 7th had the smallest intensity signals in all units. In tyrosine-like compounds, second and third signals were low and in fraction 5, there were the highest intensity values, which was higher in unit 10 compared with other units. The character of tryptophan-, fulvic- and humic acid-like compounds' fluorescence signals were similar, fractions 2 - 4 and 7 were the lowest, while 5 and 6 were the highest.

Overall, fulvic acid-like fluorescence accounted for the major contribution of the fluorescence signal of recirculating waters and make-up waters DOM (See Table 5.). In average the composition of DOM in recirculating water was: 7.4 ± 0.2 % of tyrosine-like, 21.5 ± 0.1 % tryptophan-like, 55.1 ± 0.1 % fulvic acid-like and 16.0 % humic acid-like fluorescence. Make-up water DOM character was very similar to recirculating water character.

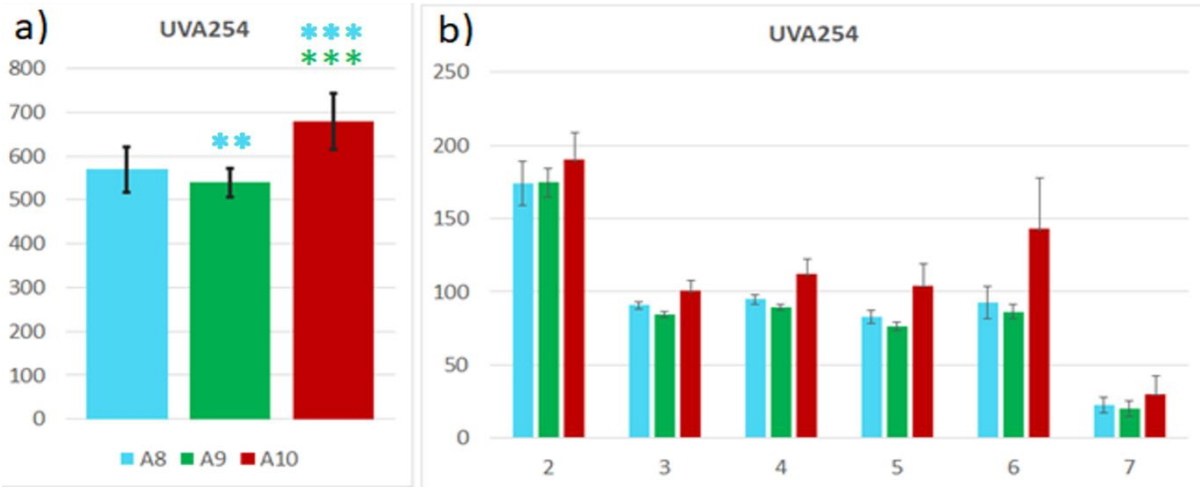

Fig. 20. a) The average total UV254 -absorbances of tanks' water samples and b) average absorbances divided into six fractions.

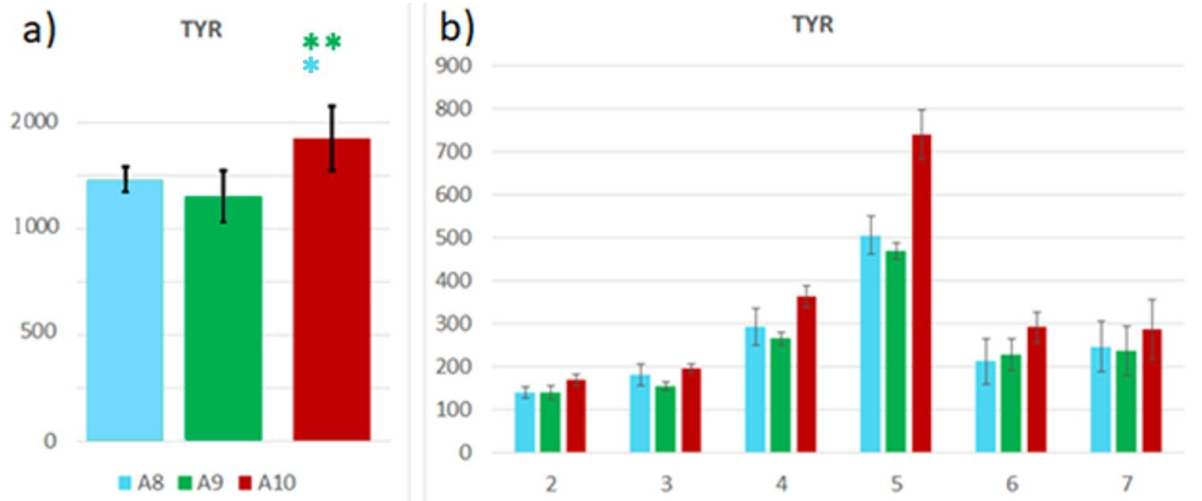

Fig. 21. a) The average total tyrosine-like fluorescence intensity of tanks' water samples and b) average intensities divided into six fractions from the whole experiment.

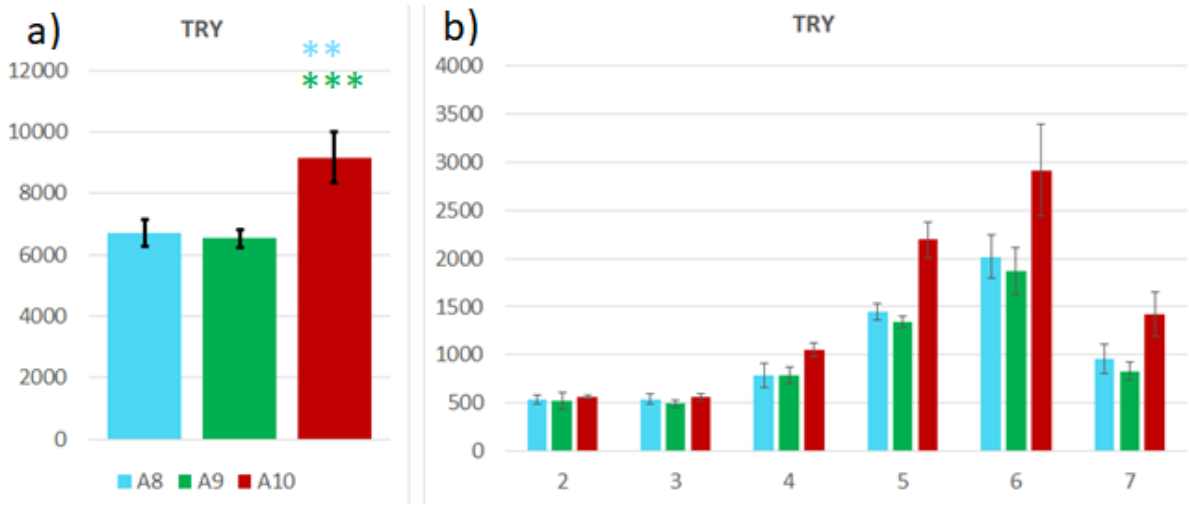

Fig. 22. a) The average total tryptophan-like fluorescence intensity of tanks' water samples and b) average intensities divided into six fractions from the whole experiment.

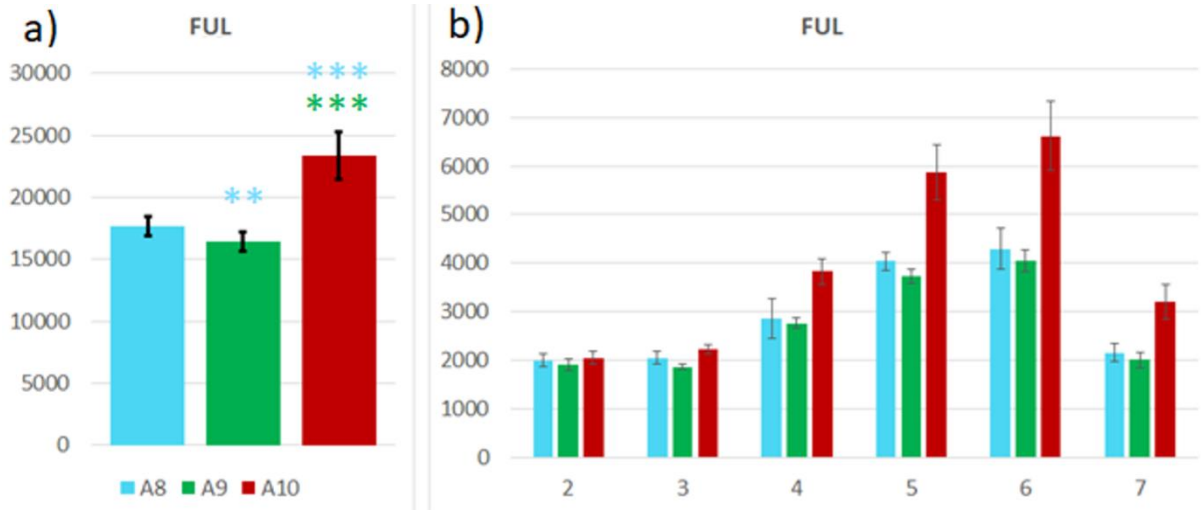

Fig. 23. a) The average total fulvic-like fluorescence intensity of tanks' water samples and b) average intensities divided into six fractions from the whole experiment.

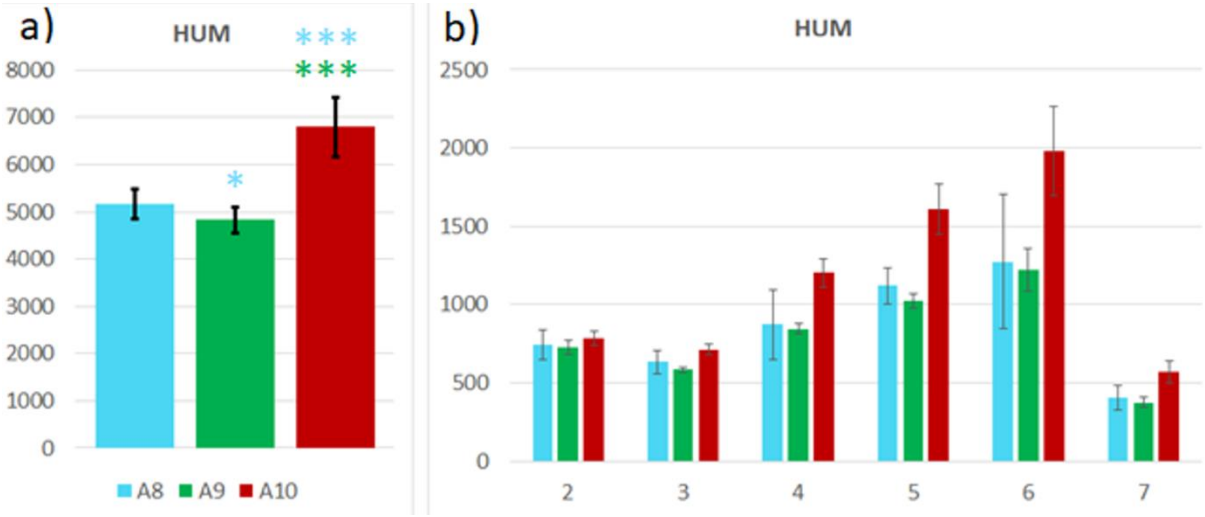

Fig. 24. a) The average total humic-like fluorescence intensity of tanks' water samples and b) average intensities divided into six fractions from the whole experiment.

Table 5. Composition of each tank fluorescence signal of all observations (n = 14) in percentage. Observations in make-up water were n = 5 and for feed only one.

Compound / **Sample**	Tyrosine-like	Tryptophan-like	Fulvic acid-like	Humic acid-like
8	7.2 ± 0.2	21.1 ± 0.1	55.5 ± 0.1	16.2 ± 0.1
9	8.1 ± 0.1	21.6 ± 0.1	54.4 ± 0.1	15.9 ± 0.1
10	6.8 ± 0.2	21.7 ± 0.2	55.4 ± 0.2	16.0 ± 0.2
Make-up water	9.4 ± 0.3	16.4 ± 0.8	58.9 ± 3.8	15.2 ± 5.1
Feed	58.2	30.5	9.1	2.1

4.1.5. Correlations between water quality parametric variables

4.1.5.1. Correlation matrix

Correlations between water quality parametric variables are shown in Fig. 25. All the observations of different tanks variable observations are combined in the matrix ($n_{variables}$ = 14, $n_{observations}$ = 36). All observations of different water quality parametric variables of tank 9 are removed from the date 17th April and 30th April because of two outlying alkalinity observations. Also, all observations of tank 8 variables are removed from 17th April because of outliers in NO_3-N and NO_2-N observations. The number of observations of each variable was n = 36. The positive coefficient is marked as bluish and negative pinkish. Two asterisks correspond very significance

($p < 0.01$) and one significant ($p < 0.05$) correlations. One should read the matrix with caution and focus only on bluish cells to find true correlations between two variables.

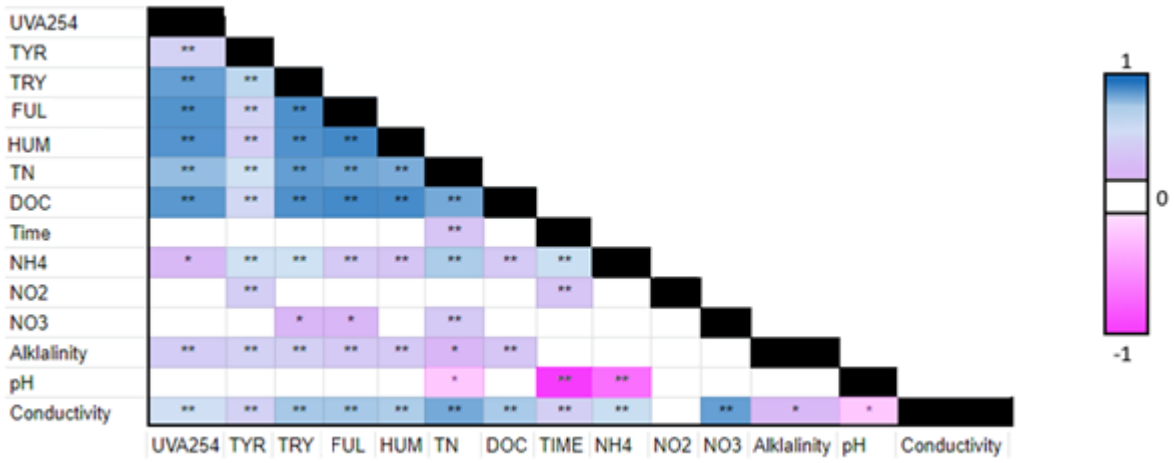

Fig. 25. Correlation matrix of water quality parameters coefficient of combined data of the tanks, where DOM-components' intensity, TN & DOC concentrations, time, nitrogen compounds' concentration, alkalinity, pH and conductivity are included (Spearman correlation, n = 14).

There was a significant, very strong correlation between DOM-components' intensities and TN and DOC concentrations, thus, correlations with tyrosine-like fluorescence were only moderate. Conductivity correlated strongly with all variables except NO_3-N, alkalinity, and pH. The correlation was significant between TN, time, NH_4-N+ and conductivity. Time had a weak negative correlation with TN, NH_4-N+, and NO_2-N. NH_4-N+ was the only nitrogen compound that correlated strongly with DOM-components.

4.1.5.2. DOC correlation with DOM-components

The correlations of DOC and different DOM-components are shown in Fig. 26.

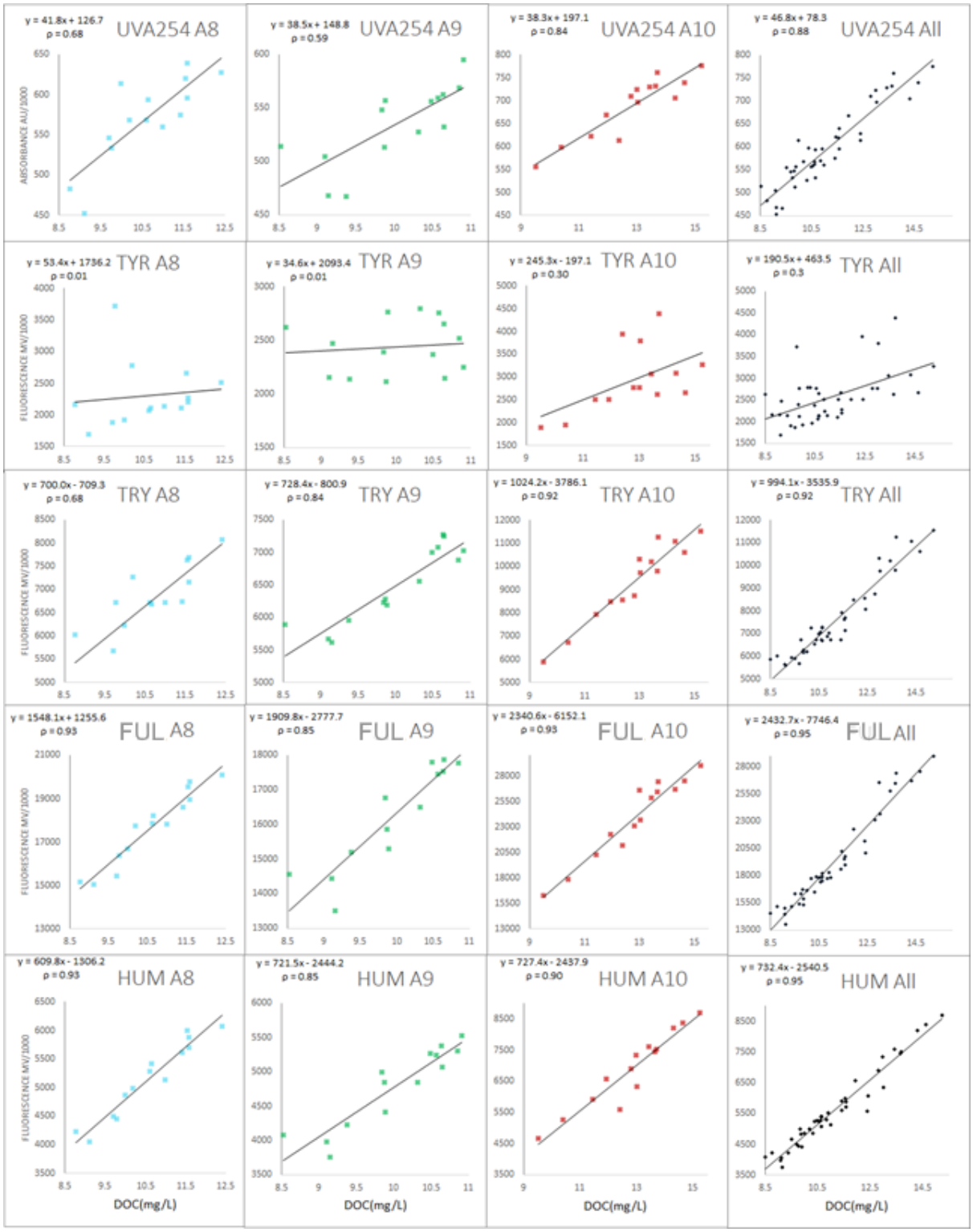

Fig. 26. Linear regression between each tank DOC (mg L), and UVA254, tyrosine-like, tryptophan-like, humic and fulvic acid-like fluorescence. The regression coefficient is marked here as "ρ". On the very right, there are graphs of certain rows combined observations named "All". Sample size was 14 for each variable observation and combined graphs 42.

An increase in DOC correlated well with the increase of UVA254, tryptophan-like, fulvic acid-like and humic acid-like fluorescence in all the tanks. Especially three last-mentioned showed a high correlation between DOC. Linear regression test showed a significant correlation between every fluorescence compound intensity and DOC in all the tanks, except with the tyrosine-like fluorescence compounds. Regression was significant for UVA254 in 8 ($df = 1$, $p < 0.001$), 9 ($df = 1$, $p < 0.01$), 10 ($df = 1$, $p < 0.0001$) and for all ($df = 1$, $p < 0.0001$). For tryptophan-like, humic acid-like and fulvic acid-like fluorescence coefficients were ($df = 1$, $p < 0.001$) very significant in all the tanks and in the combined graphs as well.

Linear regression between UVA254 and DOC were not strong in tanks 8 ($R2 = 0.68$) and 9 ($R2 = 0.52$) compared with tank 10 ($R2 = 0.83$). When all the tanks' observations were combined, the linear regression coefficient was high, with UVA254 ($R2 = 0.88$). In tyrosine-like compounds' intensities, there was no statistically significant linear regression between tyrosine-like fluorescence and DOC in any of the tanks, but in tank 10 the linear regression was almost significant. Thus, when observations were combined, the significance was found ($df = 1$, $p < 0.01$) with a quite low linear regression of $R2 = 0.3$. Correlation between tryptophan-like fluorescence compounds and DOC the linear regression coefficient was much lower in tank 8 compared with other tanks. In humic and fulvic acid-like compounds each tank coefficient linear regression was very high. Also, the combined data showed a high linear regression coefficient between DOC and tryptophan-, humic- and fulvic acid-like fluorescence.

4.2. Fish growth

Fish growth in the different tanks during the experiment from the start weighing on the 7th of February to the last weighing 29th of May is shown in Fig. 26. in kilograms. Mass of the dead fish is also included on the top of each bar. The survival rate of the fish in tanks 8, 9 and 10 were 66.3, 66.7 and 65.6 %, from those percent values infected or otherwise unhealthy fish, are removed. There were no significant

differences in FCR (Friedman test, Chi-square = 0.16, df = 2, p >> 0.05), nether in SGR% (Friedman test, Chi-square = 0.61, df = 2, p >> 0.05) between three tanks.

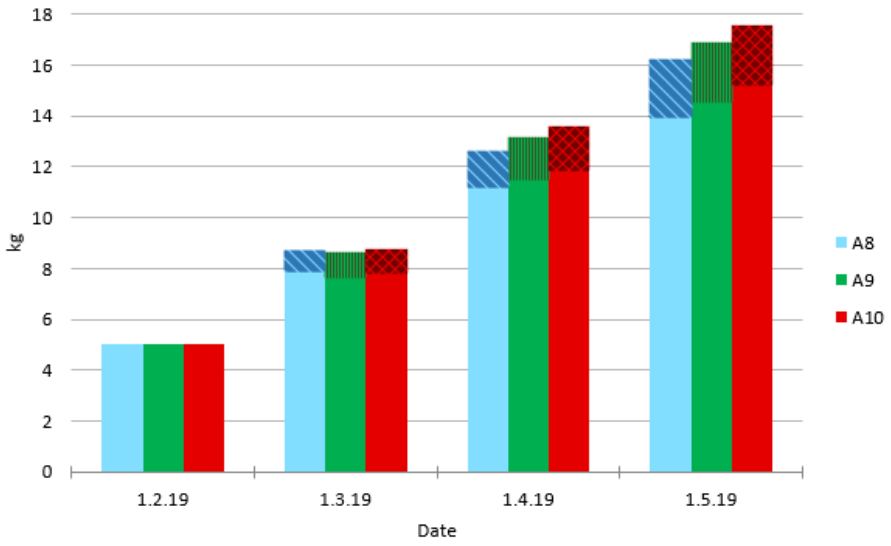

Fig. 26. Fish weighing dates on the x-axis from different tanks during the experiment. Darker patterned colors on top of the bars indicate the mass (kg) of the dead fish plus the removed fish from the period between that weighing date and the previous one.

5 DISCUSSION

5.1. Water quality

Statistical analysis for differences in units water quality parameters was done for the data of the whole experiment, where sample size was 14. Because there is a dependency in each unit's certain water parameters of different time of the experiment, repeated samples ANOVA and Friedman test had to be used. With sample size this small, there is a risk of getting type 1 error in statistical tests. With those tests, significant differences might be false positive between unit 8 and 9 water parameters, because their average water parameter values did not differ much from each other. On the other hand, unit 10 had clearly higher average values than two other units, so the values where truly highest in that unit.

Only in unit 10, where the make-up water addition was 3.9% of the total system volume per day, the water quality changed worse during the experiment significantly, when different water quality parameters such as nitrogen compounds, DOC and TN concentrations, and conductivity, and DOM-components signals were investigated. In other units increase of those parameters' values did not occur, or it was much lower than in unit 10. Water quality did not differ much between units 8 and 9, which indicates that in intensive RAS, the water quality is significantly worse than in a RAS of higher RWR. That way RWR seems to play a significant role in RAS water quality and DOM accumulation.

Water quality got better at the end of the experiment in all units compared with mid-experiment, which might be because of the more stable biofilter function of the units. Differences between each units' tank samples and fixed-bed biofilter sample parameters were minimal. Conductivity values were almost in every case higher in tank water compared with fixed-bed biofilter water of the same unit, but still, no significant differences were found between the tanks and fixed-bed biofilter conductivity values with paired samples t-test. Nether any significant differences in

DOM intensities between each RAS units' tank water and fixed-bed biofilter water were found.

5.1.1. Nitrogen compounds

Nitrogen compounds' concentrations were not analyzed from fixed-bed biofilter, and that for water samples of the bioreactors did not add much value to the data. This was not thought possible, because the online monitoring system was built only into the rearing tanks. In tank 10, where the RWR was smallest, there was significantly higher nitrogen compounds concentrations than in the other tanks. Tank 8 had higher average concentration in comparison with tank 9 in NO_3-N and NH_4^+-N; thus, the differences were small and can be caused by low sample size and because of the test used to analyze differences was rank test. Type 1 error might occur if null hypothesis of the tests were rejected, so the differences between tank 10 and the other tanks were only taken account.

NO_2-N levels did not increase towards the end of the experiment in tanks 8 and 9, while in tank 10 there was a minor increase in the concentration. NO_3-N and NH_4-N+ increased a little in tanks 8 and 9, and in tank 10 there was a bigger increase of these values, especially NO_3-N accumulated strongly, which is expected as it is the product of the nitrification process (Timmons et al. 2002). In Martins et al. study (2009), they found the accumulation of NO_2-N in intensive RAS and this has been observed in other studies as well (Schuler et al., 2010). At the end of the experiment, NO_2-N and NH_4-N+ concentrations were on a safe level for fish in tanks 8 and 9, but in tank 10 the NO_2-N concentration was relatively high. Timmons et al, (2002) wrote that NO_2-N should be below 0.1 mg L^{-1} to safe the fish health, but in tank 10 they were around 0.2 mg L^{-1} at the end of the experiment, while on one day the concentration was over 0.4 mg L^{-1}. On the other hand, some other research suggests that fish can tolerate even 1 mg L^{-1} concentration (Lawson, 1995; Pillay & Kutty, 2005). The concentrations of NH_4-N+ and NO_2-N were abnormally high on a couple of days in tank 10, but they were not risky high for the fish. It is hard to tell what the reason for the rapid increase of these concentrations could be, but they were

soon adjusted back to a tolerable level. NO_3-N concentration was well over 100 mg L^{-1} at the end of the experiment in tank 10, which should be below 80 - 100 mg L^{-1} according to Boreham et al. 2004 study. Thus, in their study, the negative effect occurred after three months of exposure. This might not cause problems, because salmonids are known to tolerate even higher NO_3-N concentrations in some study (Davidson et al. 2017) and the exposure time was much shorter than three months.

Higher concentrations of nitrogen compounds did not affect fish deaths, because there was approximately an even number of fish deaths between the RAS units, while tank 10's nitrogen compounds' concentrations were higher compared with two other tanks. Also, TAN was mostly in its safer form, NH_4-N+, according to Emerson et al. (1975) ammonia equilibrium calculations, where they calculated that in pH 7 at the temperature of 16 °C, only 0.29 % of the ammonia is in form of toxic NH_3-N. In this study, water pH was for almost the whole experiment below 7 and its temperature was between 14 to 16 °C. The results mean that it is possible, that nitrogen compounds will not accumulate in RAS operating greater than 500 L kg^{-1} feed d^{-1} of make-up water if just the biofilters are working properly. Pulkkinen et al. (2018) and Martins et al. (2009), found out that NO_2-N and NO_3-N accumulate more to the system when RWR decreases. On the other hand, ammonia level did not differ between high RWR operated RAS and intensive RAS in the previously mentioned study, while in this study unit 10's NH_4-N+ concentration was significantly the highest of the units. There is still a risk that intense RAS farming might lead to problems with fish health and growth by the increase of toxic nitrogen compounds' concentrations in water during the growing season because the season is typically longer than 105 days. This experiment was too short to explain the chronic toxicity responses of whitefish for the elevated nitrogen compounds and DOM concentrations. RAS operating over 500 L kg^{-1} feed d^{-1} could be considered stable in terms of nitrogen compounds and DOM accumulation, thus many things including feed consumption and rearing density may affect this. Also, different fish species have a different kind of tolerance towards nitrogen compounds'

concentrations, so these results can be applied only on whitefish. It is possible to add a denitrification process within the RAS, as Martins et al. (2015) did, which can increase nitrogen compounds' removal from the system, and that way help to maintain their concentrations in the lower levels. Intermittent fasting of fish might be considered also one option to stabilize nitrogen and DOM concentrations in recirculating water because the day-long pause in the feeding affected the nitrogen compounds' and DOMs concentrations. Some studies have found that the starvation of fish not necessarily decreases the growth of the fish (Moustafa et al. 2017).

5.1.2. TN and DOC concentrations

Tank 10 had the highest concentrations of TN and DOC, while tank 8 had significantly higher TN concentration in comparison with tank 9 values. Differences between the last mentioned were rather small. Considering the whole experiment, TN concentrations did not increase in tanks 8 and 9, except during the first half of the experiment and after that, they started to decline, which might be because of the stabilization of the purification process. In the tank, 10 there was a significant increase in TN concentrations, even though there was a clear fall in the concentrations at the end of April. This suggests that in RAS which is operated with RWR of 250 L kg^{-1} feed or less, the TN and DOC concentrations accumulate during the growing season. RAS operated with RWR of 500 to 750 L kg^{-1} feed, there seems not to be the accumulation of these concentrations. DOC monitoring can be used as a tool to get an idea of RASs microbial activity level since DOC concentration correlates with microbial activity (Kaplan & Newbold, 2000).

5.1.3. Other water quality parameters

The alkalinity values measured in online monitoring were the lowest in tank 8, and in 9 there was a huge variation of these values, due to three abnormal high values. If they were cropped out from the data, then tank 10 had about twofold higher alkalinity values of three units.

Online monitored pH was also the lowest in tank 8, being below pH 7 during the whole experiment, and tank 9 had the highest variation. Comparing online monitored pH to post-measured pH in the laboratory showed that post-measured values were a little bit higher, especially at the beginning of the experiment. In post-measured data, 9 had also relatively high variation. Online monitoring data is more precise from these two pH analyses, but the post-measured data confirmed the validity of the values of the online monitoring data. Monitoring pH constantly is important, because a rapid change in the values can lead to the increase of toxic NH_3-N concentration, which can then kill fish very quickly (Timmons et al. 2002). For example, in unit 9 the increase in pH within a week, from pH of 7 to 7.8 caused the NH_3-N concentration to increase in water over 6 times higher, according to Emerson et al. (1975) calculations. Relatively if the increase would have been for example, from pH of 7 to 8.6, the concentration of NH_3-N could have been 36 times higher, therefore, measuring also alkalinity is important, so the pH won't change rapidly in a short period.

Conductivity was measured also afterward in the laboratory. The values were increasing in all tank and fixed-bed biofilter samples during the experiment. Tank samples the values were always a little higher than in fixed-bed biofilter samples. This could be because moving-bed biofilter has heterotrophic bacteria living on the surface of plastic media, which are breaking down some organic compounds from water (Søndergaard M. & Middelboe M., 1995). Conductivity tells about the concentrations of total dissolved solids in water, and that way about how much DOM is present in water (Velichkova & Sirakov, 2013). Because of that, it is a good indicator that tells about the organic matter levels of water and running analyzes from it is easy to accomplish. Conductivity seemed to increase relatively to feed given and RWR affected its levels as well. At the end of the experiment, tank 10 conductivity was two-fold higher in comparison with other tanks. Tank 10 conductivity increased almost four times higher during the experiment. In all units, there was a drop in the conductivity values at the end of the experiment. In Martins

et al. (2009) study they found out also that in intensive RAS conductivity levels increase compared with RAS operating with higher RWR.

5.1.4. Accumulation of DOM in RAS

There were no significant differences between units 8 and 9 in protein-like fluorescent compounds' intensities. The differences in UV254-absorbance and fulvic acid and humic acid-like compounds were significant, thus, marginal. RWR being from 500 to 750 L kg^{-1} feed seems not to cause accumulation of DOM in RAS. The intensity signals of DOM compounds in these two units were on the same level or even below it at the end of the experiment compared with the beginning. In tyrosine-like compounds the differences were small and barely significant in comparison with unit 10 to other two units.

In the intensive RAS unit 10, DOM signals accumulated clearly comparing with other units and certain fractions were accumulating more than others. Accumulating DOM in recirculating water can be problematic since it decreases dissolved oxygen from water via increased microbial activity and fish increased respiration, which leads to an increase in DOC and BOD (Timmons et al., 2002; Pedersen et al. 2017). In this study, those variables were not measured and there were no signs of any problem caused by accumulating DOM. Also, a build-up of off flavor geosmin and MIB can occur (Blankheton et al. 2013).

The differences between tank water samples and bioreactor samples were minimal in DOM-components intensities, and it was a part reason why further statistical tests were not done for fixedbed-biofilters' data. Other reason was that processing the data would have taken too much time, where the benefits would have been small. Between the tanks and their corresponding fixed-bed bioreactors, the greatest differences were found in tyrosine-like compounds, which is considered coming into RAS via feed (Nimptsch et al. 2015). The DOM purification of fixed-bed biofilter was not prominent, and this can be explained with that heterotrophic bacteria living on biofilters can utilize only a very small portion of the available DOM in water

(Søndergaard M. & Middelboe M., 1995).

According the results in this study, accumulation of DOM seems not to occur in higher RWR units 8 and 9, but in low RWR RAS 10, there seemed to be some accumulation because intensity signals were significantly higher in unit 10 compared with other units. The increase of DOM occurred even at the very beginning of the experiment, which is strong evidence of accumulation because in that part of the time of the experiment all the units had the same feed consumption rate until the end of March. The increase in unit 10 intensity values was much higher than in other units, even though the amount of feed given were the same. This means that the purification systems in higher RWR units were able to keep the water quality stable. In earlier studies, it has been found that the lower the RWR is in RAS, the greater the organic matter accumulation is in the system (Pulkkinen et al. 2018).

Some of the DOM-components were at the lower level at the end of the experiment compared with the beginning in units 8 and 9. It is likely that because of the amount of feed given did not increase from the beginning of May, so did not the intensity signals either. The other reasons might be that organic compounds were consumed in the system by heterotrophic bacteria or the biolfilters performance simply increased during the experiment. The tyrosine-like signal seemed to increase linearly in RAS 10 until the end of the experiment and was twofold higher at the end of the experiment compared with the beginning. This might suggest that the tyrosine-like signal is the most prone to accumulate in intensive RAS, because its total fluorescence intensity had the linear increase and it was not purified within the unit 10 like the other DOM fluorescence components. It is likely, that the level of tyrosine-like DOM compounds would have stabilized on a certain level if the experiment would have been carried further. To eliminate accumulated DOM, ozonation could be used to control its level in recirculating water (Summerfelt et al., 2007 & 2008, Wang et al., 2017).

There were many data points, over 3000, in the HPLC-SEC analyses, which made the data processing slow and challenging. More specific charts and statistical

analyses could have been done, but the thesis' extent had to be framed somewhere. Differences between tanks' and the corresponding fixed-beds absorbance and fluorescence intensities could have been further studied, but because of there were only marginal differences, purification effectivity was not studied. The results were reliable, except for a few outliers which were removed from the data. It is more likely that those abnormal values' cause is analytical, because the values did not fit to previous and next weeks analyzed values of certain sampling point. On top of that, there were no replicate treatments of the RWR, so abnormal values had to have been discarded. Replicates was not used in this study, because there were not enough resources for that and HPLC -analysis would have been more difficult to organize.

Chromatogram results from 14th May were excluded from the final data because they had too high fluorescence intensities compared with other samples from different weeks. The last observation from 11th June of tank 8 water sample of tyrosine-like fluorescence, was removed because it differed significantly from the rest of the data and made the data skew. Also, samples of 13th March of tank 8's fraction 7 were removed, and samples of 8th May of the same tank has been removed due to the abnormally low intensity values. Samples of tank 8 were the firstly analyzed samples in HPLC-SEC, and that for they were the most easily contaminated, which can be seen in the number of discarded samples of the final data.

5.1.5. Regression models of DOM

The regression models of tank 10 samples were quadratic like in UVA254 and all, but tyrosine like DOM-components. In tanks 8 and 9, tryptophan-, fulvic-, and humic acid-like compounds' regression model were also quadratic. In UVA254 tanks 8 and 9 models were decreasing linearly (See fig. 13.) and those regression models were statistically significant, which means the biofilters were able to purify recirculating water from UVA254 fraction of DOM from those two units. In tyrosine-like fluorescence, any of the models were not fitting the observations.

Those observations had some outliers and weekly intensity values varied greatly. A higher make-up water addition towards the end of the trial would have explained why DOMs intensity values curved downwards at the end of the experiment, but the feed ratio to makeup water added was constant. Also, the overall volume in the system did not change significantly. The decline in DOM-components signals from the mid-experiment towards the end of the experiment might have been caused by the increased activity of heterotrophic bacteria, which can consume fluorescence DOM compounds to smaller compounds and the increased purification effectivity of the biofilters in general among the experiment. There was a decline in intensity values on the weighing days because the feed was not given that day. This partly explains the sudden decline of fluorescence intensity values. Besides, at the end of the experiment fish were not fed as much compared with the mid experiment, which made the intensity values decline.

5.1.6. Characteristics of DOM in Laukaa's RAS

About 55 % of the total signals of all fluorescence compounds were formed of fulvic acid-like fluorescence, about 22 % of tryptophan-like fluorescence, about 16 % of humic acid-like fluorescence and only about 7 % of tyrosine-like fluorescence. The difference between each units' tank water and fixed-bed biofilter water samples DOM characteristic were minimal. The relative composition of the fluorescence signals of the different compounds was somewhat different in unit 9 compared with other units. In unit 9, the amount of tyrosine-like fluorescence was higher with 8.1 ± 0.1 compared with 8 with 7.2 ± 0.2 and 10 with 6.8 ± 0.2. On the other hand, the fulvic acid-like fluorescence ratio of the total fluorescence signal was in unit 9 the lowest being 54.4 ± 0.1 compared with 8 with 55.5 ± 0.1 and unit 10 with 55.4 ± 0.2. This small difference might be caused by the higher degradation of protein-like organic compounds in lower RWR RAS units compared with unit 9, due to possibly higher microbial activity or just with higher RWR that the unit 9 had. DOM concentration in RAS correlates with microbial activity, which breaks protein-like organic matter into smaller products. Tyrosine is known to be more degraded

peptide material from the two protein-like material studied in this study (Fellman et al. 2010), which can explain the higher amount of tyrosine in high unit 9. On the other hand, tyrosine-like fluorescence seemed not to be removed from unit 10 water at the end of the experiment in comparison tryptophan-like fluorescence.

The changes in different fractions DOM characteristics were seemingly different in unit 10 compared with other units that shared similar trends in DOM-components change over time. The accumulation of each fraction can be found when examining higher intensity signals in unit 10 fractions compared with other units because that unit has the lowest RWR. UVA254 compounds fraction 2 signals decreased during the experiment in all units. There were minor or no changes in fractions 3 – 6 of UVA254 in units 8 and 9, but in A 10 those fractions increased while fraction 6 increased the most seemingly. In tyrosine-like fluorescence compounds, there were a lot of variances and in 9 all the fractions' signals decreased somewhat during the experiment. In contracts, in unit 10, all the fractions increased. This suggests that in low RWR RAS the increase in protein-like fluorescence is remarkable. In unit 8, there was an increase only with fractions 5 and 7. In tryptophan-like fluorescence nether of 2 – 4 fractions changed during the experiment in any unit. In units 8 and 10 fractions 6 and 7 increased, but in 9 only fractions 6. Fulvic- and humic acid-like fluorescence compounds change were like each other. In all units, fraction 2 decreased and 5 – 7 increased, but the trend turned down after the midpoint of the experiment. Fraction 3 decreased in 8 and 9, but not in 10. All the fractions of different DOM-components which signals were higher in RAS 10 compared with other units, were the ones that accumulated in the more intensive RAS.

In UVA254, the second fraction had about twofold higher signals in total intensities compared with fractions 3 to 6, except in unit 10 the signals of the sixth fractions were much higher in comparison with fractions 3 - 5 and in comparison with the signals of sixth fractions of the other units. Infraction seven, all the signals were rather small. Strong UV absorbance at fraction 6 might suggest increased carboxyl and amino acid formation in microbiotas biological processes, which was the case

in Jarusutthirak & Amy's research in 2007 about wastewater treatment plants microbial products. In Fig. 7. seen example chromatogram smaller fractions (6 and 7) were present in the diluted feed sample while fraction 7 signal was tenuous and fraction 6 less magnitude in recirculating water.

It has been studied earlier that small molecule size fractions (4 – 7) account in wastewater a significant amount of dissolved organic nitrogen, which contains many disinfection by-products (Pehlivanoglu-Mantas & Sedlak, 2008). That for an increase in these small molecule size fractions might indicate an increase in disinfection by-products in water. On the other hand, that study was accomplished for water treatment plants processed effluent, which differs from aquaculture wastewater.

5.1.7. Make-up water and diluted feed DOM character

Make-up water samples' DOM signals' intensities were rather high at the beginning of the experiment. The DOM signal of water taken from the lake might variate seasonally. In Spring melting snow can flush more terrestrial organic material to the lake. In the later part of the experiment, there was only one sampling of make-up water in April and others in May. The character of the make-up water was almost the same with recirculating water. In UVA254 the make-up water had a little lower absorbance values in comparison with recirculating water UV254-absorbance of DOM. In diluted feed bigger molecular weight components (fractions 2 – 4) absorbance values were much lower in comparison with recirculating water values, but smaller molecular fraction values, especially in fraction 7, the values were much higher.

In protein-like fluorescence DOM, make-up waters character was close to recirculating water which suggest that at least some of the protein-like fluorescence DOM is coming from the lake into the RAS. In the diluted feed samples, the protein-like fluorescence intensity was many tenfold higher than in recirculating water, and it consisted mostly of tyrosine-like fluorescence, while recirculating water consisted

more of tryptophan-like compounds. Feed samples proteinous DOM character is in a line with the results of Yamin et al. study (2017), where tyrosine-like compounds dominated the feed sample character. On the other hand, in that study tyrosine-like compounds were not found from the water samples and the authors suggest that tyrosine-like compounds are degraded in the system. Fractions 5 and 7 of tyrosine-like fluorescence were relatively the highest in the diluted feed sample, while in tryptophan-like fluorescence fraction 7 was the highest. There for, fraction 5 of tyrosine-like fluorescence seemed to accumulate in unit 10 via feed. In tryptophan-like fluorescence small molecular size components accumulated the most in unit 10.

In humic- like components, make-up water had clearly lower intensities of DOM. The smaller the molecular weight was, the bigger the difference was. Again, smaller molecular weight components of diluted feed sample were more present in the intensity values, but only in fraction 7 the values were higher in comparison with recirculating water values. Smaller molecular weight components accumulated the most.

Recirculating water DOM character contained the bigger share of tryptophan-like fluorescence, which proves together with feeds high tryptophan-like fluorescence content, that tryptophan-like fluorescence origin is mostly feed, which is found in other studies (Yamin et al. 2017). There are similar observations in the literature that the make-up water is the main source of fulvic/humic acid-like DOM in the RAS (Stedmon et al. 2007; Walker et al. 2009; Kothawala et al. 2013), which can be also concluded from this study.

5.1.8. Water parameters correlation

Many variables are somehow linked to each other, so the correlation with one variable does not necessarily mean that there is a true correlation between these two variables. Association does not necessarily mean a causal relationship between both variables. For that reason, one must read the correlation matrix with caution and focus on the very strong correlation coefficients ($p > 0.8$). However, some variables

didn't correlate much with other variables, so in those cases, even the moderate correlation coefficient can be taken to account.

TN and DOC correlated well with DOM-components. In Tuhkanen's and Ignatev's study about wastewater (2019), they also found a linear correlation between tyrosine- and tryptophan-like fluorescence, DOC and UVA254. In this study, TN correlated with NH_4-N+, NO_3-N, and alkalinity and strongly with conductivity. DOC correlated also with conductivity, and somewhat with NH_4-N+ and alkalinity. Two variables correlated with each other.

The strong correlation between DOC and DOM-components' intensities suggest that studying the DOC concentrations can give a picture of the overall DOM concentration, which has been found also in previous studies (Ryder et al. 2012; Lee et al. 2015). Also, on the other perspective online fluorescence monitoring can be used to study DOC concentrations (Wasswa et al. 2019).

All the fluorescence compounds intensities correlated strongly with each other, except tyrosine-like fluorescence, which didn't seem to have a strong relation to other compounds. In RAS 10, it still had a moderate correlation coefficient with tryptophan-like fluorescence. RAS 10 had a strong correlation between fluorescence compounds and feed consumption, which was not seen with the other tanks. This was also found in Hambly et al. study (2015) that the increasing feeding results in an increase of fluorescence signals. Protein-like fluorescence is mostly coming to the system from the given feed (Hambly et al., 2015). The reason for that might be that DOM was accumulating in RAS 10, which did not occur in other RAS units. DOC also had a high correlation between TN, NO_3-N and feed, and little weaker correlation with NH_4-N+ and conductivity. In Hambly et al. (2015) study, they found out also that DOC correlates linearly with feed consumption. Conductivity correlated somewhat with fluorescence compounds, DOC, TN, NH_4-N+ and NO_3-N, and in 10 also with feed consumption. DOM had a strong linear correlation with UVA254, tryptophan-, and fulvic- and humic acid-like fluorescence when all the tanks' data were combined. Tyrosine-like fluorescence had too many outliers to be

tested, and even without the outliers, the correlation coefficient would have been weak.

5.2. Fish growth

The overall fish growth in the RAS units was between 8950 to 10190 grams, being the highest in RAS 10. Nevertheless, there were no differences in whitefish growth in this study between the units. There were only three weighings during the experiment and no individual weighs were measured, so the sample size was too small to find any meaningful differences. If it would have been possible to weigh every fish separately, differences could have been found between the fish FCR and SGR. Unit 10 had the highest median weights and SGR and the lowest FCR, but the results were not even nearly significant. In Pulkkinen et al. 2018 study, they found out that in lower RWR the FCR increased and the SGR relatively decreased, which is quite an opposite result compared with this study results. One-third of the fish died or were removed due to illness during the experiment, which is quite a high number for RAS. This is too high a percentage if considering profitable RAS farming. It is unclear why the mortality rate was that high. Mortality increased towards the end of the experiment and might be caused by nitrite poisoning or pathogens. Two important factors when studying fish growth are dissolved oxygen concentration in tanks and oxygen saturation. Especially oxygen saturation level correlates with fish growth and a feed conversion rate (Mallya, 2007). In this study, these variables were not measured, but in future studies, it is important to include these variables if studied fish growth in RAS.

6 CONCLUSION

To conclude this master thesis, the water quality was significantly worse in intensive RAS unit 10 compared with the other two units. Some differences were found between units 8 and 9, but they were minimal and might be false positive due to the types of statistical tests used and small sample size. This suggests that intensive RAS farming, which was 250 L^{-1} kg in this study, causes accumulation of DOM and nitrogen compounds and DOC & TN concentrations in the recirculating water. The optimal make-up water addition is there for somewhere between 500 to 750 L^{-1}kg being close to 500 liters in terms of water quality and optimal farming. It is good to remember that intensive farming might still cause the accumulation of GSM and MIB in fish tissues as well. There might more likely to be infection outbreaks in intensive RAS or declined fish health if the growing season is longer than 105 days. In reality, the growing season is much longer than the length of this experience, so the accumulation of DOM can pose problems to intensive RAS.

The results showed that the DOC and TN concentrations and HPLC-SEC analyzed DOM-components' intensities were almost similar between samples from rearing tanks and fixed-bed biofilters. TN concentration was 2.2-fold higher in unit 10 compared to unit 8 and 2.6-fold higher compared to unit 9 concentrations, at the end of the experiment. DOC concentration and total UV254 -absorbance were about 40 % and fluorescence signals intensities about 60% higher in unit 10 in comparison with other units' values. Differences in units' 8 and 9 values in these values were minimal. The most accumulating fractions of UVA254 were fractions 3 – 6, of tyrosine-like fluorescence fraction 5, of tryptophan-like fluorescence small molecular weight components (fractions 5 – 7), and of fulvic and humic acid-like fluorescence also smaller molecular weight components (fractions 4 – 7).

It was unclear if the microbial flora changed during the experiment because the microbial analysis was not done in this study. The literature suggests that even a small increase in DOM can cause a shift in the RAS microbial community (Pinhassi

et al. 1999). The presence of high DOM in RAS 10 could have favored coexisting heterotrophic bacteria at the expense of nitrification bacteria, which could have weakened the TAN and nitrite purification efficiency of nitrification bacteria. When there is a lot of DOM in water, the heterotrophic bacteria are known to conquer the space on the biofilter much faster than nitrification bacteria (Grady and Lim, 1980).

Accumulation occurred with fluorescence DOM-components, UV254 -absorbance, NH_4-N+, NO_3-N, NO_2-N, TN and DOC in the unit of the lowest RWR, unit 10. Conductivity increased constantly during the whole experiment also in that unit. Units 8 and 9 UVA254 decreased linearly during the experiment. Tyrosine-like fluorescence did not change in these units. Other fluorescence DOM-components increased a little on the first a couple of weeks but curved down towards the end of the experiment on the level, which was lower than at the beginning of the experiment. Thus, those compounds did not change statistically during the experiment. The function models in DOM were quadratic like for unit 10, where the values increased until the mid-experiment and then curved downwards. This trend was because higher RWR units' purification system were able to stabilize the water quality during the experiment. Pause in feeding between one and two days on the weighing days caused at least DOM, UVA254, and NH_4-N+, NO_2-N to decrease temporary.

DOM characteristic was similar between the units and between each unit tank and fixed-bed biofilter samples. Make-up water shared as well similar DOM characteristics. Most of the fluorescence DOM found in RAS water was fulvic acid-like, 55.1 % on average. 21.5 % of the DOM was tryptophan-like, 16 % humic acid-like and only 7.4 % tyrosine-like fluorescence compounds. Diluted feed sample had high protein-like fluorescence character, and at least tryptophan-like fluorescence was coming to recirculating water from the feed, while the fulvic/humic acid-like fluorescence was coming from the lake via make-up water, on top of that, it was probably created within the system. It seemed that humic- and fulvic acid like DOM was also accumulating to the RAS in all the units, at least on the couple of first

weeks, because DOM signals were lower in make-up water in comparison with recirculating water.

More research is needed to study different fish species growth in differently managed RAS. Furthermore, a longer growing season than 105 days, could be studied to investigate the possible negative effects of impaired water quality due to intensive farming with low water exchange and the accumulation of DOM in intensive RAS. Also, the effect of ozonation on water quality and DOM characters change in recirculating water could be studied with HPLC-SEC analysis used in this study. The theme has economy-related possibilities to study the financial profitability of RAS farming in Finland. Online DOM monitoring, which is making its way in the market, can be used to study DOM character in real-time. This can be used to prevent some infection outbreaks or water quality declining at an early stage.

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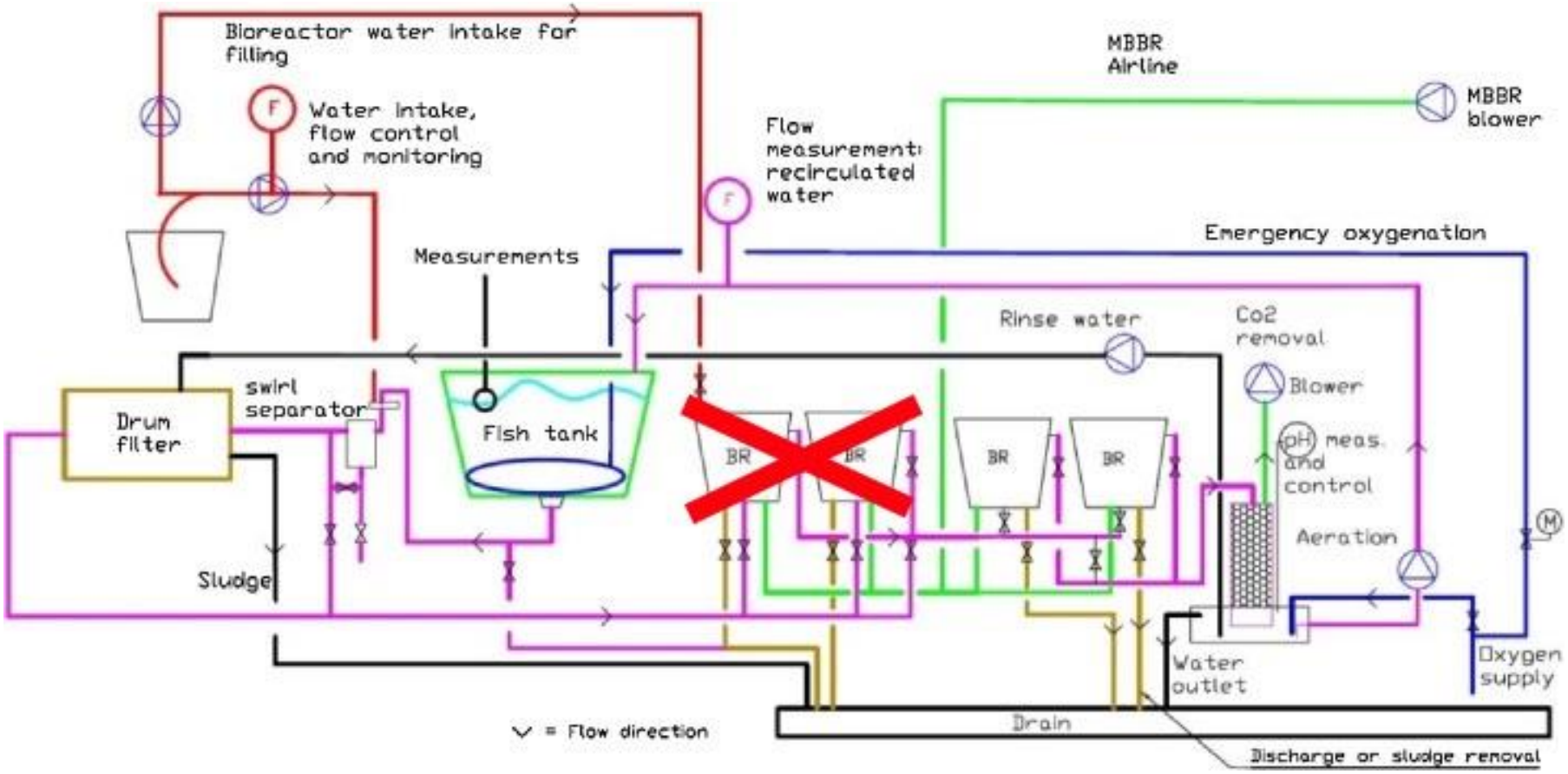
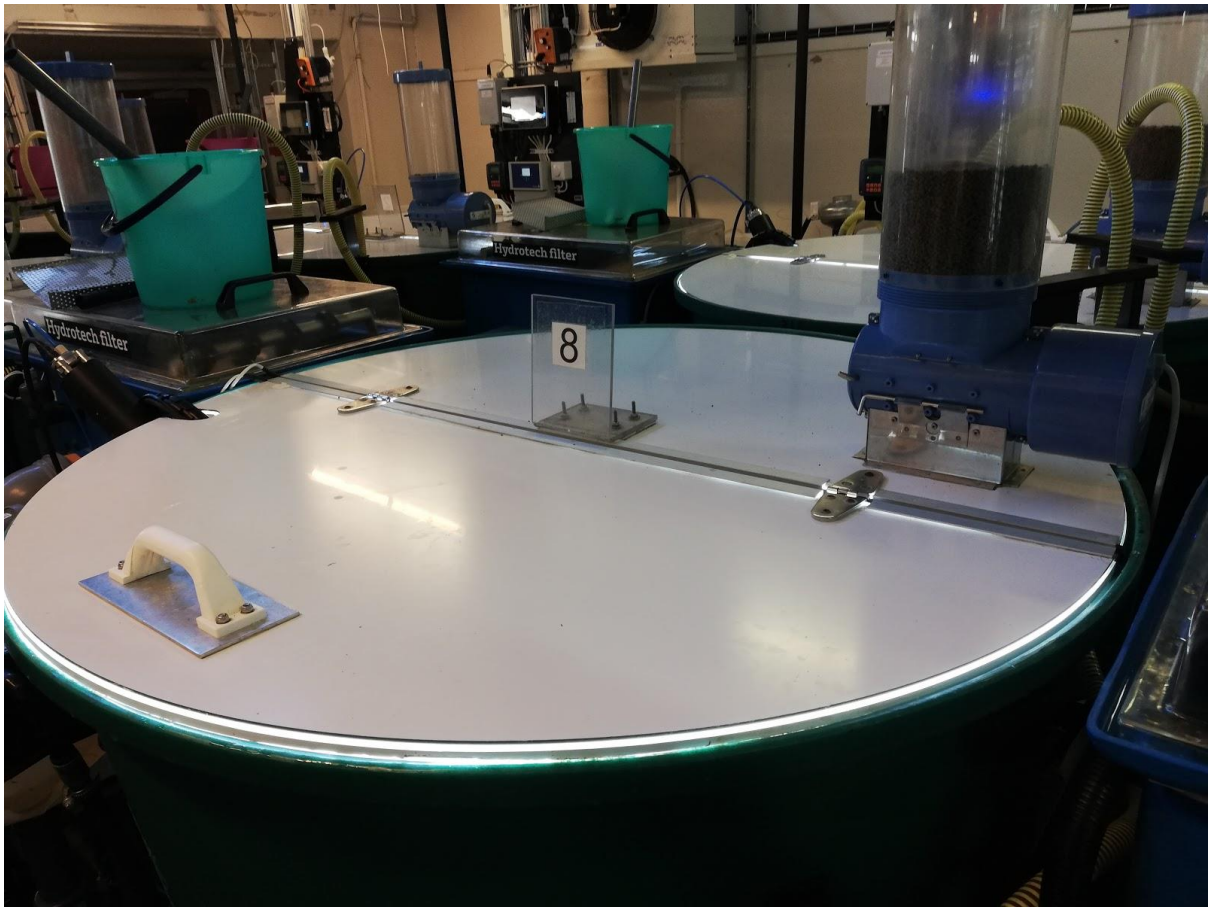
APPENDIX 1: The process chart of Laukaa's RAS facility

In the process chart, only two bioreactors were in use in this study, they were serial linked so that the first was fixbed-bioreactor and the second moving-bed bioreactor. The flow direction is marked with arrow heads.



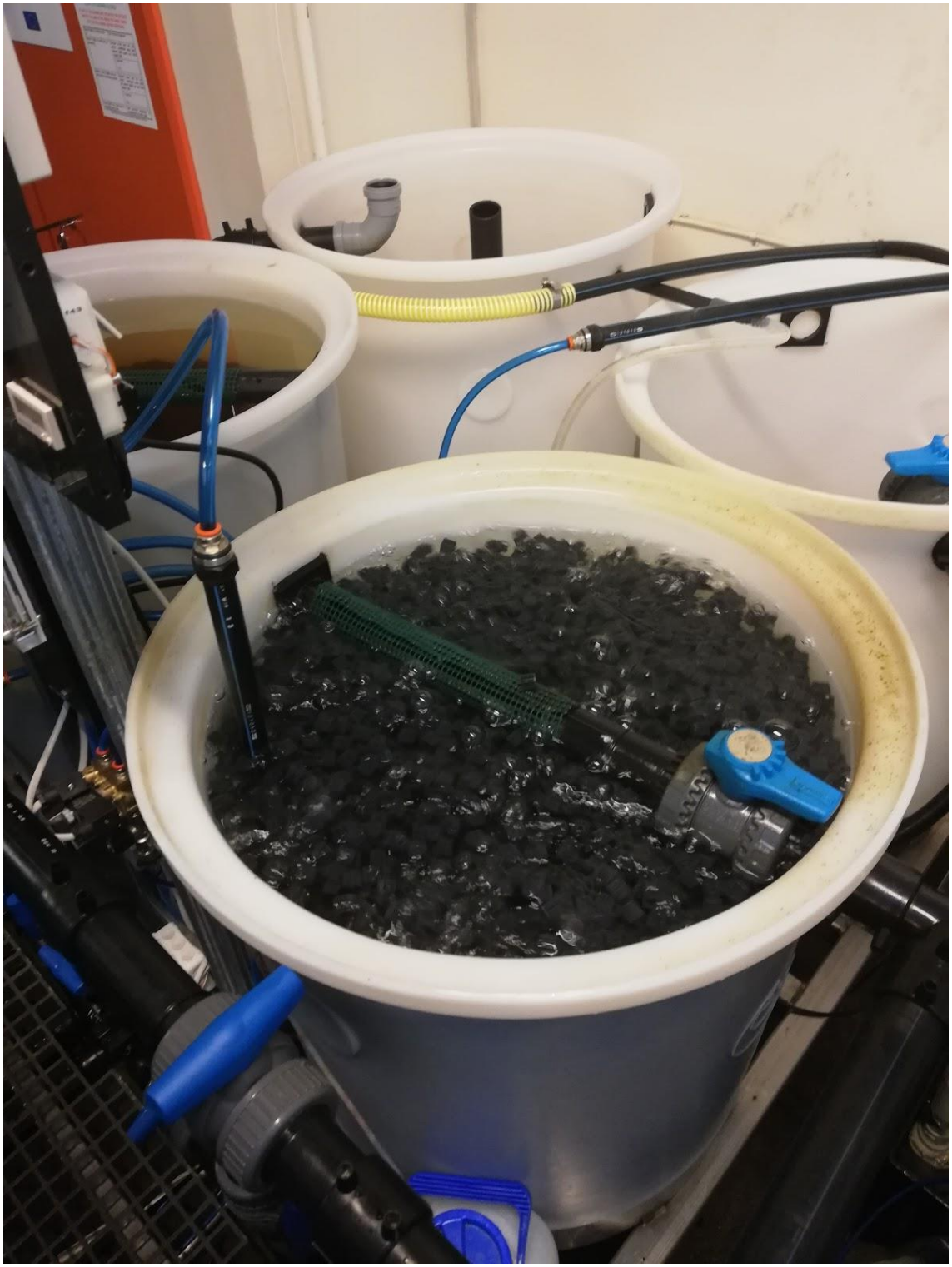
APPENDIX 2: Pictures of Luke's RAS system in Laukaa

Numbered RAS units side by side. In the front of the picture can be seen 1. a drumfilter (inside the blue container), 2. swirl separator in the middle-bottom, 3. green rearing tank with 4. an automatic feeder on its white cover and on the right 5. online monitoring system linked to the rearing tank. Bioreactors were on the right site of the online monitoring systems in the picture.



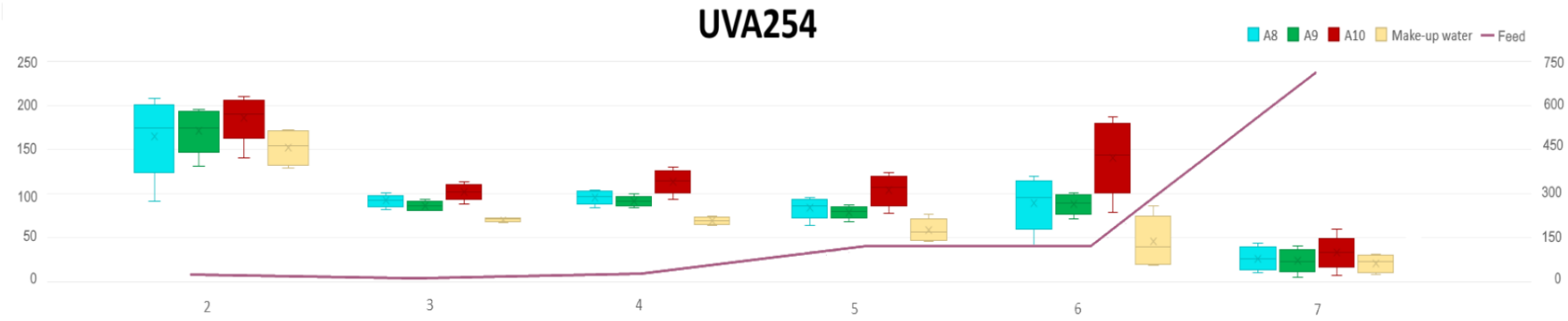
A closer picture of tank 8. The automatic feeder was emptied and feed was weighed once a week. That way the feed consumption for each week was calculated. See light coming from the gap between the cover and tank; tanks were lit 24 hours in a day.

A closer picture of the online monitoring system. Each rearing tank had its own system, so the water parameters, such as NH_4-N, NO_2-N, NO_3-N, alkalinity and pH, were in constantly monitored from one monitor. In the back, there are some bioreactors.

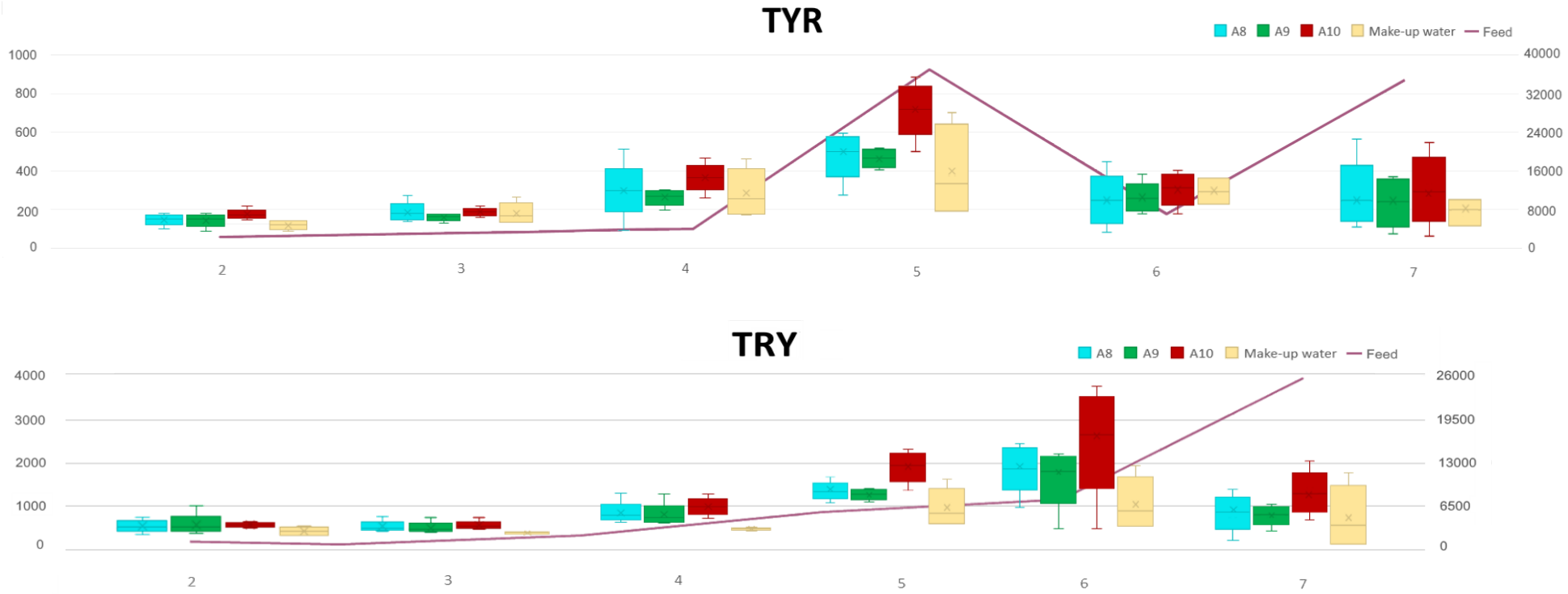


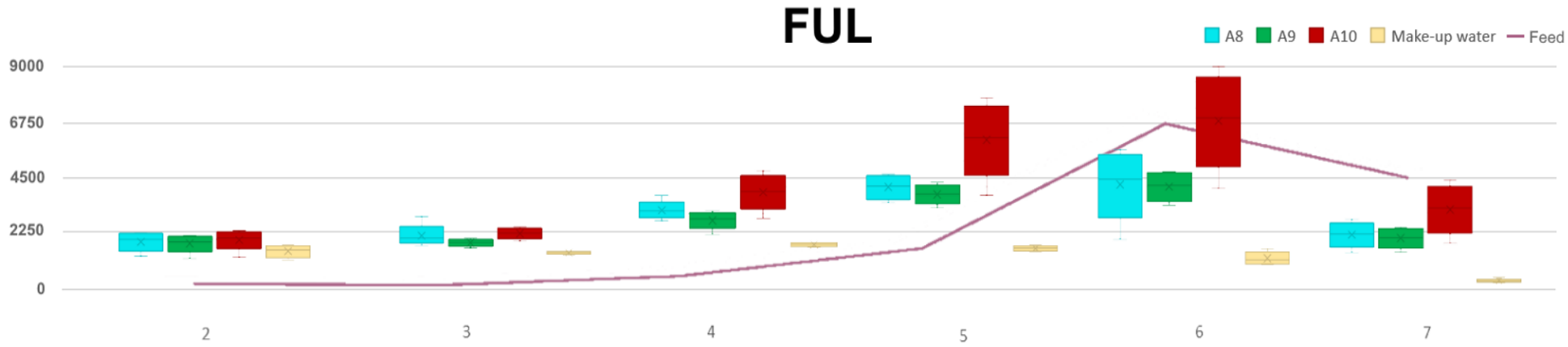
Moving-bed biofilter filled with constantly moving plastic media. On the left upper corner there is a fixed-bed biofilter, where the bioreactor water sample was taken.

APPENDIX 3: DOM character in RAS tanks, make-up water and diluted feed.

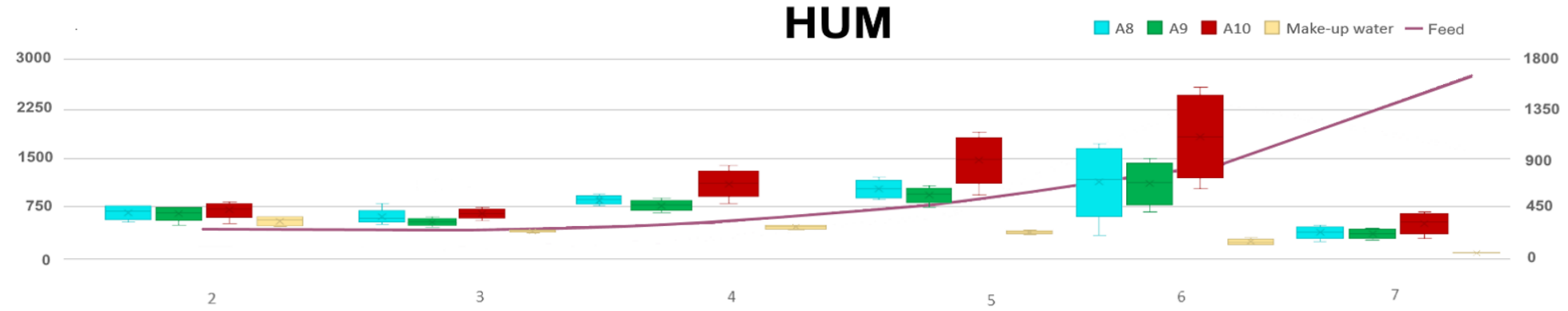


In the diagrams are shown three tanks, make-up water and diluted feed absorbance and fluorescence intensities divided into six fractions (2 – 7) measured with HPLC-SEC. Box plots consist of upper quartile (Q3), the median and lower quartile (Q1) values of the whole data (n_{weeks} = 14), and the whiskers shows the max and min values of the data. Outliers are not marked. The left y-axis shows values for box plots and right shows for diluted feed (n = 1).

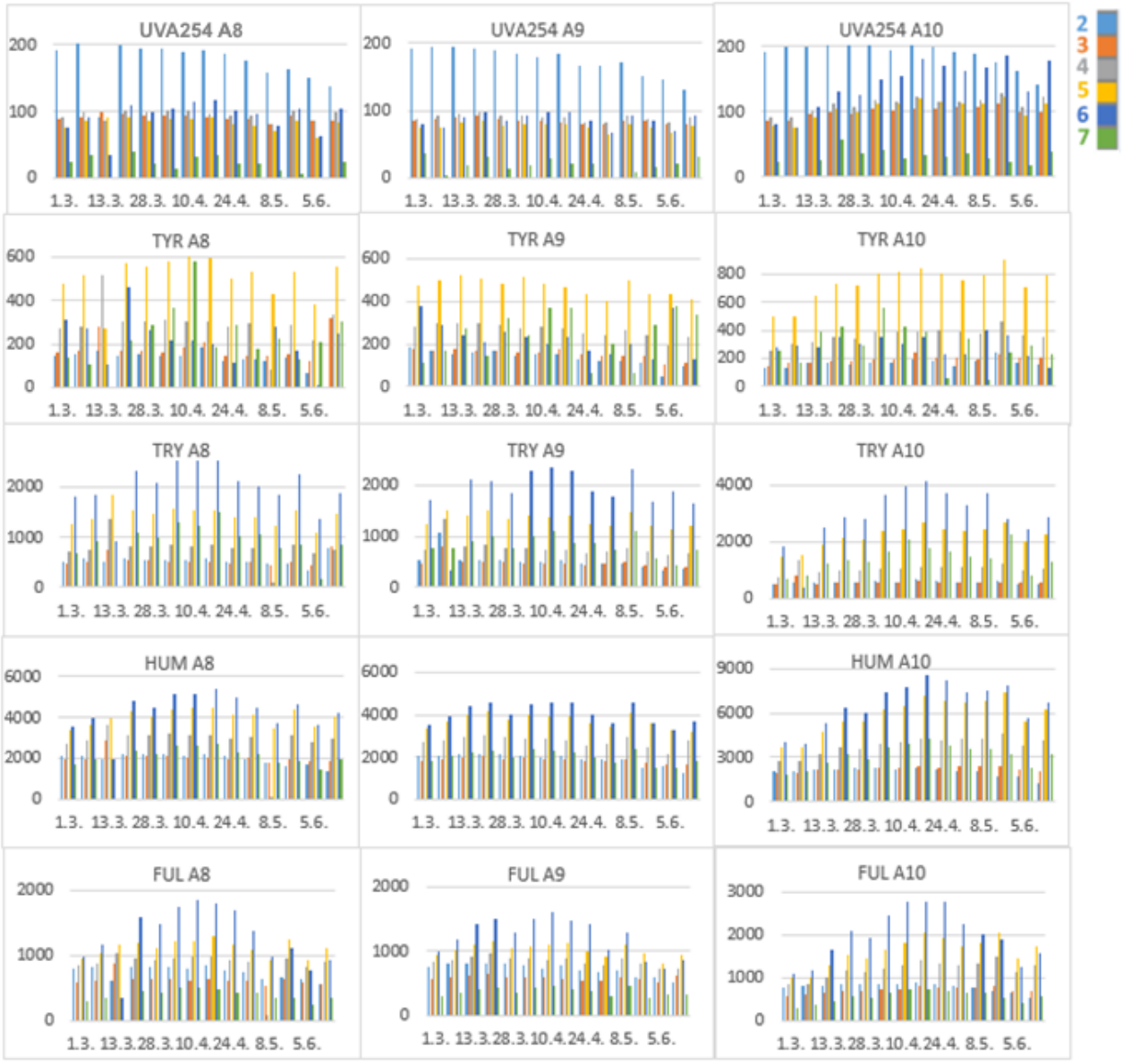

TYR
A8
A9
A10
Make-up water
Feed
1000
800
600
400
200
0
40000
32000
24000
16000
8000
0
2
3
4
5
6
7
TRY
A8
A9
A10
Make-up water
Feed
4000
3000
2000
1000
0
26000
19500
13000
6500
0
2
3
4
5
6
7



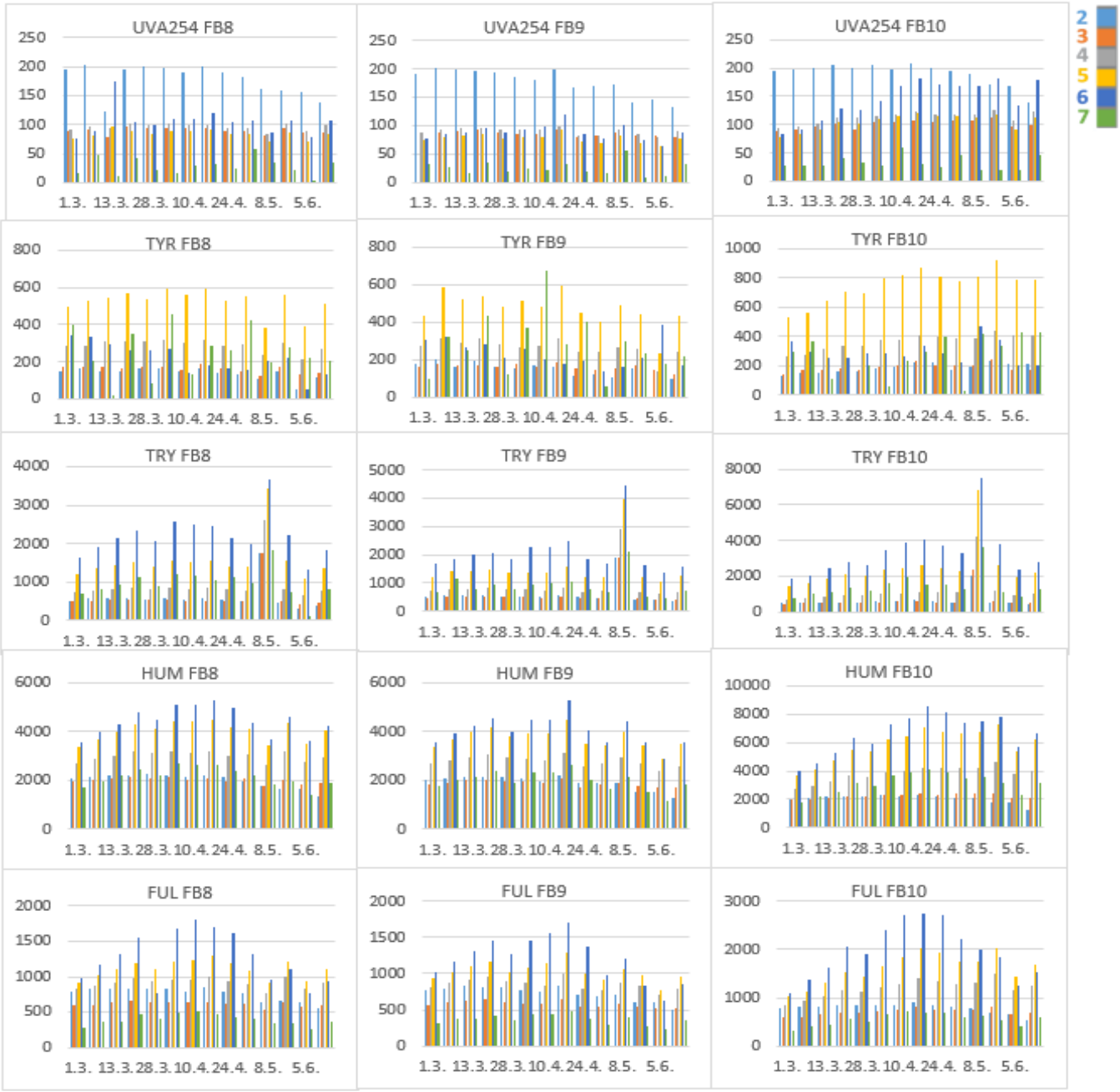
In fulvic acid-like fluorescence y-axis presents the values of box plots and the trend line of the diluted feed observations.



APPENDIX 4: The UV254-absorbance and fluorescence intensities of every fraction of every week sample.



Tanks UV254 absorbance and four different fluorescence compounds signals change over time presented in six different fractions (2 – 7, see the color codes on top right). Y-axis presents the peaks of each fraction total areas. Note that the tank 10 has different scale on y-axis. In the figure below there are corresponding fixed-bed diagrams.


UVA254 FB8
UVA254 FB9
UVA254 FB10
TYR FB8
TYR FB9
TYR FB10
TRY FB8
TRY FB9
TRY FB10
HUM FB8
HUM FB9
HUM FB10
FUL FB8
FUL FB9
FUL FB10
1.3. 13.3. 28.3. 10.4. 24.4. 8.5. 5.6.
2
3
4
5
6
7