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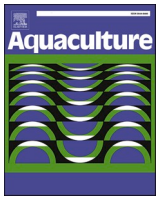

Microbiome response to foam fractionation and ozonation in RAS

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ABSTRACT

Efficient water treatment is required to maintain high water quality and control microbial growth in recirculating aquaculture systems (RAS). Here, we examined the effects of two treatment methods, ozonation and foam fractionation, separately and combined, on the microbiology in twelve identical experimental RAS with rainbow trout (*Oncorhyncus mykiss*) during 8 weeks. Microbes suspended in water and growing in biofilter biofilms were examined using flow cytometry analysis and high throughput sequencing of the 16S rRNA gene. The results showed that foam fractionation did not cause large changes in abundance or overall community composition of free-living microbes. Instead, through decreasing the organic matter availability in water, it targeted specific microbial taxa, leading to e.g. decreased potential for off-flavor production. In contrast, ozonation was found to have a profound impact on the system microbiology, by reducing the overall cell abundance, increasing microbial dead/live ratio, and changing the community composition of both free-living and biofilm microbes. Ozonation increased the abundance of certain key microbial taxa adapted to low carbon conditions, which might form a stable and more abundant community under a prolonged ozone dosing. Combining the two treatment methods did not provide any additional benefits as compared to ozonation solely, corroborating the high disinfection potential of ozone. However, ozone had only a minor impact on biofilter microbial communities, which were, in general, more resistant to water treatment than water communities. Water treatment had no effect on the overall genetic nitrification potential in the biofilter biofilms. However, foam fractionation led to changes in the nitrifying microbial community in biofilter, increasing the abundance of *Nitrospira* conducting complete ammonia oxidation to nitrate (comammox). Altogether, the results obtained indicate that although these two water treatment methods have similar outcomes on physico-chemical water quality and microbial activity, their underlying mechanisms are different, potentially leading to different outcomes under the long-term application.

1. Introduction

The concept of recirculating aquaculture system (RAS) is based on high water recirculation rate (Martins et al., 2010). A central treatment unit is a biofilter, where nitrifying microbes maintain good water quality for fish by converting toxic ammonium into less harmful nitrate (Hagopian and Riley, 1998). Biofilters host a diverse microbial community (Hüpeden et al., 2020; Schreier et al., 2010), including a high amount of heterotrophic microbes degrading organic matter. Furthermore, the presence of microbes in RAS is not limited to the biofilter, but they inhabit all RAS compartments, floating as flocs or free-living cells in the water phase or forming biofilms on the surfaces e.g. tank walls and pipes (Bartelme et al., 2019). Although a majority of these microbes is harmless or even beneficial for maintaining stable water quality conditions, RAS microbial communities can also involve harmful microbes, such as opportunistic pathogens, hydrogen sulfide or off-flavor producers (e.g. Fossmark et al., 2020; Lukassen et al., 2017). In high intensity RAS with high levels of feed loading and long retention times, high organic matter concentrations in the system promote the abundance and activity of heterotrophic microbes (e.g. Michaud et al., 2006). This can increase the need for aeration and degassing and associated operational costs of the system, as heterotrophic microbes consume high amounts of oxygen and release CO_2.

To maintain sufficient system water quality as well as to hinder

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blooms of potential harmful microbes (e.g. Moestrup et al., 2014), water treatment methods to remove microbes and other organic matter are currently searched and developed for RAS. Two potential water treatment methods for this are foam fractionation and ozonation. Foam fractionation, often termed as protein skimming, is based surface-active particles (e.g. organic matter) adsorbing to the surface of fine air bubbles injected to water, generating foam that is then removed (Timmons and Ebeling, 2010). Foam fractionators have primarily been applied in marine RAS, where they have been found to remove microbes but also reduce the overall availability of organic matter (Barrut et al., 2013). In marine RAS with abalone, applying foam fractionators led to 2.6 times lower amount of heterotrophic bacteria (Rahman et al., 2012), leading to 7% higher oxygen concentrations in system water. In marine seabass RAS, foam fractionation reduced the abundance of both large (>60 μm) and small (0.22–1.2 μm) particles, but not of the intermediate ones, and reduced the abundance of heterotrophic microbes in water by 32–88% depending on operation time and pre-filtration of water (Brambilla et al., 2008). In addition to targeting microbes and organic matter, foam fractionation can also decrease the concentrations of dissolved inorganic nitrogen (DIN), as 13–35% lower DIN concentrations were recorded after applying foam fractionator in marine abalone RAS, due to increased nitrification activity after decreased abundance of heterotrophic microbes (Rahman et al., 2012).

Ozone oxidizes organic matter, decreasing chemical oxygen demand (COD) of the water in general (Spiliotopoulou et al., 2018), but also destroys and inactivates microbes through damaging cell walls (Ramseier et al., 2011). Ozone has been found to decrease the abundance of heterotrophic bacteria in RAS water (Davidson et al., 2021; Davidson et al., 2011), while bacteria embedded in biofilm or attached to particles are expected to be less susceptible. Furthermore, a high abundance of particles reduces the oxidative effect of ozone on microbes, as ozone is consumed by other particles before attacking microbes (Hess-Erga et al., 2008). This means that the effect of ozone on the overall microbial abundance can be moderate in intensive RAS with high organic loading or when using low ozone dosage, as has been observed in marine larval RAS (Attramadal et al., 2012). However, ozone can cause significant changes in the microbial community. Previously, ozonation has been found to shift the bacterial community growing as biofilms on tank walls from Alphaproteobacteria-dominated to Gammaproteobacteria-dominated, through altering water chemistry (bacterial habitat conditions), and oxidizing complex organic molecules into more bioavailable forms (Wietz et al., 2009). When selecting for certain microbial taxa, ozonation can also open niches for potentially harmful opportunistic microbes (Dahle et al., 2020). Furthermore, in seawater, a moderate ozone dosage (≤0.15 mg/L OPO) has been found to have either no effect or even to slightly improve biofilter nitrification performance through removing organic matter and heterotrophic bacteria commonly present in the biofilter and/or by indirect liberation of oxygen (Schroeder et al., 2015). However, a detailed knowledge on the response of microbial communities in both water and biofilms to these two water treatment methods is still lacking.

In this study, we examined the effect of foam fractionation and/or ozonation on microbial communities in RAS water and biofilter biofilms in replicated freshwater RAS with rainbow trout. We hypothesized that when applied alone, foam fractionation would affect microbial abundance in water and potentially change the microbial community composition through reduced organic matter concentrations. Furthermore, ozonation alone or together with foam fractionation was expected to have a more profound effect on the microbial community composition than foam fractionation alone.

2. Materials and methods

2.1. Experimental setup

The experiment was conducted in 12 replicated, 0.8 m^3 pilot-scale freshwater RAS (Suppl. Fig. 1) at DTU Aqua in Hirtshals, Denmark. Each system was stocked with 8.05 ± 0.03 kg juvenile rainbow trout (*Oncorhynchus mykiss*). For 13 weeks before the trial, all 12 RAS were acclimatized by daily feeding of 60 g d^{-1} Efico E 920 (Biomar, Denmark), which was increased to the final feed amount 100 g d^{-1} three days before the trial started, the final feed loading being 1.66 kg feed m^{-3} make-up water. Each RAS had a 100 L biofilter filled with 40 L of new RK BioElements (injection-molded polypropylene with surface specific area of 750 m^2/m^3; Dania Plast, Skive, Denmark) operated as a moving bed biofilter with an airflow of 4 L min^{-1}. All biofilters were fully operational after the pre-acclimatization period. After taking week 0 samples, four treatments were applied in triplicate: 1) three control RAS, 2) three RAS with foam fractionator (ff), 3) three RAS with ozone (oz), 4) and three RAS with ozone and foam fractionator (oz + ff). Foam fractionators were operated with a water flow rate of 1500 L h^{-1} and an airflow rate of either 1320 L h^{-1} (ff) or 1200 L h^{-1} plus 120 L h^{-1} of ozonized air (oz + ff). Bubble columns were supplied with 120 L h^{-1} ozonized air (oz). Ozone was injected at a dosage of 20 g O_3 kg^{-1} feed, the estimated true dosage applied being appr. 7 g O_3 kg^{-1} feed, which can be considered as a low dosage level. The trial lasted eight weeks. Temperature in the system ranged between 17 and 21 °C (Table 1), due to the lack of cooling in the experimental facility. Despite being a high temperature, it is commonly achieved on commercial trout farms during summer and no negative impacts were seen on the fish during the trial.

2.2. Sampling and water quality conditions

The water quality characteristics during the last three experimental weeks are described in Table 1, and the details for sampling are given in de Jesus Gregersen et al. (2021). For microbial abundance measurements, water was collected weekly from the sump of each RAS. For the microbial community analysis, sump water was collected using syringe filters (0.22 μm Millipore Express® PLUS PES membrane) before feeding at the beginning of the experiment (week 0) and at weeks 1, 3, and 7. In addition, at week 7, eight bioelements from each MBBR were collected and microbial biofilm was detached from them by sonication of 4 min (Branson 1510). Microbiological samples were stored at −20 °C before DNA extraction.

2.3. Microbial abundance using flow cytometry

Immediately after the sampling, 10 mL of water from each system was prefiltered through a cell strainer (40 μm FisherBrand, Thermo Fisher Scientific), and 500 μL of filtrate was labelled with 5 μL of SYBR Green (100×, MilliporeSigma, Germany) and 5 μL of propidium iodide (PI, 600 μM, MilliporeSigma, Germany) for incubating at 37 °C for 10 min, after which the total abundance of cells (cells mL^{-1}) and the proportion of dead cells (%Dead) was measured with BD Accuri C6 Plus flow cytometer (Becton, Dickinson and Company, NJ, US).

2.4. Microbial community composition

DNA was extracted using the DNeasy PowerLyzer™PowerSoil DNA Isolation Kit (Qiagen, Germany) from water and biofilm samples, and the DNA quantity was measured with Qubit™ dsDNA HS assay and Qubit 2.0 Fluorometer (Thermo Fischer Scientific). Microbial community composition was studied using Ion Torrent PGM next-generation sequencing targeting the V4 region of the 16S rRNA gene with primers 515F—Y (Parada et al., 2016) and 806R (Caporaso et al., 2011). The analysis of gene sequences was done using mothur (version 1.44.3; Schloss et al., 2009) to remove sequences shorter than 200 bp, low-quality sequences, barcodes and primer sequences. The sequences were aligned using Silva reference alignment (Release 132), chimeric sequences were identified and removed (Edgar et al., 2011), and a preclustering algorithm was used to reduce the effect of sequencing errors (Huse et al., 2010). Sequences were assigned to taxonomies with a

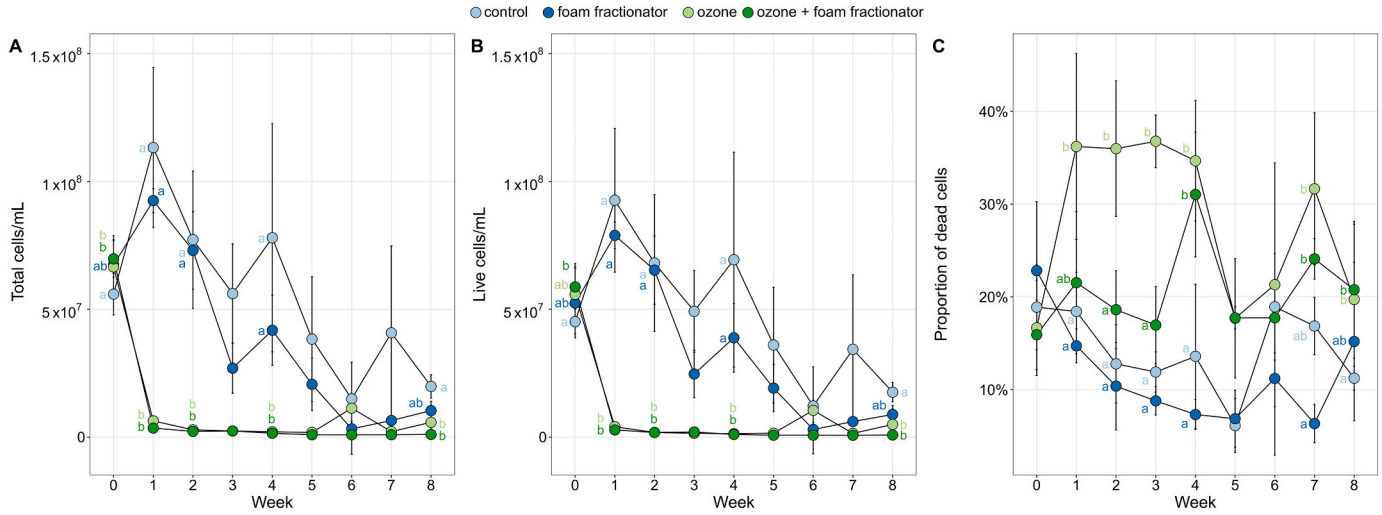

Fig. 1. The abundance of A) total and B) live cells (cells/mL), and C) the proportion of dead cells in four triplicate treatments during 8 weeks experiment. Values are reported as mean ± SD (n = 3). The letters denote for post-hoc test results within sampling time.

Table 1
Water quality characteristics in the pilot-scale recirculating aquaculture system units with either foam fractionator, ozone, ozone + foam fractionator, or unexposed control. Values are given as mean ± SD over experimental weeks 6–8 (n = 9/treatment). Modified from de Jesus Gregersen et al. (2021).

	Control			Foam fractionator			Ozone			Ozone + Foam fractionator		
Particle abundance (10^6/mL)	2.43	±	1.38	1.01	±	1.01	0.42	±	0.22	0.27	±	0.14
Turbidity (NTU)	7.02	±	2.56	2.46	±	0.83	4.34	±	1.07	1.49	±	0.43
Microbial activity k (h^{-1})[1]	0.84	±	0.24	0.33	±	0.17	0.44	±	0.27	0.08	±	0.03
BOD5 (mg O_2/L)[2]	6.09	±	1.05	2.99	±	0.89	3.45	±	0.55	1.53	±	0.24
COD (mg O_2/L	37.6	±	5.86	22.8	±	2.70	25.2	±	2.90	16.0	±	1.49
TAN (μg NH_4-N/L)[3]	74.7	±	30.0	83.8	±	17.9	88.5	±	36.7	82.9	±	11.6
Nitrite (μg NO_2-N/L)	119	±	24.5	77.5	±	20.6	104	±	24.3	70.5	±	24.3
Nitrate (mg NO_3-N/L)	57.5	±	2.57	56.7	±	2.70	57.4	±	2.33	56.6	±	2.65

[1] see Pedersen et al. (2019).
[2] BOD5 = 5 day Biological Oxygen Demand.
[3] TAN = Total Ammonia Nitrogen.

naïve Bayesian classifier (bootstrap cutoff = 80%) (Wang et al., 2007), using the Silva 132 database, and sequences classified as chloroplast, mitochondria, and eukaryota were removed. Sequences were divided into operational taxonomic units (OTUs) at a 97% similarity level, and singleton OTUs were removed. The total amount of sequences obtained was 4,531,155. For calculating alpha and beta diversities, each sample was subsampled to 25,772 sequences. To identify OTUs *Nitrospira*, we analyzed these OTU sequences using MiDAS 4.8.1 taxonomic database (Dueholm et al., 2021) and separated them into strictly nitrite-oxidizers and comammox Nitrospira (Pinto et al., 2016). Sequences have been submitted to NCBI Sequence Read Archive under BioProject PRJNA695118.

2.5. Statistical testing

The data analysis was conducted using R (version 3.6.3; R Core Team, 2020) using packages "vegan" (Oksanen et al., 2019), "phyloseq" (McMurdie and Holmes, 2013), and"ggplot2" (Wickham, 2016). The differences in the abundance of cells (alive cells) and the proportion of dead cells (%Dead) between treatments and weeks were tested with non-parametric Aligned Ranks Transformation ANOVA (ART ANOVA; Wobbrock et al., 2011), since the normality assumptions were not met. The differences in the microbial community composition between treatments were assessed with principal coordinates analysis (PCoA) and PERMANOVA based on Bray-Curtis similarities. The four main OTUs explaining the differences between treatments or between water and biofilm communities were determined with SIMPER function. The differences in the similarities (based on Bray-Curtis dissimilarity), diversity, OTU richness, and the abundance of ammonia/nitrite-oxidizing or off-flavor producing bacteria between treatments and different weeks in water samples, or between treatments and sample types (water, biofilm) in week 7 were tested with two-way ANOVA. The differences in the similarities in time within treatments were tested with one-way ANOVA.

3. Results

3.1. Microbial abundance in water

At the beginning of the experiment, the abundance of total cells ranged from 5.6×10^7 to 7.0×10^7 mL^{-1} and the abundance of live cells ranged from 4.5×10^7 to 5.9×10^7 mL^{-1} with limited variation within and between treatments (Fig. 1A, B). The abundance of both total cells and live cells decreased towards the end of the experiment, also in control RAS units, however, being still significantly affected by the water treatments (ART, Total cells: Treatment × Week, $F_{24,72} = 8.8$, $P < 0.001$, Live cells: Treatment × Week, $F_{24,72} = 8.1$, $P < 0.001$; Fig. 1A, B). Over time, large within-treatment variation was found in the control and foam fractionation treatment groups, while the ozonated units were more similar to each other. In weeks 1, 2, and 4, ozonated units had a significantly lower amount of alive cells than the control and foam fractionator units, and in week 8, control units had a higher amount of alive cells than the ozonated units (post-hoc comparisons). In week 0, the proportion of dead cells ranged from 13 to 28%. Similarly to the live-cell abundance, treatments affected the proportion of dead cells

(Treatment × Week, $F_{24,72} = 3.0$, $P < 0.001$, Fig. 1C), with a significant difference only in a few weeks, as the proportion of dead cells was higher in ozonated units in weeks 2–4, the latter including also units with both ozone and foam fractionator.

3.2. Microbial community composition

The microbial communities suspended in water and growing as biofilms in biofilters (Fig. 2) were significantly different (pseudo-$F_{1,23} = 8.4$, $P = 0.001$). Although the microbial communities evolved over time, water treatment had a significant effect on the microbial community composition both in water samples (PERMANOVA: Treatment × Week, pseudo-$F_{3,35} = 2.18$, $P = 0.002$) and biofilm samples (Treatment, pseudo-$F_{1,11} = 3.0$, $P = 0.001$) (Fig. 2A). In water samples, the communities sampled from ozone-treated units were distinct from the non-ozone-treated units in all sampling times ($P < 0.05$; Fig. 2A), and treatment explained 49–69% of the variation in the community composition. In biofilms, water treatment explained 53% of variation, control unit communities being different from the treatment unit communities. The communities suspended in the water in the ozone-treated units evolved significantly in time, as the similarity with week 0 communities decreased from 33 ± 8% in week 1 to 7 ± 1% in week 7 (Fig. 2B; Supp. Table 1). This was not observed in either control or foam fractionator units, where communities in week 7 were 41 ± 9% similar to the original week 0 communities. When comparing the similarities between treatments within sampling time (Suppl. Fig. 2), control communities were more similar to the communities from foam fractionator units than from ozone-treated units, except in week 3, when foam fractionator communities were dissimilar from the other three treatments (Suppl. Table 2). In biofilm samples, the overall similarity between treatments was higher than in water samples. There, the composition of the foam fractionator communities overlapped with both control and ozone-treated unit communities, while control communities were separated from the ozonated units (Suppl. Fig. 2, Suppl. Table 2). The similarity between water and biofilm community in week 7 was significantly lower in the foam fractionator units (15 ± 2%) than in the other units (Fig. 2A; Suppl. Table 3). Furthermore, the communities in water and biofilm were less similar to each other in control (31 ± 7%) and ozone + foam fractionator units (26 ± 6%) than in ozone units (40 ± 8%; Suppl. Table 3).

In water samples, treatment had a significant effect on the OTU richness and diversity (Fig. 3; Suppl. Table 4), while there was no significant effect of sampling time or interaction between treatment and time. The units with ozonation hosted the highest richness (4468 ± 573) and diversity (4.6 ± 0.4; Suppl. Table 4). When comparing units with ozone to the units with ozone and foam fractionation, the richness was similar (ozone + foam fractionation: 3992 ± 716), but diversity was significantly lower in the latter (ozone + foam fractionation: 3.9 ± 0.7). Furthermore, richness (3043 ± 443) and diversity (3.1 ± 0.2) were lowest in the foam fractionation units. The richness (3537 ± 635) and diversity (3.4 ± 0.4) of the control units were similar to the two latter treatments. In biofilm samples, treatment also affected both richness and diversity (Suppl. Table 4), both being significantly higher in the units with foam fractionator (5387 ± 361, 5.5 ± 0.4) or with ozone and foam fractionator (5351 ± 153, 5.6 ± 0.1) than in control units (4572 ± 409, 5.2 ± 0.1), while ozone units did not differ from the other units (5046 ± 173, 5.4 ± 0.2). Furthermore, when comparing biofilm and water samples taken in week 7, the richness was higher in biofilm than in water in the units with foam fractionator or with ozone + foam fractionator and the diversity was higher in biofilm than in water within all the treatments (Suppl. Table 5).

Throughout the experiment, the most abundant microbial class was Alphaproteobacteria (Fig. 4). When comparing the development of the microbial communities suspended in water in the ozonated units to that of the ones in the non-ozonated units, Actinobacteria disappeared and the relative abundance of Alphaproteobacteria decreased, whereas Bacteroidia and Deltaproteobacteria became more abundant towards the end of the experiment. Furthermore, class Verrucimicrobiae disappeared from the non-ozonated units before week 7. All biofilter biofilm communities had a higher relative abundance of classes Gemmatimonadetes and Nitrospira than the communities suspended in the water (Fig. 4).

Of the main ten OTUs (Table 2) explaining the differences between non-ozonated and ozonated units (46% of difference explained), OTUs assigned to alphaprotebacterial genera *Hyphomicrobium* (OTU1) and *Tabrizicola* (OTU7), gammaproteobacterial *Comamonas* (OTU6), actinobacterial *Aurantimicrobium* (OTU9), *Candidatus* Planktophila (OTU21), and bacteroidial *Lacihabitans* (OTU13) had a higher abundance in the non-ozonated units, whereas deltaproteobacterial *Haliangium* (OTU4), alphaproteobacterial *Gemmobacter* (OTU2), and bacteroidial *Flectobacillus* (OTU5) were more abundant in the ozonated units.

The main ammonia-oxidizing bacterial (AOB) genus in biofilter biofilm samples was *Nitrosomonas* (85 ± 8% of AOB sequences), while *Nitrospira* was the only nitrite-oxidizer (NOB) found (Table 3). The relative abundance of all *Nitrospira* was higher than of AOB, and the

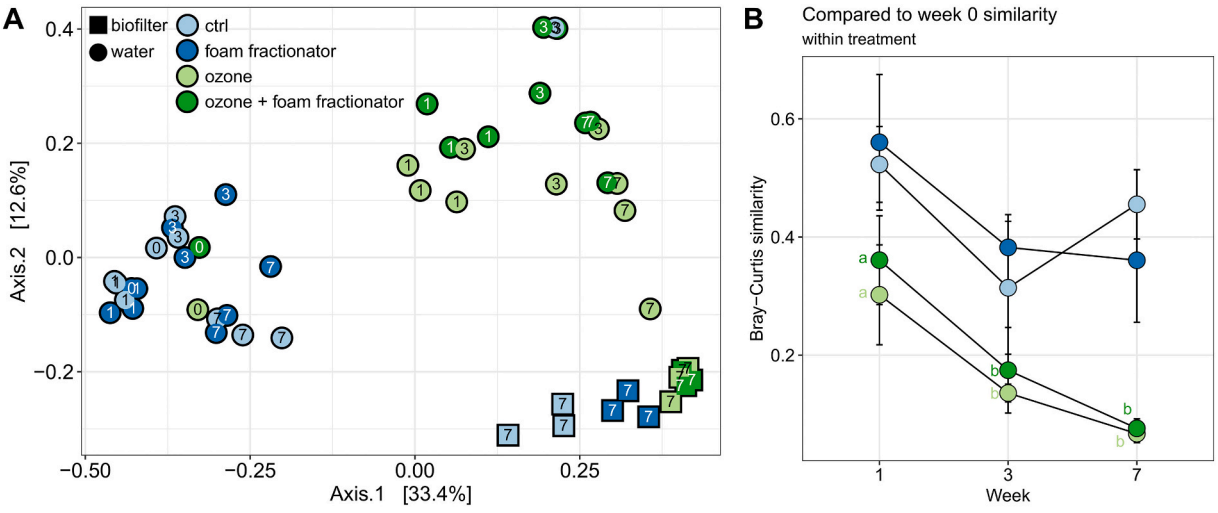

Fig. 2. A) PCoA of water samples based on Bray-Curtis similarities and B) similarities as compared to week 0 communities within treatment. Values are reported as mean ± SD. The letters denote for significant differences in the Tukey post-hoc test results between sampling times.

Table 2
The average relative abundance of the ten main OTUs of 16S rRNA genes differentiating non-ozonated (control, foam fractionator) and ozonated (ozone, ozone + foam fractionator) RAS units, their contribution to differences in the microbial community structures, and the accession number, taxonomy, isolation habitat and proposed physiology of their closest matching organisms represented in the SILVA 132 database.

OTU	Relative abundance		Contribution	Accession number	Identity percentage	Taxonomy (Genus)	Isolation habitat	Physiology
	Non-ozone	Ozone						
OTU1	14.3%	1.8%	7.8%	HM124367.1	98%	*Hyphomicrobium*	Lake sediment	Heterotroph, uses simple carbon compounds (Oren and Xu, 2014)
OTU7	11.1%	0.1%	6.5%	KU360709.1	98%	*Tabrizicola*	Lake water	Heterotroph, some strains are aerobic anoxygenic phototrophs (Tarhriz et al., 2019)
OTU4	1.9%	9.4%	5.4%	CP001804.1	91%	*Haliangium*	Coastal sand	Heterotroph, degrades biomacromolecules, lyse microbial cells (Garcia and Müller, 2014)
OTU6	9.5%	1.6%	5.3%	MT323131.1	99%	*Comamonas*	Rainbow trout	Heterotroph, degrades complex aromatic compounds (Willems, 2014)
OTU2	2.2%	8.0%	4.9%	CP028918.1	99%	*Gemmobacter*	River water	Heterotroph (Chen et al., 2013; Kang et al., 2017a)
OTU5	0.0%	7.1%	4.2%	MK402935.2	99%	*Flectobacillus*	Groundwater	Heterotroph (Sheu et al., 2017)
OTU9	5.9%	0.2%	3.4%	NR_145615.1	99%	*Aurantimicrobium*	River water	Heterotroph, ultra-micro sized (Nakai et al., 2015)
OTU16	5.2%	0.1%	3.1%	NR_136787.1	99%	*Emticicia*	Stream sediment	Heterotroph, abundant in high C:N (Yu et al., 2016)
OTU13	5.1%	0.7%	3.0%	MG780349.1	99%	*Lacihabitans*	Lake sediment	Heterotroph, degrades biomacromolecules (Kang et al., 2017b)
OTU21	4.5%	0.1%	2.7%	CP016773.1	97%	*Candidatus* Planktophila	Freshwater lake	Heterotroph (Neuenschwander et al., 2018)

Table 3
The relative (% of sequences) and absolute abundance (amount of reads) of total ammonia-oxidizing (AOB) bacteria and *Nitrospira,* the absolute abundances of AOB genera, nitrite-oxidizing (NOB) *Nitrospira* and comammox-*Nitrospira*, and the proportion of comammox of all *Nitrospira* reads (mean ± SD) in biofilter biofilms in the four treatments in week 7.The letters denote for significant differences in the Tukey post-hoc test results between treatments

	Control			Foam fractionator			Ozone			Ozone + foam fractionator		
All AOB	0.35%	±	0.13%	0.83%	±	0.17%	0.54%	±	0.31%	0.80%	±	0.22%
	89	±	34	210	±	44	138	±	77	204	±	56
Nitrosomonas	72	±	37	181	±	36	125	±	72	174	±	66
Other Nitrosomonadaceae	5	±	2	7	±	5	4	±	5	6	±	2
Nitrosomonadaceae unclassified	11	±	7	21	±	4	9	±	2	23	±	10
All *Nitrospira*	1.78%	±	0.64%	4.29%	±	0.71%	3.26%	±	1.21%	4.62%	±	1.64%
	452	±	164	1089	±	179	827	±	308	1173	±	416
Strictly NOB *Nitrospira*	357	±	97	652	±	246	585	±	213	646	±	301
Comammox *Nitrospira*	95	±	85^{a}	437	±	90^{bc}	242	±	103^{ab}	527	±	117^{c}
Comammox of all *Nitrospira*	19%	±	$12\%^{a}$	42%	±	$14\%^{ab}$	29%	±	$4\%^{ab}$	46%	±	$6\%^{b}$

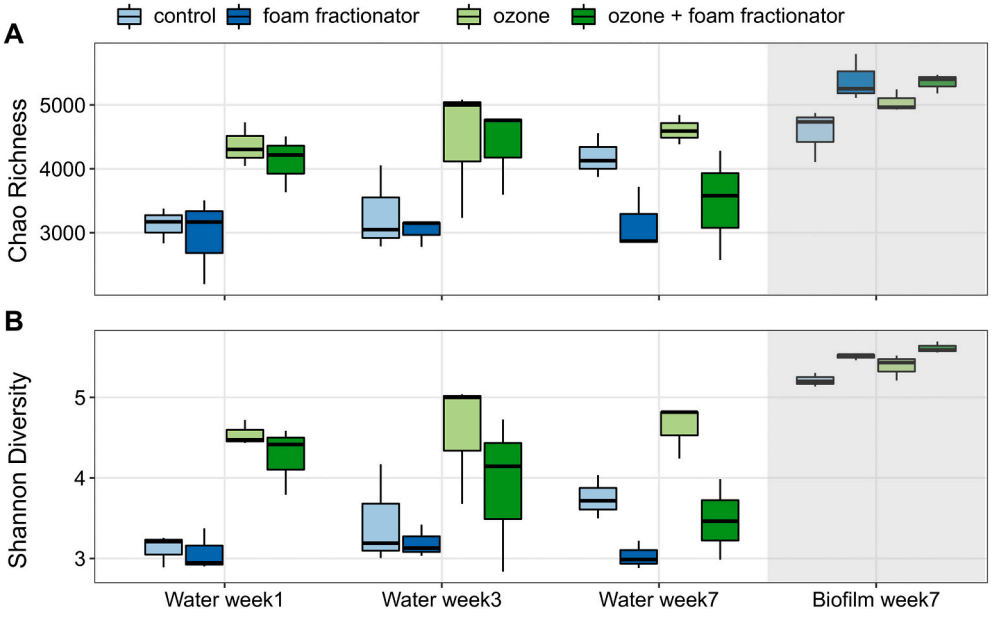

Fig. 3. A) OTU richness (chao) and B) Shannon diversity index in water and biofilm samples in four treatments in weeks 1, 3, and 7.

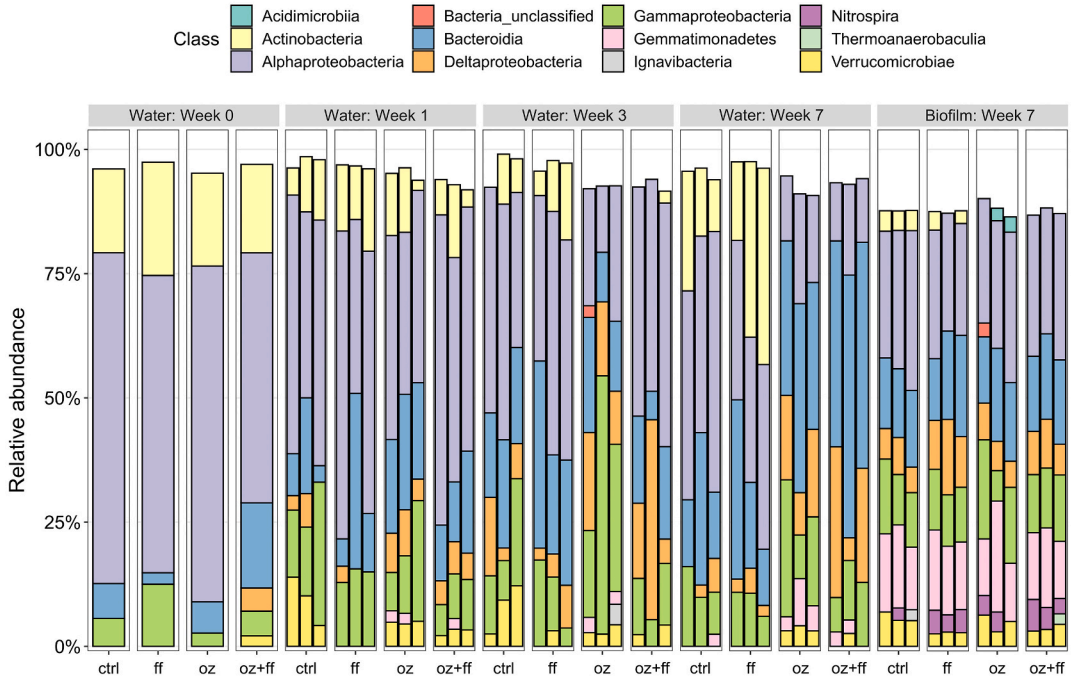

Fig. 4. The relative abundance of microbial classes in water and biofilm samples in four treatments (ctrl = control, ff = foam fractionator, oz. = ozone, oz. + ff = ozone and foam fractionator) in the beginning (week 0), and in weeks 1, 3, and 7. Only classes with an abundance of >1% are included.

relative abundance of both groups seem to be higher, yet statistically insignificant (ANOVA, AOB: $P = 0.08$, NOB: $P = 0.06$), in the units with foam fractionators. When dividing *Nitrospira* into strictly NOB-*Nitrospira* and comammox *Nitrospira*, the absolute abundance of the latter one was significantly higher in the units with foam fractionation and foam fractionation + ozonation (Suppl. Table 6). Furthermore, the relative abundance of comammox *Nitrospira* of all *Nitrospira* sequences was higher in the foam fractionation + ozonation units than in the control units.

The only potential off-flavor (geosmin or MIB) producers found were assigned to deltaproteobacterial genus *Nannocystis* and actinobacterial *Nocardia*. Treatment had a significant effect on the potential geosmin-producer abundance, as units exposed only to ozone had a significantly higher relative abundance of geosmin producers in water (0.37 ± 0.18%) than units without ozonation in all the weeks (control: 0.13 ± 0.26%, foam fractionator: 0.04 ± 0.02%; Suppl. Tables 7, 8). When comparing the relative abundances between water and biofilter in week 7, biofilters hosted a significantly higher relative abundance of potential geosmin-producers (0.69 ± 0.38%) than water samples (0.20 ± 0.24%), independently of the treatment (Suppl. Tables 7, 8).

4. Discussion

In this study, we quantified the separate or combined effects of two water treatment methods, ozonation and foam fractionation, on freshwater RAS microbiology. Each treatment was found to have different effects on and mechanisms to affect water and biofilm microbial communities. Foam fractionation had only a limited effect on the microbial abundance and community composition, while ozone caused dramatic microbiological changes recorded after only one week of the experiment. Even though we demonstrated that biofilter biofilm communities are less vulnerable to the water treatment than the communities suspended in the system water, we saw differences in the relative abundances of key microbial groups in biofilter biofilm between control and different water treatment units.

The water quality data collected from the experiment showed that ozonation and foam fractionation each improved the water quality by decreasing the amount of particles, microbial activity, and biological oxygen demand in water, and these effects were pronounced when these two treatments were combined (de Jesus Gregersen et al., 2021). Foam fractionation, when implemented solely, decreased turbidity and particle volume, but the microbiological results presented here indicate that the effect of foam fractionation on the system microbiology is less substantial and indirect. Based on the flow cytometry data (Fig. 1), foam fractionation did not lower the abundance of free-living microbes as compared to the control units, suggesting that the lower microbial activity observed under foam fractionation is not due to the reduced microbial abundance as such, but rather due to the lower amount of organic matter i.e. substrate being available for heterotrophic microbes. However, we acknowledge that samples were prefiltered to remove particles larger than 40 μm, so it is possible that foam fractionation could still have reduced the amount of large microbial flocs or larger eukaryotic micro-organisms, which are covered in the microbial activity measurements (Pedersen et al., 2019). No information on the effect of foam fractionation on total microbial abundance in RAS exists before this study, but it has previously been found to decrease the abundance of viable heterotrophic microbes (Brambilla et al., 2008; Rahman et al., 2012) and to target both small (microbial cells) and large (organic matter aggregates) particles, but not of medium-sized ones (Brambilla et al., 2008). These previous findings support our conclusion on foam fractionation controlling microbial abundance through decreasing organic matter content. Furthermore, the microbial community composition was not significantly different between the control and foam fractionation units (Fig. 2), while OTU diversity and richness were lower under foam fractionation in week 7 (Fig. 3). This indicates that the organic matter removal through foam fractionation affects the abundance of specific rare microbial taxa, not being visible in the overall abundance patterns, but potentially having a functional significance. For example, the microbial genus with very small cell size, *Aurantimicrobium* (Nakai et al., 2015), was more abundant in foam fractionation units (data not shown), indicating that the communities can adapt to the foam fractionation treatment. Furthermore, the potential geosmin producers, such as genera *Nocardia* and *Nannocystis* (Azaria and van Rijn, 2018), which abundance is known to be connected with the organic matter

availability in RAS (Guttman and van Rijn, 2008), were found to have lower relative abundance in the water of the RAS units with foam fractionation than in the other units. Even low relative abundances (<1%) of off-flavor producers can lead to significant accumulation of the produced compounds in water and fish (Lukassen et al., 2017), so this result on foam fractionation reducing their relative abundance is encouraging and needs further investigation.

Unlike foam fractionation, ozone was observed to have a strong effect on the microbial communities suspended in water already one week after application (Figs. 1-4). Ozone attacks the microbial cells directly, so even the moderate dosing used in this experiment was enough to significantly lower cell abundance and increase microbial mortality. Combining foam fractionation with ozonation did not seem to have an additional effect and the variation in the cell abundance was much lower in ozonated units than in non-ozonated ones (Fig. 1), highlighting the high disinfection efficiency of ozone. Ozonation led to significant changes in the microbial community composition, making them to deviate more and more from the communities in the beginning and from the control communities during the experiment (Figs. 2, 4). After two weeks of applying ozone, the microbial abundance had dropped by 97%, but it started to rise slightly towards the end of the experiment, indicating that the remaining microbial taxa had adapted to tolerate ozone and grow. Indeed, when inspecting the ten main OTUs explaining the differences between non-ozonated and ozonated units, the genera being previously isolated from very clean water with low carbon availability (spring, artificial fountain; *Gemmobacter*, *Flectobacillus;* Chen et al., 2013; Kang et al., 2017) or being capable to produce spores to survive through harsh conditions (*Haliangium;* Garcia and Müller, 2014) were substantially more abundant in ozonated than in non-ozonated (control, foam fractionator) units (Table 2). In contrast, the main taxa that were more abundant in non-ozonated units (control and/or foam fractionator) are known to thrive in carbon-rich conditions (Emticicia; Yu et al., 2016), degrade complex organic molecules (*Comamonas*, *Lacihabitans;* Kang et al., 2017a; Willems, 2014) or inhabit lake environments (*Hyphomicrobium*, *Tabrizicola)* with presumably variable organic matter and nutrient concentrations. Overall, these results suggest that continuing with a moderate ozone dosing could eventually lead to the increase in the microbial abundance with community consisting of the adapted key taxa. Interestingly, the relative abundance of potential geosmin producers was highest in the ozonated units, suggesting them to benefit from the higher abundance of bioavailable molecules. Ozonation has already previously shown to be ineffective in reducing the off-flavor compounds in water or fish flesh (Schrader et al., 2010), so this result indicates the low potential of ozonation in targeted control of off-flavor production. However, the overall cell abundance was very low in ozonated units (17 ± 29% of control cell abundance), so the relative increase may not have a true biological impact.

Our results corroborate the previous findings on biofilms being microbial richness and diversity hotspots (Hüpeden et al., 2020), both values being higher in biofilms than in water communities. Although the major part of the biofilter community consisted of non-nitrifying microbes, both ammonia-oxidizers (AOB) and nitrite-oxidizers (NOB) were found to be present. Nitrite-oxidizing *Nitrospira* was more abundant than any AOB in all the biofilters, as has been previously observed in freshwater and marine RAS biofilter samples (Bartelme et al., 2017; Fossmark et al., 2021; Suurnäkki et al., 2020). When inspecting *Nitrospira* sequences, 19–46% of them were affiliated with comammox Nitrospira, which conducts complete nitrification (Daims et al., 2015), suggesting that the higher abundance of *Nitrospira* than AOBs can be explained by a large proportion of them conducting complete nitrification rather than only nitrite oxidation. Only singleton sequences assigned to ammonia-oxidizing Archaea (AOA; *Candidatus* Nitrocosmicus) were found in two biofilter samples (one from the system with foam fractionator, one from ozone + foam fractionator). Since AOA have also not been found in RAS biofilters in the previous RAS studies (Hüpeden et al., 2020; Keuter et al., 2017; Suurnäkki et al., 2020), they may have low importance for nitrification in the system. Biofilm communities responded differently than water communities to the treatments applied. In general, biofilm communities were more resistant to the water treatment, exhibiting rather high similarity (50–56%) between control and treatment units. Since the effect of foam fractionation on microbes seem to be indirect, and ozone is known to have a weaker effect on particle-attached than free-living microbes (Hess-Erga et al., 2008) and in general, disappear fast when applied to freshwater (Bullock et al., 1997), this outcome could be expected. However, such slight changes in the community composition in all treatment units, also in foam fractionation units, are in contrast with the results obtained in the communities suspended in water. Interestingly, the OTU richness was higher in the biofilter biofilms of the foam fractionator units, which was an opposite trend as compared to water communities. This could be related to organic matter removal decreasing the proportion of heterotrophs and opening more niches for autotrophs e.g. nitrifiers in the biofilms. Indeed, the absolute abundance of comammox *Nitrospira* was higher and relative abundance of comammox *Nitrospira* of all *Nitrospira* sequences in the foam fractionation units, and the accumulation of nitrite was lower. These results suggest that foam fractionation potentially promotes nitrification in the biofilters by decreasing the activity of heterotrophic microbes, allowing higher abundance of nitrite oxidizers but also a shift in the nitrifying community from canonical two-step process into the complete nitrification. Previously, foam fractionation has been shown to promote DIN removal (Rahman et al., 2012), and our results seem to explain the underlying reasons. Ozone did not alter the genetic nitrification potential i.e. the abundance of nitrifiers in the bioreactors, as has already been previously observed (Schroeder et al., 2015).

5. Conclusions

Altogether, the results obtained in this study demonstrate that both foam fractionation and ozonation affect the microbial abundance, microbial activity in the water, and/or community composition in the freshwater RAS, but with different mechanisms. Foam fractionation caused only slight changes in the overall microbiology but has a targeted effect on the biofilter biofilm microbial community, suggesting that it may reduce unwanted heterotrophic growth and activity through decreasing organic matter in the system, thus promoting more stable nitrification in the biofilters. In contrast, ozonation poses a strong selection pressure by attacking the microbes directly, shaping the microbial communities in water, which may potentially open niches for specific ozone-tolerant taxa. However, more information on the long-term development of RAS microbial communities under ozonation is still needed.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737846.

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