



Impact of ozone treatment on dissolved organic matter in land-based recirculating aquaculture systems studied by Fourier transform ion cyclotron resonance mass spectrometry



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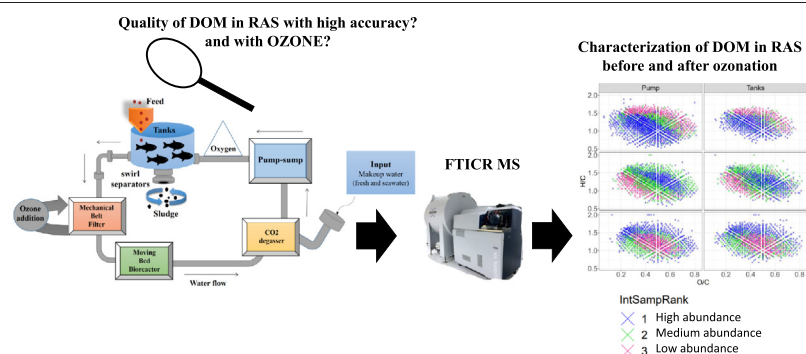
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HIGHLIGHTS

- First characterization of DOM in recirculating aquaculture systems (RAS) by FTICR MS.
- Makeup, pump-sump and tank water samples were rich in CHO and unsaturated compounds prior to ozonation.
- Humic-like and unsaturated CHO group of compounds, particularly the -CH₂- homologs of CHO-DOM, were decomposed by ozone.
- Post ozonation of RAS waters, new fulvic-like and saturated compounds were formed.
- CHON and CHONS compounds were produced during ozonation at high abundance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:

Dissolved organic matter
FTICR MS
Molecular characterization
Ozone
Recirculating aquaculture systems
Fluorescence

ABSTRACT

In land-based recirculating aquaculture systems (RAS), the accumulation of dissolved organic matter (DOM) can have detrimental effects on water quality impacting the system performance, microbial community, and consequently fish health and welfare. Ozone is used in the RAS water treatment process to improve water quality and remove DOM. However, little is known about the molecular composition of DOM in RAS and its transformation when exposed to ozone. In this study, we performed a detailed molecular characterization of DOM in RAS and explored its transformation induced by ozonation of RAS waters. Ultra-high resolution (UHR) Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) was used to characterize the DOM matrix of RAS waters (pump-sump and tanks) and to evaluate its transformation by ozonation. The analysis of DOM extracted from makeup water and feed samples allowed for the determination of DOM sources in RAS prior to ozonation. The CHO and unsaturated group of compounds were the most abundant class found in water samples. On the contrary, the DOM from feed samples was unique and consisted

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mainly of CHO, CHON and unsaturated group of compounds. After the ozonation of RAS waters, humic-like and unsaturated compounds [positive oxygen subtracted double bond equivalent per carbon (DBE-O)/C] were decomposed, particularly the CHO-DOM that contained fewer -CH₂- features. Fulvic-like compounds and several hundred saturated compounds [negative (DBE-O)/C] were formed post ozonation, particularly the CHON and CHONS group of compounds that were associated with fish diets, makeup waters and transformation products from the ozonation of the RAS waters. This study showed that the high accuracy of the ultra-high resolution FTICR MS can be applied to characterize and monitor the changes of DOM at a molecular level in RAS waters. To our knowledge, this is the first study where FTICR MS was incorporated for the characterization of DOM and its sources in RAS.

1. Introduction

Land-based recirculating aquaculture systems (RAS) are becoming a viable alternative to traditional aquaculture methods, as they intensify fish production in advanced and controlled environments (Aguilar-Alarcón et al., 2020; Lazado and Good, 2021). The benefits offered by RAS are in terms of reduced water usage, controlled water quality, and improved biosecurity and disease prevention (Ebeling and Timmons, 2012; Martins et al., 2010; Klinger and Naylor, 2012). However, one of their main drawbacks is the accumulation of dissolved organic matter (DOM). Feed spills and faecal waste are the two main endogenous sources for DOM production in RAS (Ackefors and Enell, 1994; Cripps and Bergheim, 2000). However, the percentage of natural water used for water exchange (makeup water) is regarded as the main exogenous source for DOM production (Colt, 2006; Davidson et al., 2009). The accumulation of DOM in RAS deteriorates the water quality, contributing to off-flavour, odour and colour problems, impairs biofilter performance, and promotes the growth of opportunistic pathogens that jeopardize the fish health and welfare (Summerfelt and Sharrer, 2004; García-Ruiz et al., 2018; Spiliotopoulou et al., 2018). Attempts to address these issues raise the need for further water treatment processes, which increase the production costs.

Ozone is a powerful oxidizing agent commonly used in RAS for its disinfection properties and ability to improve water quality by DOM oxidation (Summerfelt, 2003; Tango and Gagnon, 2003; Powell and Scolding, 2018; Phungsai et al., 2019). Ozone reacts with DOM by electrophilic interactions with activated aromatic systems, thioethers, olefins and amines, concurrently generating highly reactive hydroxyl (·OH) radicals able to oxidize most of the organic molecules present in water (Buffle and Von Gunten, 2006; von Sonntag and von Gunten, 2012). Ozone reacts directly with DOM, reducing the colour, odour and off-flavour problems of RAS (Bullock et al., 1997; Powell and Scolding, 2018), while improving its water quality and optimizing the rearing conditions for fish in RAS (Gonçalves and Gagnon, 2011; Good et al., 2011; Davidson et al., 2021; Lazado et al., 2021). Despite these known effects of ozone in RAS waters, the actual chemical composition and transformation of DOM remains largely unknown.

Bulk DOM measurements are currently used in RAS for monitoring changes in DOM. These include total organic carbon (TOC), chemical oxygen demand (COD), biological oxygen demand (BOD), ultraviolet (UV) absorbance at 254 nm and fluorescence excitation/emission matrix (EEM) spectroscopy (Lin et al., 2003; Guerdat et al., 2011; Fernandes et al., 2015; Spiliotopoulou et al., 2018). The ability of fluorescence spectroscopy to provide a better qualitative description of the composition of DOM has made it suitable for not only to characterize the fluorescence dissolved organic matter (FDOM) in RAS, but also to study its degradation by ozone (Spiliotopoulou et al., 2017). Nonetheless, there is still a need for the development of better analytical approaches that can provide a detailed molecular-level characterization of DOM in RAS and of its transformations when exposed to oxidants. A non-targeted screening approach by high-resolution mass spectrometry (HRMS) can characterize DOM at a molecular level without previous knowledge of DOM composition (Krauss et al., 2010; Hernández et al., 2012; Hollender et al., 2017; Gonsior, 2019). The detailed characterization of DOM can reveal useful information about alterations, changes and reaction pathways of chemicals during the water treatment processes in RAS. Non-targeted analysis coupling ultra-performance liquid

chromatography (UPLC) to HRMS was applied for characterizing changes of DOM using distinct feeds during the production of Atlantic salmon post-smolt in RAS (Aguilar-Alarcón et al., 2020). In addition, non-targeted approaches using electrospray ionization (ESI) coupled to the ultra-high resolution mass spectrometry (UHRMS) technique of Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) were used to determine the molecular composition of DOM from salmonid aquaculture effluents (Kamjunke et al., 2017). In contrast to the HRMS instrumentations, the high mass accuracy and ultra-high resolution of FTICR MS (> 400,000) allow the unambiguous determination of individual molecular formulas in complex mixtures (including DOM) with high precision (Sleighter and Hatcher, 2011; Minor et al., 2014; Zherebker et al., 2020b). In this study, the characteristics and molecular composition of DOM during the production of Atlantic salmon (*Salmo salar*) post-smolts were elucidated prior to and post ozone application to determine the sources and quality of DOM in RAS waters. To our knowledge, this is the first study where the impact of ozone on the composition of DOM in RAS was assessed by non-targeted FTICR MS.

2. Materials and methods

2.1. Experimental set-up and sample collection

Water samples were collected at the Nofima Centre for Recirculation in Aquaculture (NCRA) in Sunndalsøra, Norway (Terjesen et al., 2013). The experimental recirculating system used in this experiment (Fig. 1) consisted of three dual drain octagonal tanks (3.3 m³), which were connected to individual swirl separators, a microscreen belt filter (belt filter, 131 µm screen size), a moving bed bioreactor (MBBR), a CO₂ degasser column, a pump-sump and an oxygenation system. Additionally, an OZAT Ozone Generator, CFS-14 2G (Ozononia Degrémont Technologies Ltd., Zurich, Switzerland) was used to inject ozone into the system prior to the mechanical filtration. In the water recirculation process, the effluent water from the tanks passed through the mechanical belt filter to remove the suspended solids (SS), followed by the MBBR to remove potentially toxic nitrogen compounds (ammonia and nitrite), and thereafter passed directly to the CO₂ degasser. The mixture of makeup water that consisted of ground well water and seawater was added after the MBBR, on the top of the degassing column, while the water that derived after the water treatment process (pump-sump) was re-oxygenated and returned to the tanks. Each tank was stocked with 250 Atlantic salmon (*Salmo salar* L., 1758) with an average individual weight of 98.1 g, kept at 24 h light and fed in excess by an experimental fish feed with approximate compositions shown in Table S1. The total volume of the system was ~38 m³ with a makeup water exchange rate of 20 % per day and a system hydraulic retention time of 5 days. Prior to the ozone application, fish were acclimated to the experimental tanks for 3 weeks. Samples of the makeup, pump-sump and tank waters were collected on the last day of the acclimation period (day 0) for characterizing the water matrix of RAS prior to ozonation (initial period).

After the acclimation period, part of the RAS water flow (approximately 150 L) that derived through the mechanical filter was ozonated and returned to the system prior to the belt filter during a period of 45 days. Two experimental periods: a transition period (day 28) and a final period (day 43) were selected for sampling ozonated waters from the two experimental RAS sections (pump-sump and tanks). The addition of ozone was

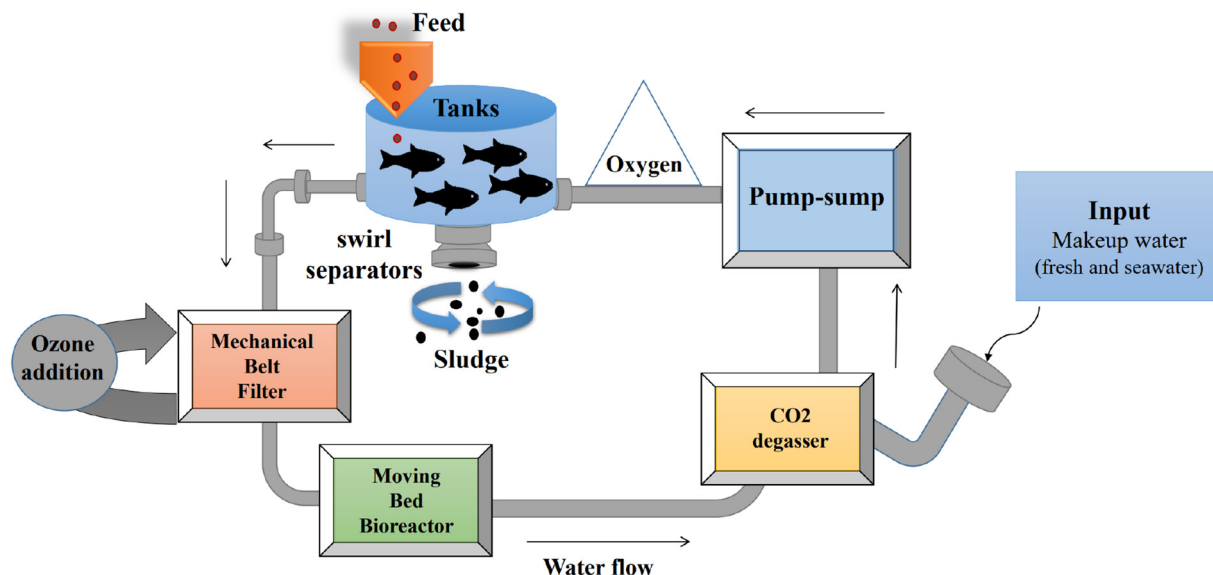


Fig. 1. Experimental set-up of the recirculating aquaculture system (RAS) at the Nofima Centre for Recirculation in Aquaculture (NCRA).

regulated using a set oxidation-reduction potential (ORP) point of 530–535 mV at the point of entrance of the ozonated water into the system. Average ORP values in the fish tanks were kept at 334 ± 22 mV throughout the trial as described by Lazado et al. (2021). The ozone dose was controlled multiple times during a single day using an ORP probe (OxyGuard International A/S ORP probes with Redox Manta controllers, Farum, Denmark) located after the MBBR point, and using an ORP meter (Multi 3620 IDS & SenTix ORPT 900, WTW, Weilheim, Germany) in the fish tanks. During the acclimation period and the whole duration of the ozonation water trial, the parameters were maintained as follows: oxygen saturation in tanks: $\geq 85\%$; salinity: 12 ng/L; temperature: 12.5 ± 0.2 °C; and pH: at 7.4–7.5. The average concentrations of ammonia ($\text{NH}_4\text{-H}$) during the trial (measured weekly 3 times) were: 0.11 ± 0.06 mg/L prior to ozonation (acclimation period) and 0.34 ± 0.10 mg/L during the ozonation period. The average concentrations of nitrate ($\text{NO}_3\text{-N}$) during the trial (measured 3 times weekly) were: 0.02 ± 0.01 mg/L prior to ozonation (acclimation period) and 0.06 ± 0.04 mg/L post ozonation. Water samples were collected in triplicates for the water chemistry (DOC and FDOM) and DOM analysis using 50 mL sterile metal-free polypropylene (PP) tubes and 2 L high-density polyethylene (HDPE) bottles (VWR Chemicals, Trondheim, Norway), respectively.

2.2. Water sample measurements

Water quality parameters such as pH, salinity and temperature were measured using a multi-parametric measuring instrument, WTW Multi 3430 (WTW, Weilheim, Germany), while oxygen was measured with a Handy Polaris TGP (OxyGuard, Farum, Denmark). DOC concentrations were measured with a Lotix combustion total organic carbon analyzer (Teledyne Tekmar, Mason, USA). Fluorescence measurements were performed using a FluoroMax-4 fluorometer (Horiba, Japan). Fluorescence excitation-emission matrices (EEM) measurements were performed selecting excitation and emission wavelengths from 280 to 450 nm and from 350 to 600 nm, respectively. The ratio of the integral intensities was calculated in the emission range of 490–515 to 370–395 nm measured at 280 nm excitation. Detailed procedures are available in Supplementary Information 1.1. (page S5).

2.3. Solid phase extraction (SPE) of DOM and ESI-FTICR MS analysis

DOM was extracted using 500 mg Agilent Bond Elut™ PPL (500 mg, 6 mL; Matriks, Oslo, Norway) cartridges following the same protocol as

Dittmar et al. (2008). The pellets from the experimental fish feed (Table S1) and the acidified ultrapure water samples (method blanks monitoring background contamination) were also extracted with the same SPE procedure. Details about sample preparation and the SPE procedures are available in Supplementary Information 1.2. (page S6).

Negative ions of DOM from all the RAS samples were produced by ESI and their complex composition was subsequently analysed using the FT MS Bruker Apex Ultra mass spectrometer equipped with a harmonized cell (Bruker Daltonics) (Nikolaev et al., 2011), 7 Tesla superconducting magnet. The mass spectra were both externally and internally calibrated: the former was performed using a synthetic carboxylated polystyrene standard (Zherebker et al., 2017) and the latter by the known residual peaks of fatty acids (Sleighter et al., 2008), reaching an accuracy value of <200 ppb. The spectra were acquired within a time domain of 4 megawords in ESI (-) and 200 scans were accumulated for each spectrum. Resolving power was 530,000 at $m/z = 400$.

Molecular formula assignment was performed using the open source browser-based application UltraMassExplorer created by Leefmann et al. (2019) (<http://dockersrv1.awi.de:3838/ume>) based on the elemental combinations $^{12}\text{C}_{>1}$, $^1\text{H}_{>1}$, $^{16}\text{O}_{>2}$, $^{14}\text{N}_{0-2}$, $^{32}\text{S}_{0-1}$ and neutral masses up to 702 Da. The ion charges were determined by measuring the m/z difference between the monoisotopic peaks and ^{13}C -isotopologues. Additionally, peaks with signal to noise >6 were considered. Only the formulas that were presented in all three replicates were considered with the average relative abundances. The double bond equivalent minus oxygen (DBE-O), which was calculated as reported by Koch and Dittmar (2006), was constrained from -10 to 10 for molecular formula assignment (Herzprung et al., 2014; Herzprung et al., 2016). Additionally, the formulas obtained in the method blanks were subtracted from all the samples. Detailed description of elemental composition restrictions is provided in Supplementary Information 1.2. (page S6).

2.4. ESI-FTICR MS data interpretation

Data interpretation was performed with the R studio (version 4.0.2). Different parameters used in FTICR MS for the interpretation of the selected formulas were calculated including the oxygen subtracted double bond equivalency per carbon (DBE-O)/C, the modified aromaticity index (AI_{mod}) and the normal oxidation state of carbon (NOSC). The molecular formulas were classified into the CHO, CHOS, CHONS and CHON group of compounds, and nine molecular classes (namely, saturates, N-saturates, aliphatics, low-oxidized unsaturated, highly oxidized unsaturated, low

oxidized aromatic, highly oxidized aromatic, low oxidized condensed, and highly oxidized condensed compounds) (Zherebker et al., 2020a) and were represented on the van Krevelen diagrams (Kim et al., 2003).

Tanimoto similarity tests were performed to provide differences between the type of RAS samples (makeup, pump-sump, tank and feed). This type of statistical analysis together with the inter-sample ranking and Kendrick mass defect (KMD) analyses were used to reflect the compositional differences of DOM extracted from the waters prior to and post the ozone treatment. Additionally, Spearman rank correlation was calculated to depict the relationships between the sum-normalized abundances of the identified formulas in the DOM extracts from RAS waters, and the water sample parameters measured during the experiment. Transformation trends in the total set of formulas determined in the pump-sump and tank DOM by ozonation were evaluated (Zhang et al., 2021). The calculation of the different DOM parameters, the classification ranges for the nine molecular classes and the description of the data visualization techniques are provided in detail in Supplementary Information 1.3. (page S7-S8).

3. Results and discussion

3.1. Molecular fingerprints of the DOM matrix in RAS

Two main DOM sources in RAS (makeup water and feed) together with the RAS waters (pump-sump and tanks), were analysed to determine the origins and molecular composition of DOM in the water matrix of RAS prior to the addition of ozone (initial period, day = 0). For this purpose, the SPE extracts from the makeup, pump-sump, tank and feed samples were analysed with FTICR MS. Spectra of makeup and non-ozonated RAS waters showed similar patterns (Fig. S1), ranging intense peaks from 300 to 500 *m/z*. By contrast, pronounced molecular differences were observed between the feed samples and the other three water samples. A total of 5189 unique elemental compositions were detected prior to the addition of ozone, from which 3825 and 1460 were identified in the pump-sump and tanks, respectively, while 1753 and 3636 were identified in the feed and makeup samples, respectively (Table S2). The low number of identified elemental compositions in feed samples was associated with high percentages of carbohydrates in its DOM. Hydrophilic saccharides are not extractable by PPL cartridges nor they have high ionization efficiency; this was indicated by the minor recoverable proportion of carbohydrates under negative ESI (Dittmar et al., 2008; Raeke et al., 2016). While considerable variance was observed in the weight average mass values between the feed samples and the other three water samples (makeup, initial pump-sump and initial tanks), an alignment with these parameters was indicated between the makeup and non-ozonated RAS waters (Table S2). These similarities in composition were more noticeable when pair-wise Tanimoto similarity scores were calculated (T-score; Fig. S2). By T-score, RAS water samples showed high similarity to each other (index of 0.96) and with the makeup water (index of 0.93) indicating a common core of DOM between the makeup water and the non-ozonated RAS waters. However, the feed samples strongly differed from all the other water samples (index of 0.06), demonstrating the uniqueness of its DOM.

The van Krevelen diagrams confirmed the similarity between the makeup water and non-ozonated RAS waters (Fig. S3A and B). For both makeup and non-ozonated RAS waters, the CHO (934–2034) group of compounds accounted for the highest number of identified formulas followed by the CHON (315–1220) group of compounds, contrary to the feed samples in which both the CHO (829) and CHOS (428) group accounted for the highest number of identified formulas (Fig. S3C). Although the similarity between non-ozonated RAS and makeup water samples was evident, the normalized abundances of the four chemical groups and nine molecular classes were represented to elucidate possible differences in their DOM composition (Fig. 2). The CHO group of compounds was the most abundant in all the samples (55 to 86 %), particularly in the makeup and RAS water samples (79 to 86 %), which corresponds well with previous studies related to DOM in marine and freshwater samples (Fig. 2A) (Hawkes et al., 2016; Yuthawong et al., 2020). Moreover, the makeup and initial pump-sump

samples showed higher abundances of the CHON group of compounds (12 to 13 %) when compared to the tank samples (7 %). This can be attributed to the continuous input of DOM from the freshwater and seawater mixture (makeup water), which is in proximity to the pump-sump (Fig. 1), while fish excretion products and water microorganisms can lead to changes in the composition of DOM in the tanks. Unlike feed samples, makeup samples shared similar proportions of CHO, CHON, CHONS and CHOS group of compounds with the RAS water samples indicating that the DOM matrix from RAS was largely dependent on the makeup water and to a smaller extent on the feed prior to ozone addition. Moreover, the O₈₋₁₂ and O₉₋₁₂N₁ species (Fig. 2B and C) were the most abundant in all the samples, particularly in the makeup waters. Such results indicated that the makeup waters can be an important contributing source that can act as a sink for nitrogen and oxygenated species in the system, influencing the composition of DOM in the water matrix of RAS (Antia et al., 1991; Berman and Bronk, 2003; Aminot and Kérouel, 2006). Unsaturated compounds (lowOC ~62 % and highOC ~30 %) were the most abundant classes found in the water samples (Fig. 2D), while unsaturated lowOC (39 %), unsaturated highOC (25 %) and aliphatics (23 %) were dominant in the experimental feed. These findings directly indicate that the water matrix of RAS has an abundant core of molecules from freshwater and seawater (makeup waters), continuously entering the system, whose composition can be an important starting point to understand the quality of the DOM matrix in RAS and its transformation during ozonation.

3.2. Overall changes in the DOM matrix of RAS during ozonation

The gradual increase of ORP values during the ozonation of tank waters (217.5 to 315.4 mV) was accompanied by changes in the water quality parameters, particularly in the DOC concentrations (Table S3) and fluorescence measurements (Fig. 3, Fig. S4). The EEM spectra of DOM (Fig. S4) showed several peaks, which their proportion changed post ozonation. Firstly, two typical humic-like (A) and fulvic-like (C) peaks (Sierra et al., 2005; Wells et al., 2022) were observed. The contribution of fulvic-like fluorescence increased in the final tank, while peak A significantly decreased post ozonation. Peak A was potentially composed of reactive species, which further contributed to peak C. It is noteworthy that peak A was expected to originate from two overlapping signals of humic-like and protein-like species, e.g., tryptophan oxidation products (Yakimov et al., 2022). Although both of these species underwent ozonation, yet tryptophan oxidation products were less reactive, and their presence was pronounced in the transition tank and they were eventually observed in the final tank. Besides the humic- and fulvic-like peaks, soluble microbial products (SMP) at 388 nm emission were detected in the transition and the final tank (Wells et al., 2022). Likely, SMP are resistant to ozonation, but in the original water their contribution was negligible compared to humus. The decrease in the contribution of humic-like species was also observed in the fluorescence band shape asymmetry indexes (Fig. 3), which decrease significantly with the ozone dosage ($r = -1.0$, p -values <0.05) (Fig. S5). The higher asymmetric index observed in the initial tank waters was related to the more red-shifted spectra, indicating a higher humification index, and hence a higher conjugation in its DOM composition. After the ozonation of RAS waters, a shift in the fluorescence emission spectra from red to blue and a gradual decrease in the asymmetric index (Fig. 3) was observed. These results suggested that the unsaturated humic-like fraction of DOM from the water matrix of RAS was decomposed post ozonation. The fluorescence band shape asymmetry indexes were negatively correlated with the DOC concentrations ($r = -0.95$, p -values <0.05), indicating that the increase of DOC in the system was dependent on the decomposition of the unsaturated fraction of its DOM (Fig. S5). The increase of the DOC values and the consistent reduction of the fluorescent asymmetric indexes post ozonation indicated the breakdown of unsaturated humic aromatic moieties and the production of saturated DOM and fulvic-like products without further mineralization (Miao and Tao, 2008; Roydhouse et al., 2013; von Sonntag and von Gunten, 2012; Agbaba et al., 2016). Indeed, the comparison of the fluorescence spectra at 280 nm excitation of tank

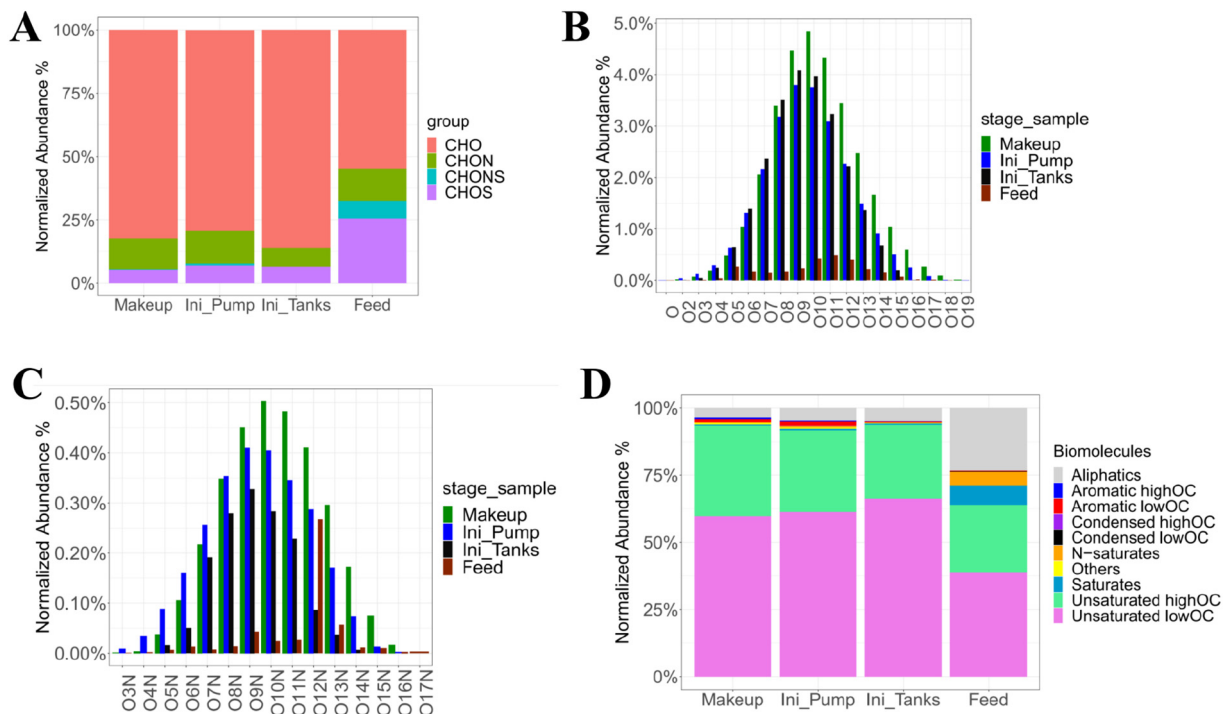


Fig. 2. Comparison of the sum-normalized abundances of DOM (in percentages) in the makeup, feed and non-ozonated RAS samples [pump-sump and tanks at the initial period, day = 0 (Ini_Pump and Ini_Tanks)] after three weeks of acclimatization period. A) Groups: CHO, CHON, CHONS and CHOS; B and C) O_x and O_xN_1 species based on the intensity sum of all identified elemental compositions at the initial period; and D) Nine molecular classes.

DOM to humic and fulvic acid (Fig. S6) showed a blue-shift of fulvic acid and final tank relative to humic acid and initial tank, respectively. Low reactivity of the newly formed saturated and fulvic-like compounds with ozone could induce their accumulation in the tank waters, denoting the need to be removed by biological filtration (Von Gunten, 2003). While ozonation could decompose the unsaturated fraction of DOM without mineralization, the concurrent input of new DOM from fish excretion products and makeup water could enhance the accumulation of DOC in RAS during the experimental trial.

3.3. Molecular transformation of DOM in the RAS matrix post ozonation

Ozonation of RAS waters resulted in lower similarities with the makeup water [(T-scores ranged from 0.93 to 0.74 in the pump-sump (Tran_Pump) and from 0.93 to 0.63 in the tanks (Tran_Tanks) at the transition period)] and these differences increased over time [(T-scores ranged from 0.93 to 0.58 in the pump-sump (Fin_Pump) and from 0.93 to 0.54 in the tanks (Fin_Tanks) at the final period)] (Fig. S7). This finding suggested that great changes in the molecular DOM from tank waters occurred during

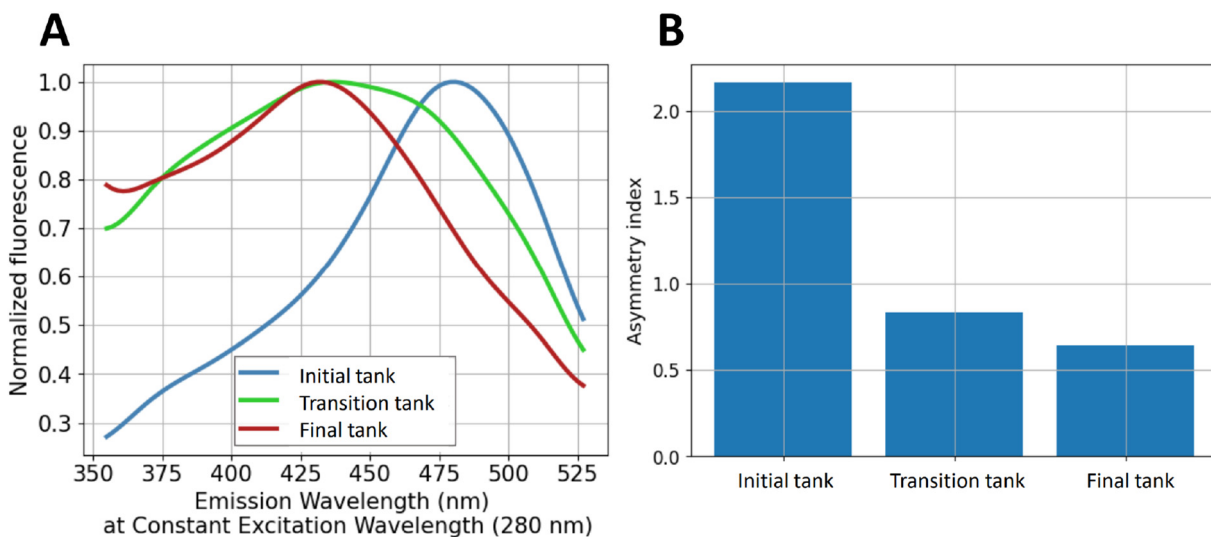


Fig. 3. A) Fluorescence emission spectra of the non-ozonated tank samples analysed in the initial period and the ozonated ones analysed in the transition and final periods at 280 nm excitation; B) Asymmetry index.

ozonation. Changes in the composition of DOM from the RAS waters could be attenuated by the nutrients washed out from the feed samples (T-score 0.12 for the transition and final tanks, and for the final pump-sump with feed). The gradual decrease of T-score index, AI_{wa} and $(DBE-O)/C_{wa}$ values observed in the DOM from tanks were accompanied by an increase in its H/C_{wa} values (Table S2). These observations agreed with the fluorescence measurements, indicating a higher degree of saturation in the DOM from RAS as the experiment progressed (Fig. S5). Although the ozone dosage was higher at the point of entrance (ORP: 524.4 ± 9.6 mV, pump-sump) than in the tanks (ORP: 308 ± 19.8 mV), the calculated T-score and molecular-level parameters for the pump-sump waters did not show significant changes prior to and post ozonation when compared with the tanks. Higher ozone dosages in the pump-sump (than in the tanks) potentially led to a higher oxidation of its DOM with high percentages of oxidized transformation products non-extractable by conventional SPE columns (Phungsai et al., 2016, 2018; Varanasi et al., 2018; Phungsai et al., 2019; Liu et al., 2020).

The sum-normalized compound abundances were correlated with ORP values using Spearman rank correlations (Fig. S8). Overall, 973 compounds in the RAS matrix were significantly correlated (p -value < 0.05), from which 766 were negatively correlated with ORP values (blue dots in the correlated plots). These results indicated that when the ozone dosage increased, the DOM from the water matrix of RAS underwent changes in the low-oxygen ($O/C < 0.5$) fraction of its DOM, particularly in the unsaturated lowOC region. The large number of identified formulas during the ozonation of RAS waters were displayed in van Krevelen diagrams (Fig. S9) and their sum-normalized abundances were described to evaluate differences in their DOM composition (Fig. 4). CHO (86 to 66 %) and unsaturated lowOC (66 to 46 %) compounds significantly decreased post ozonation, while CHON (7 to 13 %), CHOS (7 to 15 %), CHONS (0 to 8 %), N-

saturated (0 to 8 %) and aliphatic (5 to 14 %) compounds increased. During the oxidation of DOM, unsaturated CHO-DOM formulas can be substantially decomposed forming more saturated compounds through cyclization (Diels and Alder, 1928; Harris and Wamser, 1976; Devine and Oh, 1992) and radical polymerization (Matsumoto et al., 1996, 1998) processes, which was in agreement with the reduction of the CHO compounds and fluorescence asymmetry in the tank waters, since the cleavage of carbon double bonds decreases the conjugation of compounds and possible electron transfer (Yakimov et al., 2021). These results were in agreement with those observed in the photodegradation of terrestrial DOM by Chen et al. (2014) and the hydroxyl radical degradation of lignins by Waggoner et al. (2015), where DOM was altered to form relatively saturated molecules matching the compounds produced post ozonation of RAS waters. Although the alteration of fluorescence asymmetry was indicative of the decomposition of carbon double bonds in organic molecules, the unique information obtained from the molecular-level analysis of DOM by FTICR MS highlights the importance of this technique to assess DOM changes during the water treatment processes of RAS.

3.4. DOM decomposition and formation post ozonation

The demonstrated power of the FTICR MS technique in the characterization of the overall DOM changes in RAS was used to study the impact of ozone on its DOM. For this purpose, the identified elemental compositions in RAS waters were classified into decomposed, formed and resistant compounds and were divided into four classes based on the positive and negative values of $(DBE-O)/C$ and NOSC as shown in Fig. 5 and Table S4. From the four classes identified in RAS waters, the highest proportion of decomposed compounds (60.63 to 76.36 %) had positive $(DBE-O)/C$ and negative NOSC values confirming that the unsaturated and reduced

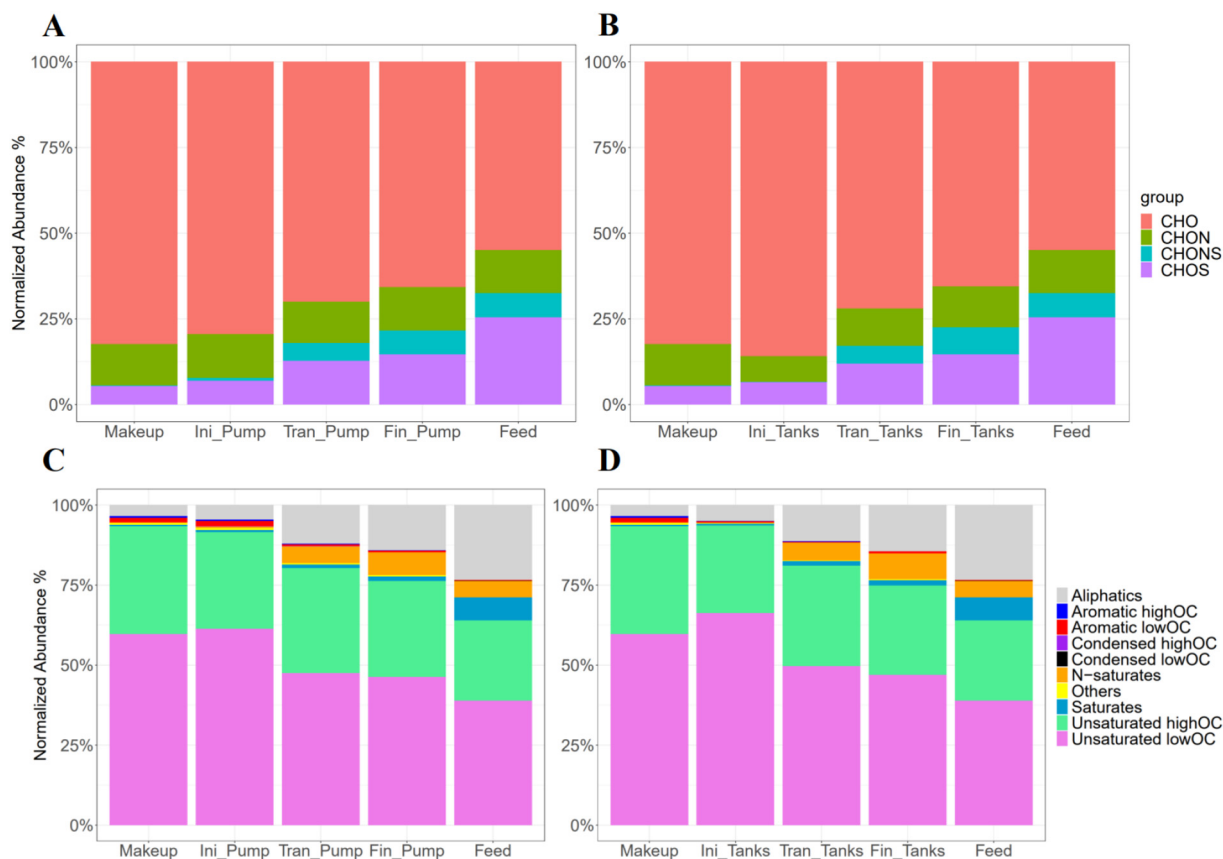


Fig. 4. Comparison of the sum-normalized abundances of DOM (in percentages) based on CHO, CHON, CHONS and CHOS of A) makeup, feed and pump-sump samples (in the initial, transition and final periods); and B) makeup, feed and tank samples (in the initial, transition and final periods). In addition to the sum-normalized abundances of the nine molecular classes in the: C) makeup, feed and pump-sump waters; and D) makeup, feed and tank waters in the initial, transition, and final periods.

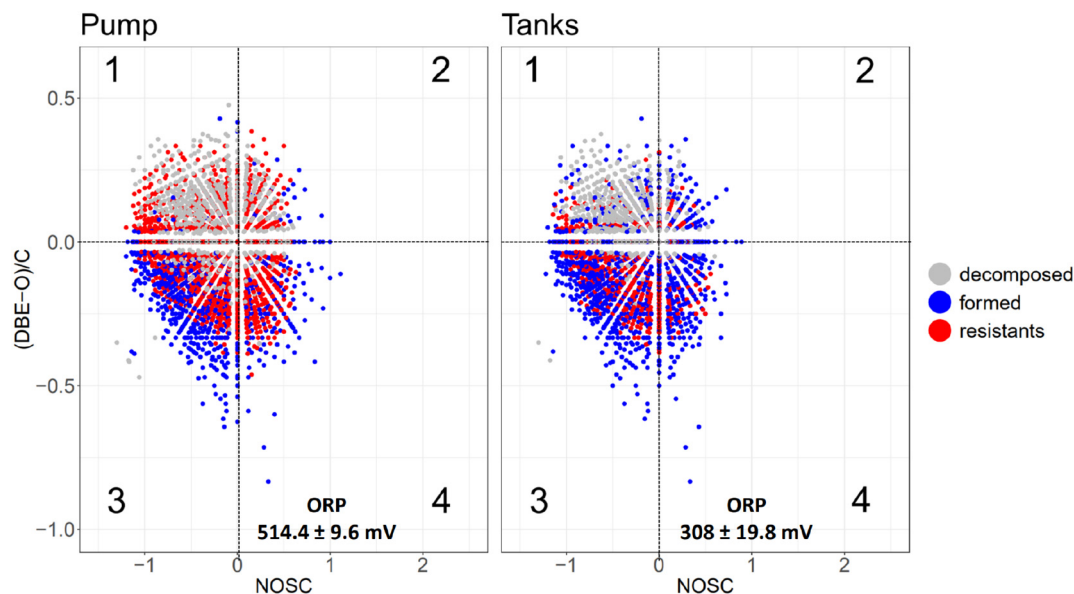


Fig. 5. Molecular transformation of DOM extracted from the pump-sump and tank waters during the 43 days of the trial at different ozone dosages (ORP values). The four delimited regions represent: 1) unsaturated and reduced compounds; 2) unsaturated and oxidized compounds; 3) saturated and reduced compounds; and 4) saturated and oxidized compounds.

compounds (e.g., with carbon double bonds or activated aromatic rings) were decomposed during ozonation of RAS waters at two ORP values (Table S4). The unsaturated compounds containing multiple double bonds or activated aromatic rings can be available to react with ozone (Von Gunten, 2003). On the contrary, the highest proportion of formed compounds were saturated (reduced: 55.30 to 69.95 %; and oxidized: 23.90 to 24.36 %) (Fig. 5 and Table S4). Saturated compounds can be by-products from the reaction of ozone with DOM. During the ozonation processes, the oxidation rate of saturated compounds can be lower than their formation rate inducing their accumulation in the water matrix of RAS (Von Gunten, 2003), which alongside the newly added fish feed and excretion products at low water exchange rates can further justify the increase of DOC during the ozonation of RAS.

Ozone mainly decomposed the CHO group of compounds (Fig. S10), mostly from the makeup waters, which were the major contributors of CHO-DOM compounds in the system (Fig. S11 and Fig. S12). By T-score (Fig. S13), a decrease in the similarity index prior to and post ozonation of RAS waters was observed, when compared with the makeup waters (T-score ranged from 0.94 to 0.61 in the pump-sump and from 0.94 to 0.57 in the tanks) confirming the transformation of the CHO fraction of DOM post ozonation of the water matrix of RAS, which decreased in abundance (Fig. 6A). To further study the decomposition of CHO compounds in the RAS water matrix, a KMD analysis based on the $-CH_2-$ homologs of CHO-DOM was performed (Fig. 6B). Ozone preferentially reacted with the CHO-DOM that has more $-CH_2-$ groups (higher KMD and $m/z > 580$) in the pump-sump at higher ozone dosages (ORP: 514.4 ± 9.6 mV), while the CHO features with fewer $-CH_2-$ groups were decomposed in the tank and pump-sump waters at both ozone dosages. Such results indicated that the CHO features with fewer $-CH_2-$ groups can be more reactive to ozone oxidation than those with a higher number of $-CH_2-$ groups (Phungsai et al., 2019). This corroborates the preferential ozone attack to unsaturated and aromatic compounds, which have low KMD (Waggoner et al., 2015). The decomposition of $-CH_2-$ homologs in CHO-DOM by ozonation of waters was previously reported and it can be attributed to unsaturated CHO compounds with electron donating substituents (e.g. $-CH_2$, $-OR$, $-O$), which can make the carbon double bond richer in electron density and in turn more reactive to ozone (Phungsai et al., 2019).

The compounds formed post ozonation in the pump-sump were mainly transformation products occurring in the RAS water matrix (68 %) since few compounds were introduced from the main endogenous (feed pellets,

21 %), exogenous (makeup waters, 7 %) or other common sources (4 %) of DOM (Fig. S11). However, in the tank waters, a higher proportion of compounds from the makeup waters (35 %) and fewer from the feed (14 %) were introduced post ozonation, indicating that the water matrix of RAS was not only dependent on the transformation products formed post ozonation (formed compounds: 38 %), but also on their main endogenous and exogenous sources of DOM. These findings highlight the importance of characterizing the molecular composition of the DOM extracted from the makeup and feed samples since changes in their quality and quantity can have clear implications in the efficiency of ozonation in RAS. Post ozonation, a higher number and abundance of CHON and CHONS group of compounds was observed (Figs. S10 and S14). The CHON compounds were mainly introduced from the makeup waters in the tanks (55 %), while in the pump-sump they were mainly originated in their waters (87 %) (Fig. S15). The CHONS compounds were mainly formed in RAS waters (71 % tanks and 77 % pump-sump), where they were found in high abundance (Fig. S10). The high number and abundance of CHON and CHONS compounds were positively correlated with the increase of nitrates and ammonia in the system (see Section 2.1). This can be attributed to the ozone reaction with amines or amino-groups, which can lead to an increase of nitrates and ammonia concentration in the system, and new CHON and CHONS by-products (Zahardis et al., 2008; Sharma and Graham, 2010; Krasner et al., 2013; de Vera et al., 2017; Lim et al., 2019; Essaïed et al., 2022). Nonetheless, further research should be performed to assess the relationship between ozone and the formation of CHON and CHONS group of compounds in RAS. In both system locations, a higher number of unsaturated CHO-DOM compounds was decomposed during the ozonation of RAS waters, while N-saturates and aliphatic compounds were formed (Fig. S16). The significant changes in the chemical composition of DOM in RAS waters post ozonation observed in this study demonstrated the suitability of the FTICR MS technique for characterizing DOM at a molecular level and tracking its changes during the ozonation processes in RAS.

4. Conclusions

The non-targeted approach using FTICR MS was successfully used for the first time to characterize and identify the sources of DOM in the RAS water matrix. Furthermore, the fluorescence measurements together with FTICR MS revealed changes in the composition of DOM post ozonation in RAS. The DOM composition was clearly differentiated on each system

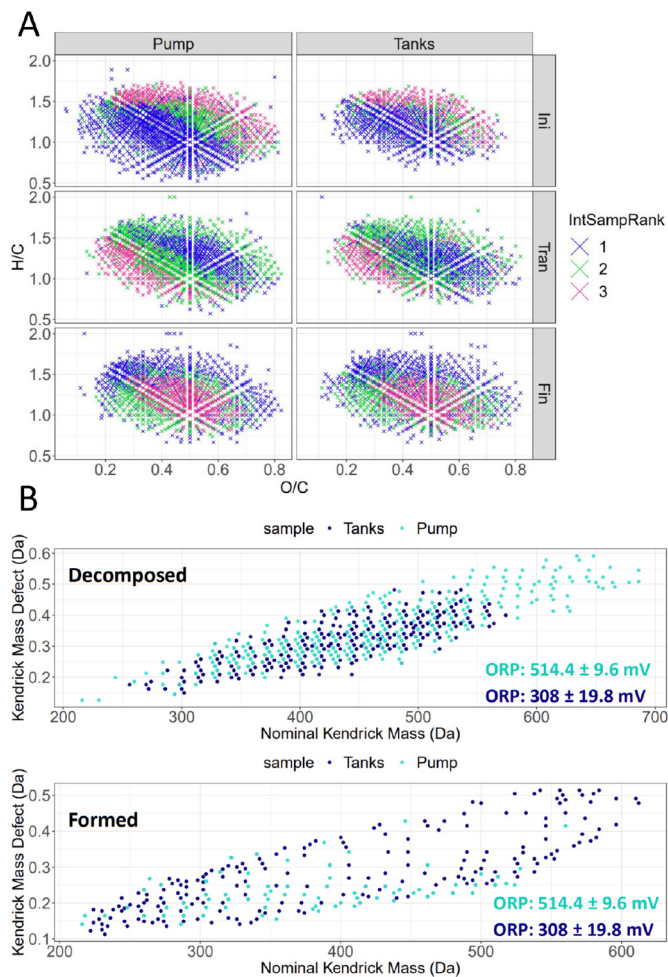


Fig. 6. A) Inter-sample ranking analysis of the CHO-DOM sum-normalized abundances (1 = highest abundances and 3 = lowest abundances) from the common elemental compositions found in the non-ozonated tanks, and pump-sump waters (initial period) and ozonated ones (transition and final periods); B) Kendrick mass defect diagrams for CHO compounds that were: decomposed and formed post ozonation in the pump-sump (ORP: 514.4 ± 9.6 mV) and tank waters (ORP: 308 ± 19.8 mV).

location, and prior to and post ozonation. The molecular fingerprints of DOM in RAS allowed the understanding of the transformation processes of DOM under ozonation. In total, 5189 elemental compositions were detected in the non-ozonated RAS waters at two system locations (pump-sump and tanks) and in their exogenous (makeup water) and endogenous (fish feed) sources with the aim to characterize the DOM from the RAS water matrix at the initiation of the experiment. The RAS waters (pump-sump and tanks) consisted of four main chemical groups, including CHON, CHONS, CHO, and CHOS, from which the CHO group was the most abundant. The calculation of the Tanimoto similarity index and van Krevelen diagrams revealed that the compounds found in non-ozonated RAS waters had high similarity to those found in the makeup water, and showed low similarity to those found in the feed. The DOM fraction of the pump-sump, tank and makeup waters was dominated by the abundance of CHO and unsaturated compounds, whereas unsaturated, aliphatics, CHO and CHOS compounds were more abundant in the feed. Ozonation of RAS waters induced large changes in the DOM composition. Ozone decomposed mainly humic-like and CHO compounds, specifically unsaturated compounds (positive (DBE-O)/C), and formed relatively saturated molecules (negative (DBE-O)/C) and fulvic-like compounds. The KMD indicated that ozone selectively decomposed some $-CH_2-$ homologs of CHO-DOM in the RAS waters and promoted the decomposition of more CH_2 groups (higher KMD and $m/z > 580$) at higher ozone dosages (ORP: 514.4 ± 9.6 mV).

Post ozonation, CHON and CHONS group of compounds were formed in the pump-sump and tanks. The accumulation of CHON and CHONS compounds post ozonation was associated to fish diets and makeup waters rich in nitrogen compounds, and to the possible reaction of amines or amino-groups with ozone, which can further promote the increase of nitrates and ammonia in the system. This study demonstrated that the high accuracy of the ultra-high resolution FTICR MS can be used to characterize and track the changes of DOM at a molecular level in RAS waters.

CRedit authorship contribution statement

Patricia Aguilar-Alarcón: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Alexander Zherebker:** Methodology, Validation, Data curation, Formal analysis, Resources, Software, Writing – review & editing. **Anna Rubekina:** Formal analysis, Visualization. **Evgeny Shirshin:** Resources, Formal analysis. **Mads Adrian Simonsen:** Methodology, Data curation, Formal analysis, Writing – review & editing. **Jelena Kolarevic:** Conceptualization, Resources, Writing – review & editing. **Carlo C. Lazado:** Conceptualization, Resources, Writing – review & editing. **Evgeny N. Nikolaev:** Resources, Funding acquisition, Project administration. **Alexandros G. Asimakopoulos:** Funding acquisition, Methodology, Resources, Project administration, Supervision, Validation, Writing – review & editing. **Øyvind Mikkelsen:** Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This project was part of CtrlAQUA SFI, Center for research-based innovation, funded by the Research Council of Norway and the partners of the center (project # 237856/O30). The FTICR MS study was supported by the Russian Science Foundation grant 21-47-04405. Optical measurements were supported by the RF President's scholarship for PhD students and Young scientists (AR scholarship no. SP-4410.2021.4). AZ, EN acknowledge the partial support of European's Horizon 2020 Research and Innovation Program under grant agreement No. 731077. The authors would like to thank Dr. Susana Villa González and Dr. Ana R. Borrero Santiago for the guidance and Kristin Skei Nerdal, Dag Egil Bundgaard, Britt Kristin Megård Reiten and May Britt Mørkedal at Nofima Sunndalsøra for technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2022.157009>.

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