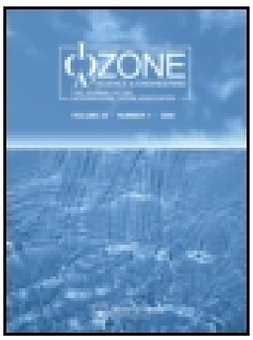

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Ozone: Science & Engineering
The Journal of the International Ozone Association

ISSN: 0191-9512 (Print) 1547-6545 (Online) Journal homepage: http://www.tandfonline.com/loi/bose20

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To cite this article: Desislava Bögner, Frederike Schmachtl, Björn Mayr, Christopher P. Franz, Sabine Strieben, Gregor Jaehne, Kai Lorkowski & Matthew J. Slater (2018): Sludge Pre-Treatment through Ozone Application: Alternative Sludge Reuse Possibilities for Recirculating Aquaculture System Optimization, Ozone: Science & Engineering, DOI: 10.1080/01919512.2018.1510765

To link to this article: https://doi.org/10.1080/01919512.2018.1510765

OZONE: SCIENCE & ENGINEERING
https://doi.org/10.1080/01919512.2018.1510765

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ABSTRACT

Recirculating Aquaculture Systems (RAS) reduce water consumption by efficient filtration to maintain appropriate levels of accumulating compounds and sludge. Sludge is mechanically separated by drum filters and disposed of to the detriment of overall system water budgets. Dissolved nitrogen compounds are reduced via nitrification–denitrification filters, requiring commercial external carbon sources. The reuse of sludge after ozone pre-treatment may represent the next step in RAS optimization. The present study analyzes the content of sludge from RAS and tests ozonation as a pre-treatment for recycling as carbon source. The dissociative effect of ozone and the physicochemical changes due to ozonation lead to a significant increase in soluble carbon availability. Predominantly long-chain fatty acid (FA) (saturated and unsaturated) with 16 and 18 carbon atoms independently of the treatment were found in the profiles. Saturated FA concentrations in solution increased after 20, 40, and 60 min ozonation. The solid content of the sludge was practically unaffected by ozonation in terms of FA profile: only saturated FA slightly increases after 40 min treatment. The implications of these findings for denitrifying bacteria are discussed.

Abbreviations: Recirculating Aquaculture Systems (RAS); Advanced Oxidation Processes (AOPs)

ARTICLE HISTORY
Received 10 April 2018
Accepted 17 July 2018

KEYWORDS
Ozone; Denitrification; *Dicentrarchus labrax*; Natural organic matter; Ozonation; RAS; Recycling; Sludge; Wastewater treatment; Water reuse

Introduction

Recirculating Aquaculture Systems (RAS) make extremely efficiently use of water resources compared to other culture systems such as pond or cage-based forms of aquaculture (Timmons and Ebeling 2013). However, sludge elimination is still an essential process in which water is needed to backflush filters. The removal and disposal of solid aquaculture wastes, mainly containing organic matter and nitrate, incurs additional production costs in RAS (Letelier-Gordo et al. 2017). However, sludge may be a valuable source of biodegradable carbonaceous compounds to be used by denitrifying bacteria, replacing the additional requirements for external commercial carbon sources (Vergara, Nickel, and Neis 2012). The impact of ozone as pre-treatment of sludge to solubilize available carbon and other compounds remains, however, unexplored.

Recycling in aquaculture requires the integration of filters to remove soluble accumulating metabolites such as nitrogenous organic and inorganic compounds and sediments/solid wastes. Most RAS have biological filters for nitrification and denitrification and drum filters, microscreen, granular filters, and sedimentation tanks for the separation of solid wastes with particle sizes >20 µm (Timmons and Ebeling 2013). With these conventional methods, nitrogen inorganic compounds, and settleable and filterable solids can be removed, but fine colloidal solids (from 0.001 to 20 µm particle size) remain in the system and require additional treatments like foam fractionation or DE/Cartridge filtration (Timmons and Losordo 1994). Sludge in RAS is mainly composed of feces and uneaten feed. Sludge biofilms are composed of active 2.5-µm-diameter bacterial micro-colonies linked by inactive biomass, mainly exo-polymers, which form subunits of 10–20 µm diameter that associate with refractory organic materials and mineral compounds into flocs of around 125 µm diameter (Déléris et al. 2000). For a system rearing seabass (*Dicentrarchus labrax*), the total solid composition within the rearing system consisted of 74% particles of 1.2–60 µm average size, 18% of particles in the range 0.2–1.2 µm, and only 7% particles >60 µm

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Supplemental data for this article can be accessed here.

(Brambilla et al. 2008). Removal rates of 96.8% and 100% can be achieved by foam fractionation for averaged particle size of >60 µm and between 0.2 and 1.2 µm, respectively, but only up to 19% removal was possible by this method for the larger percentage of total solids composition of the system. By accumulating fine, dissolved organic and inorganic substances, as well as colloidal solids and non-biodegradable refractory organic compounds within RAS, the risk of system impairment and stress for the rearing animals rises, while lower efficiency of the filters, less operational stability, and lower productivity emerge (Lekang 2013).

Sludge in RAS is produced, quantitatively and qualitatively, in a continuous predictable manner, and it is a source of particulate carbon equivalent to ca. 20% of the feed input to a system (Lekang 2013; Letelier-Gordo et al. 2017). Boyd (1985) stated that for each kg live channel catfish produced in ponds, 1.32 kg of feed are required which releases 51.1 g nitrogen, 7.2 g phosphorus, and 3.69 kg chemical oxygen demand (COD) in the form of metabolic wastes. Ghaly, Kamal, and Mahmoud (2005) reported aquaculture wastewater effluents (containing liquid and solid wastes) having total COD values of 240 mg L^{-1} of which 150 mg L^{-1} were in soluble state; 60 mg L^{-1} total Kjeldahl nitrogen (TKN) concentration, 50.4 mg L^{-1} total phosphorus, 6.6 mg L^{-1} orthophosphate, and 110 and 5 mg L^{-1} NO_3-N and NO_2-N concentrations, respectively. Total solids and suspended solids concentrations of 1000 mg/L and 100 mg/L were reported for the effluent used by these authors. For a system rearing 200 kg red drum (*Sciaenops ocellata*) of 15 g average weight in a 15 m^3 tank, the particulate organic matter production by fish ranged between 15.9 and 23.5 g h^{-1} (Barrut et al. 2013). In general, fish culture alone may retain 20–50% feed nitrogen (N) and 15–65% feed phosphorus (P) which can be increased (by the combination of fish culture with phototrophic conversion) or decreased (via the addition of herbivore consumption and the conversion of nutrients into bacteria and detrivorous worm biomass) according to general nutrient losses from one trophic level to the next one (Schneider et al. 2005). As stated by these authors, the retention may face limitations related to uptake kinetics, nutrient preferences, unwanted conversion process, and the influence of abiotic factors.

There are different measures for the organic load present in sludge samples including total organic carbon (TOC), COD, and 5-day biological oxygen demand (Davies 2005). These are mainly used by wastewater treatment plants and allow the control of the biological nitrogen removal and the enhanced biological phosphorus removal processes. In wastewater treatment plant sludge, the COD could be split into 10–20% soluble fraction and 80–90% colloidal fraction (not passing 0.45 µm membrane filtration) and requires pretreatment (e.g., sonication and fermentation) in order to increase bioavailability by modifying the hydrolysis rate of the particulate endogenous carbon compounds (Vergara, Nickel, and Neis 2012).

An alternative to enhance biodegradability, solubilize, or eliminate solid wastes and breakdown organic and inorganic compounds is the use of oxidizing agents like ozone (O_3) and hydrogen peroxide (H_2O_2). Advanced oxidation processes (AOPs), chemical processes commonly used for wastewater and drinking water treatment, make use of oxidizing agents or their combination with techniques leading to the formation of highly reactive oxygen species (e.g., hydroxyl radicals), at a sufficient concentration to enable a non-selective reaction with organic and inorganic compounds and finally their mineralization (Oturan and Aaron 2014). These kinds of treatments also reduce or eliminate bacterial loads of water samples. Among the methods and techniques used in combination with oxidizers, UV irradiation, Fenton, photo-Fenton, semiconductor photocatalysis, electrolysis, sonication, and wet air oxidation have been successfully used for water/wastewater treatment (Antonopoulou et al. 2014; Oturan and Aaron 2014) and some have been tested at lab-scale for compounds removal and disinfection purposes in aquaculture facilities to lower the incidence of illness and off-flavor compounds and to improve the water quality (Brazil 1997; Klausen and Grønborg 2010; Nam-Koong et al. 2016; Pedersen and Pedersen 2012; Yao et al. 2017). Ozonation has been used to increase anaerobic digestion of waste-activated sludge for wastewater treatment with promising results in terms of increasing the soluble COD (Silvestre et al. 2015), but excluding disinfection purposes, there is a gap on the use of ozone in aquaculture facilities.

The production of ozone is energy intensive and compounds with multiple bonds along with negatively charged atoms of elements, such as nitrogen, phosphorous, oxygen, sulfur, and nucleophilic carbons, offer sites for initial reactions with ozone and ozone-derived radicals (Brazil 1997). Ozone used in RAS facilities for disinfection purposes is generated on-site and immediately used within contactors and the systems are equipped with sensors to control ozone doses and to avoid overexposure of bacterial communities within the filters, reared organisms, and working staff (Timmons and Ebeling 2013). Ozone is also applied in protein skimmers to separate suspended solids of the liquid phase which is then disposed in the form of foam (Sander 1998). This combines fractionation and disinfection in one step and helps maintain the pH by removing organic acids and bacteria from the water (Timmons and Losordo 1994). Foam fractioning eliminates mostly suspended solids and proteins acting as surfactants (Timmons and Ebeling 2013), but may

be adversely affected by anti-surfactant properties of feeds coated with fish oil for a better palatability (Weeks, Timmons, and Chen 1992). Weeks, Timmons, and Chen (1992) found that foam fractionation concentrates volatile solids, TKN, and total suspended solids in the condensate foam collected out of fractionation columns used in fish culture systems.

According to the literature, AOPs, foam fractioning, or the combination of these techniques have not been used as pre-treatment method for increasing sludge biodegradability in RAS. As ozonation has fast reaction rates and may serve to dissociate particulate organic matter out of RAS increasing the bio-availability of different compounds contained in sludge, and it is possible to control the process by means of simple measurements already performed in RAS facilities, we selected it as a treatment method for our study. The aims of the present study were as follows:

(i) to explore the impact of ozone-treatment on sludge in terms of carbon and nitrogen balances and fatty acid compositions,
(ii) to evaluate the required exposure for decomposition, solubilization, and/or mineralization of the particulate organic matter in order to obtain the highest levels of putative biodegradable intermediates in solution.

Materials and methods

Sludge sampling and processing

Sludge for all experiments was gathered with a polyethylene cylindrical sludge collector (127.5 cm height, 31.5 cm diameter, with 11 cm inlet from the RAS drum filter, and an 11 cm outlet to the canalization) equipped with a pump for homogenization. The sludge was produced from three tanks (1 m^3 each) containing a total of 300 kg fish (European seabass *D. labrax*) reared and fed *ad libitum* in the Centre of Aquaculture Research in Bremerhaven, Germany. Ozonation was performed using Ozone generators Certizon C300 (Sanders, Capacity: 300 mg ozone h^{-1}) 1 L min^{-1} and dry air in 500 ml Duran borosilicate gas-washing bottles (Drechsel pattern) equipped with borosilicate glass gas pipes and filter plates (porosity 1, 100–160 µm) and fixed with a screw cap and a silicon sealing ring. In this manner, an ozone dosage of 5 mg O_3 L^{-1} min^{-1} was possible and according to the sludge specific weight, an effective ozone dosage of 0.01 mg O_3 per mg sludge (solid and liquid phases together) per min (or in terms of solids present in the sample of 0.85 mg O_3 per mg solid sludge per min) was used. Each bottle filled with 400 ml sludge sample (average net tared sludge weight of 414.6 mg from which 5.85 mg were solids) had an inlet flexible ozone-resistant pipe connected to an ozone generator and an outlet to degas. Bottles filled with 400 ml sludge without connexion to the ozone generators and in contact with air were used as controls for initial (T0) conditions (labeled as control in the results of fatty acids profiles). Subsamples of not-treated sludge were taken from additional control bottles to account for possible further disintegration occurring due to natural decay after each exposure period (DOC, TDN, NH_4-N; NO_2-N in the liquid phase, as well as C and N content of the solid phase were measured; supplementary data). Additionally, three bottles were filled with distilled water and used as blanks to discard any possible contribution not belonging to the samples. After ozonation, each bottle was separated from the aeration unit, homogenized, and four subsamples (45 ml volume) per bottle were collected in 50-ml Falcon tubes (Rotilabo, Germany) previously weighted (Sartorius CPA 224S, Max. 220 g, d = 0.1 mg) and washed within an HCl bath (10%) for 72 h and distilled water. The rest of the sample was used for measuring physiochemical parameters. The 45-ml collected samples were centrifuged (Eppendorf Centrifuge 5810R) at 4000 rpm at 3°C for 30 min. After centrifugation, the liquid phase was decanted into a 60-ml HDPE bottles previously washed (72 h in HCl bath 10% and distilled water) and 15-ml Falcon tubes (Rotilabo, Germany) for further analyses of total dissolved organic carbon (DOC) and total dissolved nitrogen (TDN), nutrient content and fatty acid profiles. The solid and liquid phases were wet- and dry-weighted (Sartorius CPA 224S, Max. 220 g, d = 0.1 mg), lyophilized, and prepared for the determination of carbon, nitrogen, and fatty acid contents.

Experimental setup

To determine the influence of ozonation on the outputs of treated sludge, we performed an experiment in which sludge was taken from a RAS rearing individuals feed *ad libitum* with commercial feeds Supreme 22 (44% protein, 22% fat) from Coppens International. Sludge exposure to ozone was conducted from 0 to 60 min (bottles sampled each 10 min, six replicates x six treatments, and six not-treated bottles for control). DOC, TDN, and the sludge fatty acid profiles of solid and liquid phases, as well as the available carbon and nitrogen contents of the solid phase in treated and non-treated samples, were determined.

Analytical methods

Physicochemical parameters and nutrient content determination

Salinity, pH, and temperature were determined using a digital Multiparameter Multi 3630 IDS (WTW, Germany)

and sensors for pH (SenTix 940, WTW), and salinity (TetraCon 925, WTW) calibrated before use with the corresponding standard solutions. DOC and TDN in the liquid phase were determined with a Shimadzu TOC-VCSH + TNM-1 Analyzer equipped with an ASI-V Auto-sampler, and carbon and nitrogen content of the solid phases were measured with an Elementary Analysator EuroEA (HEKA tech GmbH). For this, triplicate subsamples of 1–1.2 mg were weighted (Mettler Toledo XP6U Comparator, Max = 6.1 g, d = 0.1 µg) and encapsulated in tin capsules 5 × 9 mm (HEKA tech, GmbH). For the determination of Ammonium-N (NH_4-N) and Nitrite-N (NO_2-N), we used a Hach Lange DR2800 Spectrophotometer and the Salicylate and Diazotization methods, respectively. Nitrate-N (NO_3-N) was measured with a DIQ/S-182 WTW Universal Transmitter with IQ-Sensor Net for Nitrate-N.

Fatty acids extraction and GC measurements

Fatty acid extraction was performed in duplicates of pooled samples of the same treatment (a pool was made out of all replicates per treatment per phase). Liquid and solid phases were analyzed independently. Lipid extraction was carried out following Folch, Lees, and Stanley (1957) as modified by Koch (2012) and Wildförster (2014). Briefly, a total 0.1 g of the sample was weighed in a 12-ml culture tube with screw cap, and 5 mg ml^{-1} or 0.1 mg ml^{-1} of internal standard FAME 23:0 (Methyl tricosanoate, Sigma Aldrich, USA) were added to the solids or liquid phases of the sludge samples, respectively. Then, 2 ml of methanol (MeOH, Carl-Roth GmbH & Co. KG)-dichloromethane (DCM, Carl-Roth GmbH & Co. KG) mixture, (2:1) (MeOH/DCM) was added, and the sample was homogenized and centrifuged (2 min at 2000 rpm). Next, the liquid phase of the sample was transferred to a separating culture tube with a Pasteur pipette. This process was repeated three times and the residual solid sample of the first tube was disposed of. Two ml of 0.88% KCl solution were added to the obtained liquid phase. The sample was shaken for 30 sec, vented multiple times, and centrifuged (5 min at 2000 rpm) for extracting the lower lipid phase with a Pasteur pipette into a third culture tube previously balanced and tested for tightness of the screw cap. This extraction step was repeated twice adding 2 ml DCM. The solvent was then removed by evaporation with N_2 gas. The remaining lipids were prepared for derivatization by the addition of 500 ml of n-hexane and 2 ml derivatization reagent (50 ml MeOH and 1.5 ml 96% sulfuric acid (Carl-Roth GmbH & Co. KG)). The samples were placed overnight in a heating block, maintained at a temperature of 80°C for 4 h, and then cooled to room temperature (21°C). Then, 4 ml of Milli-Q filtered water was added and the whole extracted with 2 ml of n-hexane homogenized and centrifuged (2 min at 2000 rpm) for the separation of the upper phase of the sample to a new culture tube. This process was repeated two times with 2 ml n-hexane. Again, the solvent was removed by evaporation with N_2 until 1 ml volume remained in the tube which was then transferred into a balanced gas chromatograph (GC) vial. The lipids were dissolved in 1 ml of n-hexane and measured with the GC. Fatty acid profiles of both phases were determined using a GC Agilent 6890 N gas chromatograph provided with a Combi PAL Autosampler (CTC Analytics), a SPB-1 Capillary Column 30 m x 0.25 mm x 0.1 µm (Agilent) and with split/splitness injector (split 75:1 for solid samples and splittless for liquid samples), using a FAME-MIX standard (Supelco 37 Component FAME, Supelco) for peak identification by comparison of the retention times. Percentage of individual fatty acids was calculated in relation to the total area of the chromatogram and weighted sample.

Statistical analysis

An exploratory data analysis was performed and the data analyzed for normality and homoscedasticity (Shapiro–Wilk's Test/QQ-plot and Levene's Test/Fligner Killeen Test, respectively). For parameters following a normal distribution and accomplishing the homoscedasticity assumption, the treatment's means were compared using one-way ANOVA. Non-normal distributed or heteroscedastic data were analyzed using a Kruskal–Wallis H Test. Significant differences between treatments and between test and control conditions were located using post hoc tests (Tukey's HSD test, Dunn's Test/Conover-Iman Test). All statistical analyses were performed using R Version 3.5.0 (R_Core_Team 2018). A confidence interval of 95% was used.

Results

Physicochemical parameters and nutrient content determination

DOC and TDN differed significantly between treatments (DOC, $\chi^2 = 26.1$, df = 6, $p < 0.05$; TDN, $\chi^2 = 15.7$, df = 6, $p < 0.05$, Kruskal–Wallis Rank Sum Test, $N = 42$) and ozonation with respect to control also differed (DOC, $\chi^2 = 9.56$, df = 1, $p < 0.05$; TDN, $\chi^2 = 12.4$, df = 1, $p < 0.05$, Kruskal–Wallis Rank Sum Test, $N = 42$) both increasing with higher exposures (Figure 1). For comparison, measurements of control samples collected at the end of each exposure period are presented as supplementary data. Ammonium significantly increased with an increase in ozone exposure ($F_{(6,35)} = 4.67$, $p < 0.05$, One-way

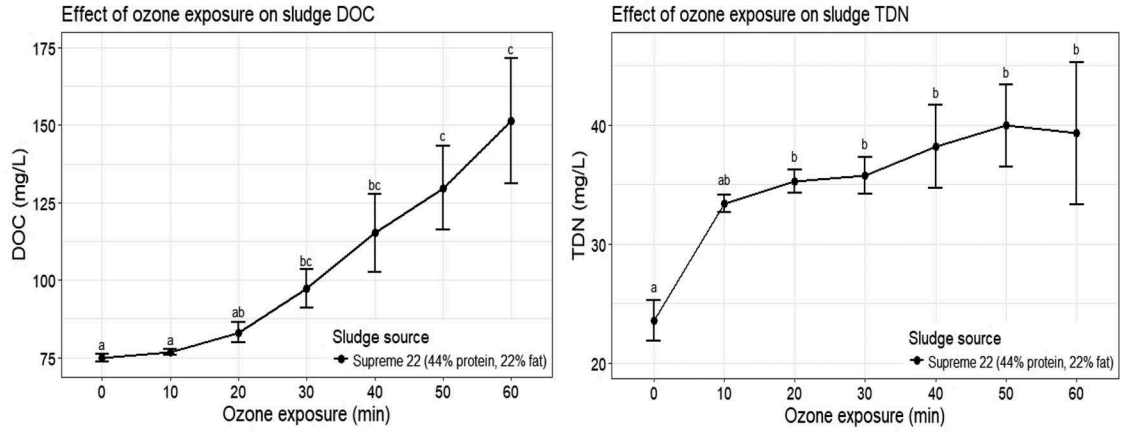

Figure 1. Effect of ozonation on the DOC and TDN contents of the liquid phase of the sludge. Values represent mean ± SE and different letters significant differences detected by Dunn's Test for multiple comparisons after a significant Kruskal–Wallis result.

ANOVA) while nitrite was completely exhausted (χ^2 = 23.47, df = 6, $p < 0.05$, Kruskal–Wallis Rank Sum Test, $N = 42$) and nitrate showed only marginal changes ($F_{(6,35)}$ = 2.31, p = 0.06; Test vs. Control: $F_{(1,40)}$ = 6.25, p = 0.02, One-way ANOVA) decreasing at 20 and 30 min treatment after an initial burst (Figures 2–4). The ozonation process was associated with an increase in pH (Figure 5). Details on the associated variations in temperature and salinity during the experiment are shown in Table 1.

The collected sludge contained a small proportion of particulate matter. From 45 ml samples, an average of 0.66 ± 0.12 ml was in solid state after centrifugation. The solid phase of the samples was analyzed for carbon and nitrogen content showing stable carbon concentrations not differing between the treatments used (C, $F_{(6,35)}$ = 1.44, p = 0.23). Nitrogen showed no differences between treatments but test samples had significantly reduced amounts of nitrogen when compared with control (N, $F_{(6,35)}$ = 1.75, p = 0.14; Test vs. Control: $F_{(1,40)}$ = 10.06 p = 0.003, One-way ANOVA). The carbon concentration, calculated as measured µg/µg sample*100, averaged 29.05 ± 1.72%, whereas the nitrogen concentration achieved 4.40 ± 0.22% in average.

Fatty acids profile

The analysis of fatty acid content of the samples revealed a higher lipid proportion in the liquid phase compared with the solid phase (Figure 6). Short-chain fatty acids were not found in the samples. A detailed fatty acid profile of the ozone-treated samples is listed in Table 2. The liquid phase had higher proportion of saturated fatty acids (SAFA) (represented by palmitic acid (16:0) and stearic acid

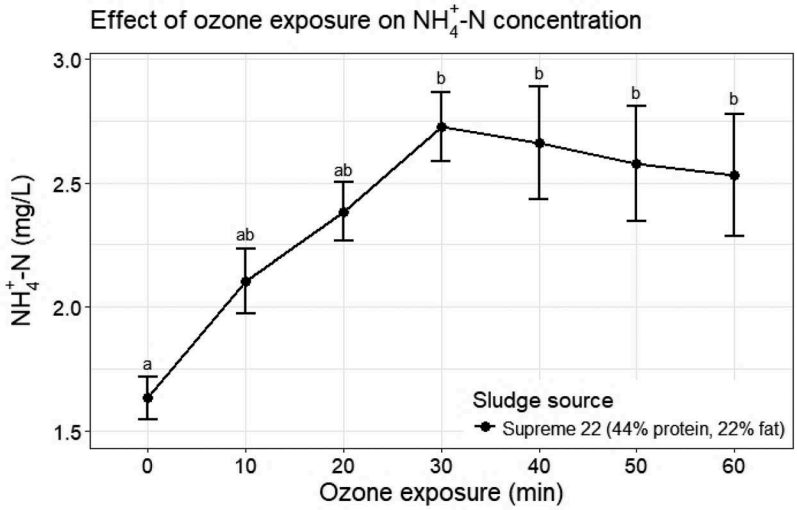

Figure 2. Variations in ammonium concentration due to ozone exposure. Values represent mean ± SE and different letters significant differences detected by multiple comparisons with Tukey contrast after significantly different means detected with one-way ANOVA.

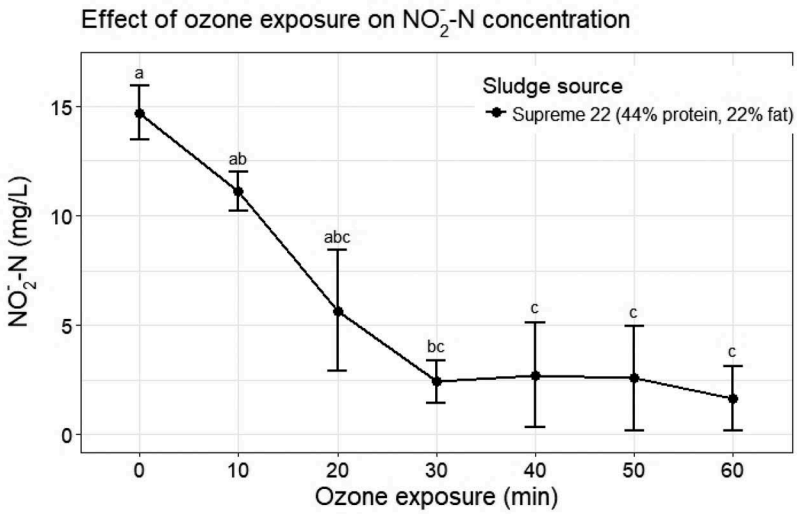

Figure 3. Variations in nitrite concentration due to ozone exposure. Values represent mean ± SE and different letters significant differences detected by Dunn's Test for multiple comparisons after a significant Kruskal–Wallis result.

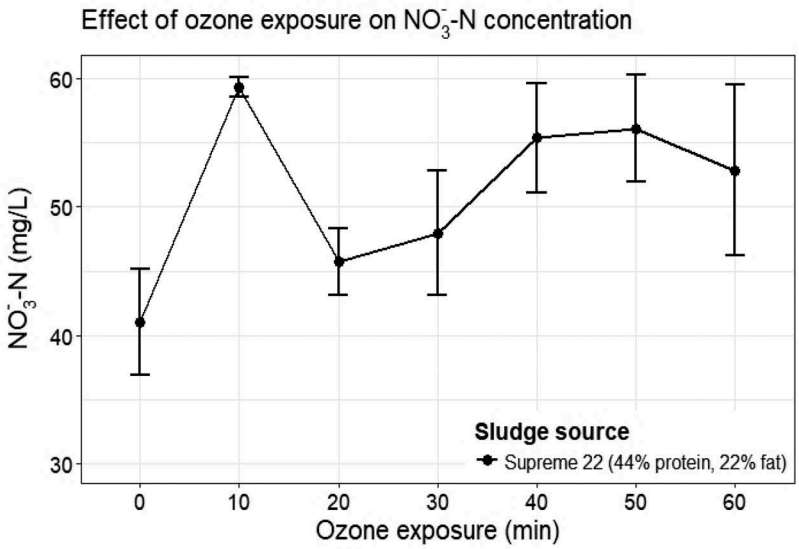

Figure 4. Variation in nitrate concentration due to ozone exposure. Values represent mean ± SE. No differences found when comparing all treatments with one-way ANOVA. Separate comparison of ozonation vs. control conditions showed marginal significant differences ($F_{(1,40)} = 6.25$ $p = 0.02$; Tukey contrast: $t = 2.50$ $p = 0.02$).

(18:0)) compared to monounsaturated fatty acids (MUFA) (represented mainly by oleic-elaidic acids (18:1) and palmitoleic acid (16:1)) and polyunsaturated fatty acids (PUFA) (mainly linoleic acid (18:2)). After 20, 40, and 60 min ozone exposure, a marked disproportion among SAFA and MUFA of the liquid phases of the sludge can be seen with a maximum ratio at 40 min. Fatty acids with odd number of carbon atoms such as heptadecanoic acid (17:0), which is a common fatty acid present in denitrifying Pseudomonas species, when present in the liquid phase, were found in small concentrations (4–6%). Yet, in the solid phase the concentration of this fatty acid slightly increased with increasing exposure to ozone. There were relatively similar proportions of SAFA and MUFA/PUFA in the solid phases before treatment (37% SAFA, and 33% MUFA/PUFA) but up to 20 min treatment and especially after 40–60 min exposure, the ratio SAFA:MUFA/PUFA increased. Heptadecanoic acid (17:0), while being in higher proportions in solution, was also found in small proportions (lower than 1%) in the solid phase where it tended to increase with an increase in ozone exposure. The feeds were rich in palmitic (16:0), oleic and elaidic acids (18:1),

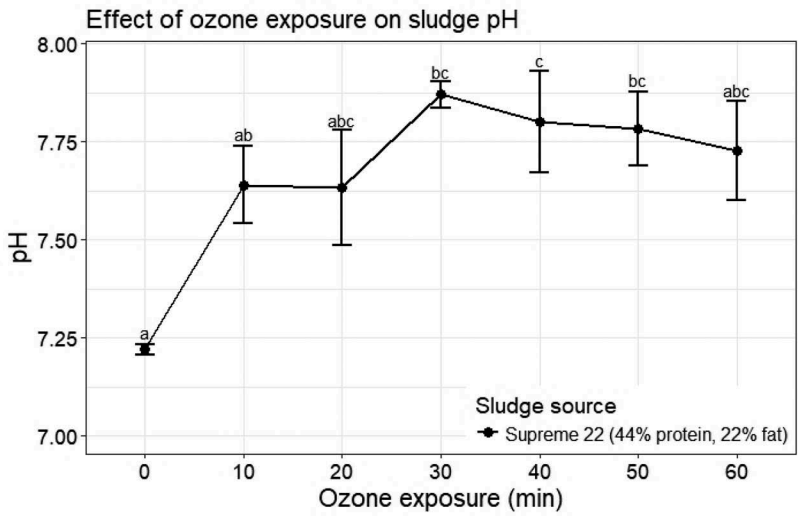

Figure 5. Relationship of pH and ozone exposure. Values represent mean ± SE and different letters significant differences detected by Dunn's Test for multiple comparisons after a significant Kruskal–Wallis result.

Table 1. Physicochemical parameters (Mean ± SD, $N = 24$). Different letters represent significant differences tested by multiple comparison.

Ozone exposure	pH	Temperature (°C)	Salinity
T0	$7.22 \pm 0.03^{(a)}$	$20.5 \pm 0.5^{(bc)}$	$31.6 \pm 0.1^{(a)}$
T10	$7.76 \pm 0.09^{(ab)}$	$20.0 \pm 0.1^{(a)}$	$31.6 \pm 0.1^{(ab)}$
T20	$7.86 \pm 0.09^{(abc)}$	$20.0 \pm 0.1^{(ab)}$	$31.5 \pm 0.1^{(abc)}$
T30	$7.90 \pm 0.04^{(bc)}$	$20.3 \pm 0.1^{(ac)}$	$31.5 \pm 0.1^{(abc)}$
T40	$7.95 \pm 0.03^{(c)}$	$20.4 \pm 0.2^{(bc)}$	$31.5 \pm 0.1^{(bc)}$
T50	$7.89 \pm 0.04^{(bc)}$	$20.5 \pm 0.1^{(c)}$	$31.6 \pm 0.1^{(ab)}$
T60	$7.88 \pm 0.05^{(abc)}$	$20.3 \pm 0.2^{(bc)}$	$31.5 \pm 0.1^{(c)}$
Statistical analysis	Kruskal–Wallis Per treatment: $\chi^2 = 14.33$, df = 6 $p = 0.03$ Dunn's test for multiple comparisons	One-way ANOVA Per treatment: $F(6,35) = 6.15$ $p < 0.05$ Tukey test for multiple comparisons	Kruskal–Wallis Per treatment: $\chi^2 = 14.02$, df = 6 $p = 0.03$ Dunn's test for multiple comparisons

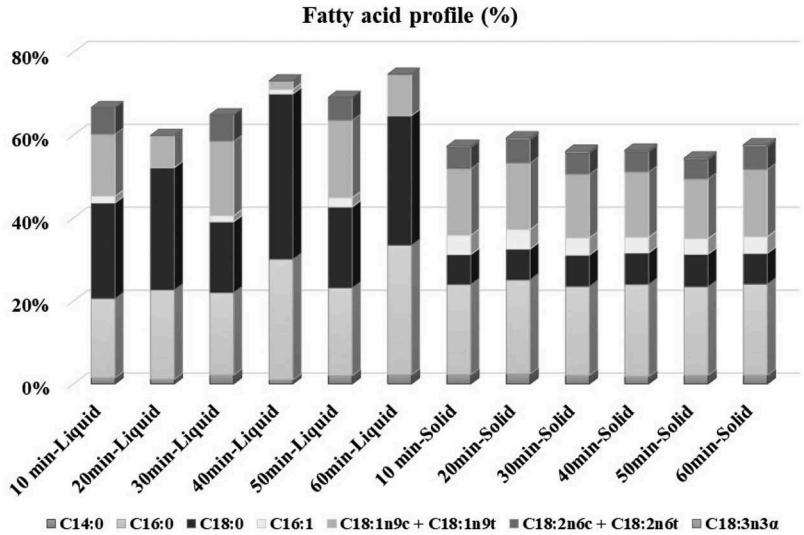

Figure 6. Fatty acid profiles (%) of ozone-treated sludge. Fatty acid in black marks the transition between saturated and unsaturated fatty acids. Only fatty acids having a concentration with more than 5% are shown.

Table 2. Fatty acid profiles of the solid and liquid phases of the sludge treated with ozone for different exposure periods. Mean concentration expressed in µg FA/g sample ± SD. In bold: fatty acids with >5%.

Lipid number	%	Mean concentration (µg/g)	%	Mean concentration (µg/g)	%	Mean concentration (µg/g)	%	Mean concentration (µg/g)	%	Mean concentration (µg/g)	%	Mean concentration (µg/g)	%	Mean concentration (µg/g)
Probe	Control Solid phase		10 min Ozone Solid phase		20 min Ozone Solid phase		30 min Ozone Solid phase		40 min Ozone Solid phase		50 min Ozone Solid phase		60 min Ozone Solid phase	
C10:0	0.04%	12 ± 0	-	-	-	-	-	-	-	-	0.03%	7 ± 0	0.03%	8 ± 0
C12:0	0.21%	33 ± 16	0.23%	30 ± 9	0.31%	45 ± 6	0.20%	29 ± 7	-	-	0.17%	22 ± 8	0.14%	20 ± 8
C13:0	0.04%	13 ± 0	-	-	0.03%	9 ± 0	0.06%	9 ± 1	-	-	0.04%	9 ± 0	0.02%	5 ± 0
C14:0	2.18%	344 ± 51	2.21%	293 ± 32	2.39%	354 ± 36	2.04%	287 ± 30	1.76%	238 ± 56	2.06%	268 ± 18	2.09%	301 ± 10
C15:0	0.80%	125 ± 9	0.73%	97 ± 4	0.75%	110 ± 6	0.71%	100 ± 3	0.64%	86 ± 11	0.69%	90 ± 1	0.76%	110 ± 3
C16:0	**22.20%**	3487 ± 176	**21.76%**	2902 ± 55	**22.69%**	3367 ± 473	**21.45%**	3008 ± 110	**22.23%**	3003 ± 317	**21.38%**	2787 ± 33	**21.98%**	3160 ± 75
C16:1	**5.10%**	801 ± 49	4.66%	621 ± 13	4.72%	700 ± 99	4.16%	583 ± 22	3.79%	512 ± 63	3.77%	491 ± 22	4.03%	580 ± 29
C17:0	0.58%	91 ± 1	0.48%	65 ± 6	0.49%	72 ± 12	0.63%	88 ± 5	0.60%	81 ± 8	0.71%	93 ± 18	0.68%	97 ± 6
C18:0	**7.64%**	1199 ± 38	**7.15%**	955 ± 4	**7.38%**	1094 ± 126	**7.50%**	1051 ± 59	**7.52%**	1016 ± 76	**7.76%**	1013 ± 6	**7.34%**	1055 ± 37
C18:1n9*	**16.02%**	2516 ± 64	**16.05%**	2144 ± 57	**16.04%**	2374 ± 210	**15.36%**	2152 ± 124	**15.76%**	2128 ± 171	**14.36%**	1872 ± 31	**16.20%**	2330 ± 17
C18:2n6*	**5.80%**	911 ± 28	**5.12%**	684 ± 12	**5.62%**	833 ± 86	**5.14%**	719 ± 84	4.91%	663 ± 25	4.73%	616 ± 9	**5.52%**	793 ± 32
C18:3n3α	0.56%	87 ± 2	0.36%	48 ± 10	0.49%	74 ± 22	0.46%	64 ± 8	0.37%	49 ± 3	0.40%	52 ± 3	0.48%	69 ± 1
C20:0	0.96%	151 ± 4	0.72%	96 ± 3	0.81%	120 ± 17	0.92%	129 ± 6	0.87%	118 ± 11	0.91%	119 ± 13	0.95%	137 ± 4
C20:1n9	0.74%	116 ± 0	0.67%	90 ± 16	0.70%	105 ± 18	0.82%	115 ± 16	0.60%	81 ± 0	0.69%	90 ± 5	0.72%	103 ± 3
C20:2	0.09%	28 ± 0	0.11%	29 ± 0	-	-	0.10%	29 ± 0	0.08%	22 ± 0	-	-	-	-
C21:0	0.93%	147 ± 17	0.28%	78 ± 0	0.62%	92 ± 2	0.79%	111 ± 10	0.59%	80 ± 12	0.68%	88 ± 3	0.68%	97 ± 30
C20:3n3	0.70%	109 ± 8	-	-	-	-	-	-	0.34%	46 ± 0	0.52%	68 ± 10	0.46%	66 ± 18
C20:4n6	1.14%	179 ± 5	1.49%	199 ± 9	0.95%	137 ± 43	0.60%	84 ± 0	1.03%	139 ± 6	1.12%	146 ± 13	1.29%	185 ± 3
C22:0/C20:5n3	0.36%	56 ± 10	-	-	0.42%	131 ± 0	1.25%	175 ± 3	-	-	-	-	0.12%	34 ± 0
C22:1n9	-	-	0.11%	29 ± 0	-	-	0.24%	34 ± 13	-	-	0.23%	30 ± 7	0.13%	37 ± 0
C23:0	**30.94%**	4855 ± 47	**35.33%**	4721 ± 134	**33.10%**	4878 ± 24	**34.92%**	4902 ± 0	**36.66%**	4951 ± 49	**37.41%**	4878 ± 24	**34.09%**	4902 ± 48
C24:0	1.16%	183 ± 4	0.88%	118 ± 25	0.92%	136 ± 0	1.03%	145 ± 7	1.03%	139 ± 21	1.11%	144 ± 9	0.97%	140 ± 5
C22:6n3	0.93%	147 ± 15	0.89%	120 ± 9	0.79%	116 ± 7	0.86%	122 ± 9	0.68%	92 ± 2	0.70%	91 ± 13	0.70%	101 ± 17
C24:1n9	0.89%	139 ± 4	0.79%	106 ± 0	0.79%	116 ± 3	0.74%	105 ± 15	0.54%	73 ± 4	0.55%	72 ± 9	0.63%	91 ± 11
SAFA	37%		34%		36%		35%		35%		36%		36%	
MUFA	23%		22%		22%		21%		21%		20%		22%	
PUFA	10%		8%		8%		8%		7%		7%		8%	
Probe	Control Liquid phase		10 min Ozone Liquid phase		20 min Ozone Liquid phase		30 min Ozone Liquid phase		40 min Ozone Liquid phase		50 min Ozone Liquid phase		60 min Ozone Liquid phase	
C10:0	1.22%	4 ± 1	0.84%	3 ± 1	1.49%	4 ± 0	1.20%	3 ± 0	1.46%	5 ± 1	0.56%	2 ± 0	0.78%	4 ± 0
C12:0	-	-	-	-	-	-	-	-	-	-	-	-	0.57%	3 ± 0
C14:0	1.31%	4 ± 2	1.44%	5 ± 1	1.10%	3 ± 0	2.12%	6 ± 0	0.95%	4 ± 0	1.98%	7 ± 3	2.20%	12 ± 2
C16:0	**18.46%**	63 ± 12	**19.13%**	73 ± 24	**21.56%**	54 ± 2	**19.89%**	56 ± 2	**29.05%**	105 ± 37	**21.17%**	80 ± 29	**31.19%**	165 ± 7
C16:1	2.62%	9 ± 2	1.62%	6 ± 3	-	-	1.46%	8 ± 0	1.06%	8 ± 0	2.24%	17 ± 0	-	-
C17:0	**5.35%**	19 ± 4	**6.17%**	23 ± 4	-	-	-	-	-	-	4.51%	17 ± 0	**5.63%**	30 ± 1
C18:0	**20.53%**	72 ± 1	**23.03%**	90 ± 45	**29.44%**	73 ± 2	**17.04%**	47 ± 5	**39.92%**	145 ± 40	**19.46%**	73 ± 7	**31.28%**	167 ± 6
C18:1n9*	**15.27%**	51 ± 26	**14.95%**	56 ± 11	**7.79%**	19 ± 2	**17.97%**	50 ± 0	2.10%	15 ± 0	**18.67%**	72 ± 41	**10.01%**	53 ± 7
C18:2n6*	**5.08%**	32 ± 0	**6.44%**	24 ± 4	-	-	**6.41%**	18 ± 0	-	-	**5.59%**	21 ± 15	-	-
C20:0	-	-	0.97%	6 ± 0	-	-	-	-	-	-	-	-	-	-
C22:2	0.24%	1 ± 0	-	-	0.07%	0.4 ± 0	-	-	-	-	-	-	-	-
C23:0	**26.93%**	95 ± 0	**25.41%**	93 ± 1	**38.55%**	96 ± 2	**33.92%**	95 ± 2	**25.45%**	94 ± 0	**25.82%**	97 ± 1	**18.34%**	98 ± 1
C24:0	2.99%	24 ± 0	-	-	-	-	-	-	-	-	-	-	-	-
SAFA	49%		51%		52%		39%		70%		47%		71%	
MUFA	18%		17%		8%		20%		3%		21%		10%	
PUFA	5%		6%		0%		6%		0%		5%		0%	

(-) = not present.
C18:1n9* = combination of the peaks C18:1n9c + C18:1n9t which could not be separated during measurement
C18:2n6* = combination of the peaks of C18:2n6c and C18:2n6t which could not be separated during measurement
C23:0 = Standard used for identification.

and linoleic and linoleadic acids (18:2) with more unsaturated (74% from which 49% comprised MUFA and 25% PUFA) than SAFA (19%) fatty acids (Wildförster 2017).

Discussion

Ozone-treated sludge could be used as alternative internal carbon source for denitrification

Large amounts of sludge are produced in RAS systems and its disposal is costly both in economic terms and in its impact on overall water use. In the current study, sludge composition was determined with regard to applicable nitrogen and carbon availabilities in response to ozonation with a long-term view to sludge recycling as a carbon source, e.g. for denitrification. Ozonation resulted in an increase on the SAFA: MUFA-PUFA fatty acid ratio in solid or liquid phases profiles, in significant increases in available dissolved carbon and nitrogen organic compounds, as well as ammonium, and otherwise resulted in a total depletion of nitrite and a marked reduction in the turbidity of the samples due to a reduction in solid contents.

Different pre-treatment methods have been applied to sludge with the purpose of increasing bioavailability. Most of these studies have been performed on wastewater (i.e., sewage) treatment plant sludge, which is not representative of the sludge obtained from aquaculture facilities. Sonication results in a significant improvement of digestibility and can break down cellular structures, release endogenous biodegradable materials, partially remove nitrate, reduce foaming, and enhance dewatering (Khanal et al. 2007; Vergara, Nickel, and Neis 2012). Meriac et al. (2015) sonicated feces of rainbow trout increasing the carbon bioavailability by 7–10% in comparison to an increase of 140% (initial DOC concentration of 75 mg in untreated sludge to a final concentration of 180 mg after 60 min treatment with ozone) under ozonation in the current study. Kampas et al. (2007) applied mechanical disintegration of thickened surplus activated sludge from a wastewater treatment plant increasing the soluble carbon in the form of volatile fatty acids (VFAs) to a maximum concentration of 850 mg L^{-1}, reducing in addition the particle size from 65.5 to 9.3 μm after 15 min of disintegration with the simultaneous release of proteins (1550 mg L^{-1}) and carbohydrates (307 mg L^{-1}) via floc disruption and cellular breakage. Other authors have used fermentation (Merzouki et al. 2005) which increases mainly the concentration of VFAs and thereby enhances nitrogen removal. Waste-activated sludge from a wastewater treatment pilot plant facility was also treated with a combination of ozonation and cavitation exhibiting 1.5 times higher sludge disintegration capacity than the individual technologies applied alone, and in terms of soluble carbon the combination proved to be the most efficient method, thus cavitation alone did not show any significant release, while ozonation yielded higher disintegration but only at longer exposures (Kumar 2012). Ahn et al. (2002) stated that sludge ozonation can be more cost-effective than incineration as a method for disposal at small- and medium-sized wastewater treatment plants because in the supernatant of the ozonized sludge solubilized organics and micro-particles are formed which are effective as carbon source for denitrification.

In the current study, in terms of the amount of carbonaceous compounds prone to be used as energy source, the ozonation process proved to be efficient taking into account that the produced sludge from aquaculture farms is relatively under-concentrated when compared with wastewater treatment plants remains. Using ozonation improved the available soluble carbon pool to final levels between 150 and 200 mg L^{-}. While this amount is promising, it may overestimate the usable carbon pool, as the detection by means of DOC comprises stable organic compounds that cannot be broken down biologically beside a chemically oxidizable carbon pool. In a system like the one used for this study, with an average wastewater disposal of 93 L per day representing 1.8% of the system volume, ozonation and reuse of the sludge as carbon source may be translated in around 13.95–18.59 g L^{-1} total DOC which could substitute or supplement commercial carbon sources administered to the system. An economic evaluation of the savings for the system in terms of energy invested in the production of the required ozone is still needed and has to take into account feed conversion ratio, as well as age and health status of reared fish which may influence the sludge composition and concentration. For disinfection and water treatment purposes, Timmons and Ebeling (2013) calculated that 13–24 g ozone/kg feed is required in RAS. The current results are in agreement with previous studies and support the ozonation method as an economically feasible and profitable way to treat sludge produced in RAS.

pH may serve as control parameter for ozonation in RAS

Water quality standards for RAS involve the control of physicochemical parameters such as pH, alkalinity, temperature, and salinity according to the species reared, the concentrations of inorganic nitrogen species, dissolved oxygen, phosphate and also the amounts of solid, refractory organic substances, and accumulating surface-active compounds and metals (Colt 2006). Physicochemical

parameters actively influence the availability of the different inorganic nitrogen species, as well as the use of the chosen carbon source for the process of denitrification. The pH of the solution significantly influences ozone decomposition in water. In fact, the main factors affecting ozonation performance during processing industrial wastewater are pH, the nature and concentration of oxidizable organics, ozone dose, competition between the target compound and biodegradable by-products, the presence of oxidant scavengers, and the efficiency of ozone mass transfer (Alvares, Diaper, and Parsons 2001).

Basic pH between 7 and 10 causes an increase in ozone decomposition and the typical half-life time of ozone increases from 15 to 25 min when ozonide and atomic oxygen lead to the formation of free hydroxyl radicals, which do not form at acidic to neutral pH (Kasprzyk-Hordern, Ziółek, and Nawrocki 2003). Kumar (2012) referred that the pH of sewage sludge treated with ozone tends to decrease. In our study as well as in Rahmadi and Kim (2014), the opposite trend was observed, probably due to different initial sludge concentrations present in solution and ozonation procedure (e.g., concentrations and exposure periods). Rahmadi and Kim (2014) explain their increase in pH through the partial decay of ozone into OH-radicals in water, which being dominant in solution, cause further ozone decay and induce additional reactions or organic matter with ozone at faster rates. It is expected that in a closed system, as mineralization takes place, the concentration of carbon dioxide increases (Bougrier et al. 2007), leading to an acidification of the solution which might be enhanced by the depletion of alkalinity and the formation of carboxylic acids from organic material oxidation (Bougrier et al. 2007; Kumar 2012). In the current study, relatively diluted sludge samples were used compared to sewage sludge (which composition may include much more complex substances), or to the treatment of filtered seawater containing TOC in the form of dissolved glucose used by the former authors. Ozone concentrations (5 mg L^{-1} at a flow of 1 L min^{-1}) used herein were lower than Kumar (2012) (35–40 mg L^{-1} at a flow of 3 L min^{-1}) and Bougrier et al. (2007) (30 mg L^{-1} at 1 L min^{-1}) but higher than those used by Rahmadi and Kim (2014) (0.04–0.23 ppm). In addition, in the current study experimental vials were intentionally not completely sealed, allowing the outstripping of excess carbon dioxide and ozone. Even when mineralization may have been taking place (reflected in lower turbidity/less suspended solids in the samples with increasing ozonation exposure), no acidification of the media was observed, which is an advantage for the application of this method in order to feed bacteria in RAS biofilters.

In the current experiment, pH increased steadily with a rise in ozone exposure, indicating the value of pH as a control parameter for the process. Further studies are required for "fine-tuning," this regulation mechanism taking into account different ozone and sludge concentrations in order to optimize the process or to scale-up to commercial RAS.

Ozonation as a disintegration method has pros and contras

Bougrier et al. (2007) stated that only 5% of sludge is recalcitrant to ozonation and that the presence of easily oxidizable soluble substances delays particle solubilization, hindering the ozone dosage. Short-chain fatty acids are among the pool of carbonaceous substances that were expected to be increased after the ozone-induced degradation of fat contained in the sludge samples. In fact, they were not found in the samples probably due to fast mineralization. In the current study, at the levels of ozone used, the carbon pool represented by SAFA seems to be relatively resilient in the solid phase, whereas unsaturated fatty acids tend to be depleted. It is known that ozone has a strong influence on the double bounds of unsaturated fatty acids (Silvestre et al. 2015) and this explains well the findings of higher concentrations of SAFA in solution after ozonation compared to the levels observed in untreated sludge. Depending on the sludge concentration used, it is possible to obtain a linear correlation between ozone doses and the concentration of long-chain fatty acids in the effluents of ozone-treated sludge with yields dependent on the sensitivity of the fatty acids present (Silvestre et al. 2015).

Fatty acids with odd carbon atom number are not common among bacterial groups, but 15, 17, and 20 carbon atoms fatty acids have been reported to be present in Pseudomonadales, including denitrifying bacteria (Doumenq et al. 1999; O'Leary 1962). In this study, 17-carbon fatty acids were found even when not present in significant amounts within the feeds (0.13%). This indicates that denitrifying bacteria present in the sludge are destroyed by ozonolysis, leading to cellular release of this fatty acid in solution. While the fatty acids found in treated samples in this study are essential for bacterial life, they will probably not be used directly as carbon source for bacterial growth as short-chain fatty acids are primarily used via the TCA/glyoxylate cycle (Cherchi et al. 2009). Nevertheless, the bacterial population of the filters may utilize more complex fatty acids to in situ synthesize others via oxygen-dependent desaturation pathways or through the anaerobically introduction of double bounds into fatty acyl chains by fatty acyl desaturases (Aguilar and De Mendoza 2006; O'Leary 1962).

The use of ozone for increasing solubility and availability of carbonaceous substances such as fatty acids may

lead to unwanted carbon-based compounds toxic to bacteria. Vranitzky and Lahnsteiner (2010) stated that aromatic compounds including polycyclic aromatic hydrocarbons and other micro-contaminants can be easily degraded by ozonation. Ozonation of industrial wastewater processes leads to the formation of recalcitrant organic compounds, generally halogenated heterocyclic, nitrogenous aromatics, aliphatic polymers and of intermediate and end-products including ketones, aldehydes, and organic acids with a higher biodegradability than the reactant compounds (Alvares, Diaper, and Parsons 2001). Silvestre et al. (2015) referred that in an aqueous media, the degradation products of long-chain fatty acids due to ozone lead to the formation of short-chain aldehydes of six to nine carbon atoms and hydroxyhydroperoxide which are known to be toxic for anaerobic microorganisms reducing methanogen bacteria in wastewater plants. The toxicology of harmful by-products formed due to ozonation was out of the scope of this study and needs further research.

Higher protein-to-energy ratio diets lead to higher production of fecal nitrogen waste (Letelier-Gordo et al. 2017). The characterization of the carbon and nitrogen pools in the current study did not include proteins which are expected to be a great proportion of sludge composition. Protein decomposition leads to the formation of biodegradable intermediates and inorganic nitrogen species. While Khuntia, Majumder, and Ghosh (2013) found that ozone microbubbles were quite effective in oxidizing ammonia at high pH and high ozone generation rates, current results indicate that ammonium concentrations tend to increase with longer ozone exposure. This could indicate the release of amine groups after ozone-induced protein hydrolysis (Bougrier et al. 2007; Silvestre et al. 2015). Maximum concentrations of ammonium were found after 40 min exposure suggesting that under current conditions, this is the maximum exposure period from which ammonium upsurge out of protein degradation takes place. Published results on oxidative effect of ozone on proteins vary. Cataldo (2003) found that only cysteine and the aromatic amino acids tryptophan, tyrosine, and phenylalanine are oxidized while polyamide bonds of the protein main chain remained resilient to oxidation even when denaturation of the proteins was indicated by changes in secondary and tertiary structures. On the other hand, Silvestre et al. (2015) stated that all amino acids are prone to be oxidized by ozone with a linear decrease with increased ozone doses for some amino acids while similar reductions independently of the ozone doses and higher reductions at lower ozone doses can be found with others. These authors inferred a differential sensitivity to ozone oxidation capacity depending on the amino acid composition of the proteins present.

Khuntia, Majumder, and Ghosh (2013) tested the oxidative potential of ozone on artificially prepared ammonium salt solutions in iron-free tap water, while Cataldo (2003) used five different proteins dissolved in distilled water. Under current experimental conditions, other organic compounds, salts, and metals were present in the samples. These compounds may act as catalysts and lead to concurrent oxidative processes favoring the degradation of complex molecules instead of ammonium or, alternatively, to a parallel oxidation of proteins and ammonium with faster rates for the degradation of proteins resulting in an apparent increase of ammonium ions in solution. Moreover, for ammonium exposed to ozone concentrations between 0.05 and 0.23 ppm, an oxidation rate in saltwater of 0.65 ± 0.28 mg L^{-1} h^{-1} has been mentioned (Rahmadi and Kim 2014). These authors needed 12 h under these conditions to deplete 5 ppm ammonium in saltwater and found a protective action of ammonium inhibiting the formation of bromate during the exposure to ozone. It is known that ammonium nitrogen can be oxidized to nitrate by ozone in a process giving rise to an over-ozone consumption and an increase in pH values (Domenjoud et al. 2011) but at very slow reaction rates in freshwater, making this conversion very ineffective (Tanaka and Matsumura 2003). However, in the presence of high bicarbonate concentrations, pH, and alkalinity, as in our samples, the formation of nitrate is enhanced by the direct oxidation of ammonium by ozone (Tanaka and Matsumura 2003).

Nitrite can act as ozone scavenger (Castro 1996) being converted into nitrate by ozone (Rahmadi and Kim 2014). Nitrite became rapidly depleted to negligible levels after 40 min exposure to ozone, in accordance to what was found by Rahmadi and Kim (2014) who referred a fast removal within the first 30 min of exposure at a rate of 4.45 ± 0.21 mg L^{-1} h^{-1}. Ozone has a weak oxidative potential upon nitrate because both have the same number of active oxygen molecules (Rahmadi and Kim 2014) and in our experiments nitrate seems not to be strongly affected by ozonation. Rahmadi and Kim (2014) obtained a depletion of 1 ppm out of 5 ppm nitrate in 24 h ozone exposure using five different ozone concentrations with faster rates at the beginning of exposure. In general, the concentrations of all three nitrogen species were far below the referred limiting values for RAS, indicating that the process of ozonation should not represent a problem for RAS in terms of accumulating and potentially toxic inorganic compounds. The presence of organic by-products is still an open issue which needs further research.

In general, it can be concluded that ozonation is a valid step toward reduced solid sludge volume from RAS by recycling, rendering water use for sludge filter cleaning unnecessary. The reuse of ozone-activated sludge as a

supplementary carbon source may represent a next step in the optimization of RAS. It offers the advantage of increasing the pH levels of the water and the reuse of long-chain fatty acids and other by-products, otherwise lost after fish digestion. Further research is needed to test the denitrification rates that can be achieved with treated sludge and further insights on the control of the process are necessary in order to apply it at commercial scales. In particular, the acclimation of denitrifying bacteria to this new carbon source may challenge aquaculture facilities/farmers and using ozone-activated sludge as the only carbon source in commercial RAS appears unlikely.

Acknowledgments

There is no conflict of interest related to this study. The authors would kindly thank Prof Boris Koch and Prof Stefan Wittke for allowing them to use their laboratory facilities, to Jana Geuer, Claudia Burau und Dieter Jansen, for their invaluable support in DOC measurements and their expert technical assistance, to Lukas Wildförster for introducing them in the fatty acids determination. They are also grateful to Ute Marx und Timo Hirse for supplying important materials for the experiments and for measurements support.

Funding

This study was supported by the DBU and is part of the ACOMACS project ("Activated Particulate Organic Matter as Carbon Source for Denitrification in RAS", under Grant 31367/01-23).

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