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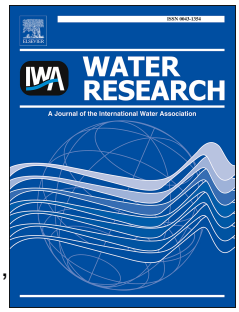
Published in:
Water Research

Link to article, DOI:
10.1016/j.watres.2018.01.032

Publication date:
2018

Document Version
Peer reviewed version

Accepted Manuscript



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PII: S0043-1354(18)30046-0

DOI: 10.1016/j.watres.2018.01.032

Reference: WR 13511

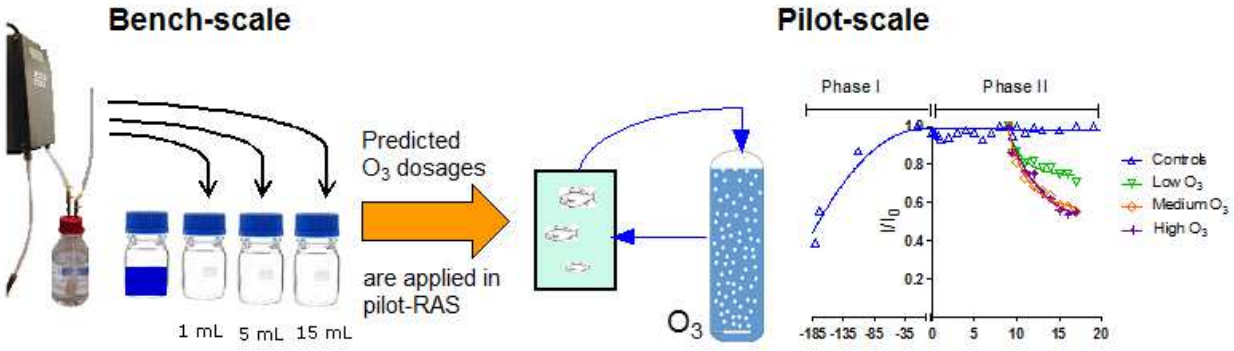
To appear in: *Water Research*

Received Date: 31 October 2017

Revised Date: 12 January 2018

Accepted Date: 13 January 2018

Please cite this article as: Spiliotopoulou, A., Rojas-Tirado, P., Chetri, R.K., Kaarsholm, K.M.S., Martin, R., Pedersen, P.B., Pedersen, L.-F., Andersen, H.R., Ozonation control and effects of ozone on water quality in recirculating aquaculture systems, *Water Research* (2018), doi: 10.1016/j.watres.2018.01.032.


Bench-scale
Pilot-scale
Predicted
O_3 dosages
are applied in
pilot-RAS
1 mL
5 mL
15 mL
O_3
Phase I
Phase II
I/I_0
Controls
Low O_3
Medium O_3
High O_3

Ozonation control and effects of ozone on water quality in recirculating aquaculture systems

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Abstract

To address the undesired effect of chemotherapeutants in aquaculture, ozone has been suggested as an alternative to improve water quality. To ensure safe and robust treatment, it is vital to define the ozone demand and ozone kinetics of the specific water matrix to avoid ozone overdose. Different ozone dosages were applied to water in freshwater recirculating aquaculture systems (RAS). Experiments were performed to investigate ozone kinetics and demand, and to evaluate the effects on the water quality, particularly in relation to fluorescent organic matter. This study aimed at predicting a suitable ozone dosage for water treatment based on daily ozone demand via laboratory studies. These ozone dosages will be eventually applied and maintained at these levels in pilot-scale RAS to verify predictions. Selected water quality parameters were measured, including natural fluorescence and organic compound concentration changes during ozonation. Ozone reactions were described by first order kinetics. Organic matter, assessed as chemical oxygen demand and fluorescence, decreased by 25% (low O_3), 30% (middle O_3) and 53% (high O_3), while water transmittance improved by 15% over an 8-day period. No fish mortality was observed. Overall, this study confirms that ozone can improve RAS water quality, provides a better understanding of the ozone decay mechanisms that can be used to define further safe ozone treatment margins, and that fluorescence could be used as a monitoring tool to control ozone. This study might be used as a tool to design ozone systems for full-scale RAS by analysing water sample from the specific RAS in the laboratory.

Keywords

Ozone, water quality, RAS, pilot-scale, laboratory study, fluorescence

35 **Abbreviations**

36 Recirculating aquaculture system (RAS), Dissolved organic carbon (DOC), Non-volatile organic
37 carbon (NVOC), Ultraviolet absorption (UVA), Ultraviolet transmittance (UVT), Total ammonium
38 nitrogen (TAN), Oxidation reduction potential (ORP).

1. Introduction

Land-based recirculating aquaculture systems (RAS) have become increasingly important, as they consume less water per kilogram of fish produced, ensure stable conditions and allow solids removal and effluent treatment, among others (Piedrahita, 2003). In such systems, organic and inorganic compounds accumulate that potentially deteriorate water quality and create favourable conditions for opportunistic bacteria. Various chemicals, namely formalin, hydrogen peroxide, peracetic acid and sodium chloride, are used to control microbial profusions and prevent disease outbreaks (Noble & Summerfelt, 1996; Pedersen et al., 2010; Pedersen & Pedersen, 2012; Pedersen et al., 2013; Verner-Jeffreys, 2015). However, high concentrations of chemotherapeutants might impair biofilter performance, affect fish welfare, jeopardize worker safety and place the ecosystem at risk when non-degraded residuals are released into nearby aquatic sources (Hohreiter & Rigg, 2001; Masters, 2004; Wooster et al., 2005; Pedersen et al., 2010).

To address the need for environmentally friendly disinfectants, ozone has been widely implemented as a supplementary water treatment technology (Von Gunten, 2003, Tsolaki & Diamadopoulos, 2010; Hansen et al., 2010; Hansen et al., 2016; Hansen, et al., 2016). It has been proven to enhance water quality, since it oxidises various deteriorating agents such as carbon-based compounds and nitrite, natural organic matter (NOM), chemical oxygen demand (COD), colour and suspended solids (Summerfelt & Hochheimer, 1997; Summerfelt et al., 2009; Davidson et al., 2011). It has been also reported to reduce geosmin, bacteria and miscellaneous fish pathogens (Bullock et al., 1997; Tango & Gagnon, 2003; Summerfeltet al., 2009), resulting in improved growth (Good et al., 2011) while enriching the water with oxygen, which is formed during ozone degradation.

Although ozonation has been applied for years in aquaculture, there is still a knowledge gap regarding how to predict the optimal ozone dosage for a system, known as “ozone demand.” In a non-meticulously designed system, residual ozone (an over-dose) will reach culture tanks, thereby

potentially affecting farmed species (Bullock et al., 1997; Summerfelt et al., 2004; Davidson et al., 2011; Powell & Scolding, 2016), while electricity consequently is wasted, having a significant monetary impact. The control of dissolved ozone is a major issue. Currently, there are several companies which supply dissolved ozone sensors which are either expensive and somewhat unreliable or not specific (Bullock et al., 1997). Dissolved ozone probes will not tell the ozone dosage to the water as the ozone is consumed very fast by reaction with dissolved organic matter in the low dosages applied in aquaculture and they also do not detect changes in ozone demand of the system.

A widely used method to control the delivery of ozone into water is the oxidation reduction potential (ORP; Bullock et al., 1997; Summerfelt et al. 1997; Summerfelt et al. 2009; Davidson et al., 2011; Li et al. 2014; Powell & Scolding, 2016), which measures a balance between the concentrations and willingness of substances in solution to give up or receive electrons. The ORP sensor is placed in the RAS system at a point where ozone is completely consumed downstream of the ozone treatment as free ozone damage ORP sensors (Bullock et al., 1997). In fully aerated aquaculture water, the dominant oxidant will be oxygen at the equilibrium concentration defined by the atmosphere and therefor the reading of the ORP will be an unspecific measure of the reducing solutes. As ozone quickly oxidised these reducing species the effect of ozone is measured indirectly but not specifically by the difference in the ORP reading before and after the ozone treatment. Wenk et al. (2013) suggested that mediated electrochemical oxidation (MEO) could be used in water treatment applications to determine the DOM oxidation in chemical oxidation processes since the electron donating capacity was highly sensitive to DOM changes. Ozone applied in wastewater showed a correlation with changes in UV absorbance at 254 nm (Bahr et al., 2007; Nanaboina & Korshin, 2010; Wenk et al., 2013) or at 272 nm (Hansen, et al., 2010). Nevertheless, a recent study

set the basis for a highly sensitive and accurate method to control ozone, based on the natural fluorescence removal of organic matter upon ozonation in an RAS (Spiliotopoulou et al., 2017).

There is therefore a need for a practical study to investigate ozone demand and kinetics in actual RAS water. The added ozone should be suitable to ensure a realistic "safety window" that is system-specific, does not exceed system demand and is nonetheless effective in promoting hygiene and water quality (Muller & Milton, 2012).

This study aims to reveal a more direct approach to describe the removal of carbon-based compounds and the control of ozone dosages in RAS. This approach could be also used to predict the required ozone dosage in RAS based solely on water quality parameters analysed in the laboratory. To achieve this aim, water samples were collected from a pilot-scale system and then, subjected to ozonation. The project objectives were i) to investigate the probability of predicting the effects of continuous ozonation in pilot-scale RAS on water quality (laboratory-scale experiments), ii) to determine the optimal ozone dosage in freshwater pilot-scale RAS, to ensure improved water quality without compromising fish health, and iii) to analyse the effects of different ozone dosages on resulting water quality parameters, including by-product formation and toxicity risk and iv) to investigate fluorescence sensitivity in ozonated RAS.

2. Material and methods

2.1 Reagents

All chemicals used in this study were purchased from Sigma Aldrich Denmark ApS and used as received.

107 2.2 Sample management

108 Samples were collected from the pump sump of the pilot RAS by siphoning. Depending on the
109 pending analysis (Table S1), the samples were filtered according to standard operational procedures
110 and stored at either 4°C, when the analysis occurred the same day, or at -20°C, when samples were
111 analysed at the end of the experiment.

112

113 2.3 Quantification

114 2.3.1 Ozonation

115 The laboratory ozone set-up was based on a 20 g/h ozone generator from O_3-Technology AB
116 (Vellinge, Sweden), supplied with dry oxygen gas. The generated ozone was dispersed through a
117 diffuser in a pressurised collection bottle containing ultra-pure water, to create the ozone stock
118 solution. To increase ozone solubility further, the bottle was submerged in an ice bath, while a
119 manometer and a valve were placed after the collection bottle at a pressure of 1.2 barG. Ozone
120 concentration in the stock solution ranged between 70 and 110 mg/L. The pilot ozonation set-up
121 was based on a 500 mg/h generator (Sander, Germany) and supplied with dry air (Flairmo ApS,
122 Denmark).

123

124 2.3.1.1 Determination of ozone concentrations

125 The concentration of ozone in both the aqueous and the gaseous phases was determined daily.
126 Ozone concentration in the water was determined utilising the indigo method (Bader & Hoigné,
127 1981), while the absorbance of the unreacted indigotrisulphonate was measured at 600 nm with a
128 spectrophotometer (Hach Lange). Ozone concentration was determined by comparing the
129 absorbance of a blank to the sample, and by using ΔA = -20000 l/(cm mol ozone added per L).

Ozone gas concentration was determined with a flow cell connected to a spectrophotometer, measured at 254 nm (Hansen et al., 2010) utilising the Beer-Lamberts law (Eq.1).

$$C_{O_3} = \frac{A}{l \cdot \varepsilon} \cdot M_{w,O_3} \quad \text{Equation 1}$$

Where A is the absorbance of gas, l is the light path in cm (l=1.00 cm), ε is the ozone molar absorption coefficient at 254 nm (ε=3000 NL/(mol·cm)) and $M_{w,O3}$ is the molar mass of ozone ($M_{w,O3}$=48 g/mol).

2.3.1.2 Determination of ozone demand

Fluorescence spectroscopy was used to determine indirectly the dosage of ozone delivered into water, as described by Spiliotopoulou et al. (2017) utilising a fluorimeter (Cary Eclipse, Varian). Two excitation/emission wavelength transitions were included in this study, namely Ex275/Em340 and Ex3355/Em450, representing protein and humic-like substances contained in water, respectively (Hudson et al., 2007). Miscellaneous wavelength transitions were studied and can be found in the Supporting Information (SI 3.1).

2.3.2 UV absorbance (UVA)

Water clarity was determined in terms of UVA and/or UV transmittance (UVT%), measuring directly the absorbance of water samples at 254 nm using a 10 mm quartz cuvette.

$$UVA = A_{254nm} = -Log(I / I_0) \quad \text{Equation 2}$$

$$\%UVT = 100 \times 10^{-UVA} \quad \text{Equation 3}$$

Where I = light intensity at the detector (light out) and I_0 = intensity of the light incident before the sample (light in).

152 2.3.3 Non-volatile organic carbon (NVOC) determination

153 A Shimadzu ASI-V UVC/Persulphate analyser quantified the non-volatile organic carbon (NVOC)
154 of the filtered samples (0.45 μm). The injected sample volume was 3.00 mL and a calibration curve
155 with potassium hydrogen phthalate standards from 50 to 2000 μg/L was determined (R^2=0.9994)
156 with a quantification limit set at 50 μg/L.

157

158 2.3.4 Chemical Oxygen Demand (COD)

159 Total (raw samples), dissolved (filtered with 0.45μm) and particulate (COD_{PART} =COD_{TOT} -COD_{DIS})
160 chemical oxygen demand (COD) was determined by utilising test-kits (LCK 1414, Hack Lange,
161 Germany) according to ISO standards (ISO, 2012).

162

163 2.3.5 Nitrogen- and phosphorous-based compounds

164 Samples were filtered (0.22 μm) and analysed for total ammonia nitrogen (TAN), nitrite NO_2^--N,
165 nitrate NO_3^--N and ortho-phosphate (dissolved phosphorous, P). TAN was determined
166 colourimetrically, accordingly to Danish standards (DS 224, 1975), while the remaining parameters
167 were analysed by utilising the auto-sampler San $^{++}$, SKALAR.

168

169 2.3.6 Water parameters

170 Water temperature, pH and dissolved oxygen (DO) were determined by probes (Hack HQ40d
171 instrument, Hack Lange, Germany), while reduction oxidation potential (REDOX) and DO were
172 monitored by a Pacific unit (Oxyguard International AS, Farum, Denmark).

2.3.7 Acute toxicity

Ozonated water samples were subjected to the Microtox (ISO, 2007; Chhetri et al., 2017) toxicity test, which utilises bioluminescent bacteria (*Vibrio fischeri*) to investigate whether toxic ozonation by-products are formed.

2.3.8 Data treatment

Obtained data were analysed using MS Excel and Prism Graph Pad 5.0. Although the systems were identical, they did not cease being autonomous systems hosting living organisms, and they therefore had their own loading and steady state thresholds. It was observed that the zero values (prior to ozonation) for several parameters varied considerably. Thus, normalisation (C/C_0) was necessary, to compare better the effect of the different treatments (SI 4.2). However, raw data can be found in SI 4.1.

2.4 System configuration

The replicated experimental set-up, located in Hirtshals, Denmark, was designed to mimic commercial RAS. Twelve identical 1.7 m^3 pilot-scale RAS (described by Rojas-Tirado et al., 2016) were each stocked with rainbow trout at a density of 40 kg/m^3/tank (Funderholme Dambrug, Silkeborg, Denmark). Belt feeders were loaded every morning with a 125 g feed/system (EFICO Enviro 3 mm; Biomar, Denmark). Water flowed from the rearing tank into the swirl separator (1.5 m^3/h) and then through to the pump sump. From the pump sump, water at a flow of 3 m^3/h was transferred to biofilters. Water excess from the trickling

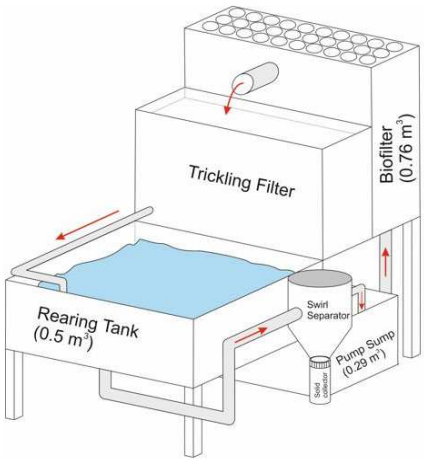


Figure 1: Schematic representation of pilot-scale RAS set-up in Rojas-Tirado et al., 2016.

197 filter overflowed back into the pump sump. Biofilters were not backwashed during the experiment,
198 while any settled solids (particulate matter such as any uneaten feed pellets and faeces) were
199 collected in the swirl separator (Fig. 1) and removed daily. Sodium bicarbonate was added to
200 compensate for alkalinity loss due to nitrification, thereby ensuring a relatively stable pH ranging
201 from 7.4 to 7.5. Water temperature ranged from 16 to 19^{o}C during the trial, while diurnal variations
202 were negligible. The daily photoperiod ranged from 07:30 to 22:00.
203
204 2.5 Experiments
205 2.5.1 Laboratory-scale experiments
206 Water samples were collected randomly from one of the twelve RAS during the stabilisation period,
207 kept at <4^{o}C and transported to the laboratory for further analysis. The purpose of the batch
208 experiment was to characterise the water matrix, define the ozone demand, determine the optimal
209 ozone dosage, which ensures improved water quality and its lifetime, and to test ozonation capacity
210 by indicating the critical range in which ozonation can occur safely in such systems.
211 Several ozone dosage amounts, ranging from 0 to 10 mg O_3/L, were spiked in 50 mL RAS water
212 samples, as described in Hansen et al. (2016), who conducted a similar study in wastewater. To
213 control better ozone concentration in the RAS water samples, the same ozone dosage was added to
214 acidified MilliQ water (50 mL) containing a 5 mL phosphate buffer and a sufficient amount of
215 potassium indigotrisulphonate (Antoniou et al., 2013).
216 To predict optimal ozone dosage in pilot-scale RAS at a given feed loading, water samples were
217 repeatedly ozonated after ozone depletion, to investigate ozone reactivity and its sensitivity to
218 optimal ozone dosage, using the indigo colorimetric assay (Section 2.3.1.1), and to quantify ozone
219 concentration profiles over time, utilising a spectrophotometer. A RAS water sample was divided
220 into five subsamples (50 mL each), four of which were ozonated with, for example, 2 mg/L ozone

221 (1^{st} ozone dosage), while the fifth set was used as a control. In one of the four subsamples, ozone
222 concentration was measured over time. When ozone was depleted, a further dosage was applied (2
223 mg O_3/ L; 2^{nd} ozone dosage), albeit only for the remaining three samples. Subsequently, ozone
224 concentration was monitored over time in one of the samples. The same procedure was carried out
225 until all the subsamples had been subjected to ozonation. All samples, including the control, were
226 measured with a fluorimeter to define the ozone effect on natural fluorescence degradation (Section
227 2.3.1.2). Repeated ozonation occurred for every examined ozone dosage.
228
229 2.5.2 Pilot-scale experiments
230 The laboratory study was followed by injecting predetermined ozone dosages into pilot-scale RAS
231 (Fig. 2). The pilot-scale investigation lasted 2.5 weeks, and it was divided into three distinct phases.
232 Phase I was the pre-ozonation period (185 days), while Phases II and III represented the two
233 ozonation periods. During Phase II, two replicated trials occurred (II_A and II_B). In Phase III, 50 g
234 O_3/ kg of feed, equivalent to twice as much as the highest applied ozone dosage in Phase II, was
235 tested (High O_3 x2). The control values consisted of the average for the three individual systems.
236 A back-up system remained untouched (operating as described in Section 2.4), to provide fish in
237 case of any mortality. Four RAS were used for each trial, one of which was operated as a reference,
238 where no ozone was added. The three remaining RAS were each equipped with one ozone reaction
239 tank (18 L) per system. In a side-stream, water was pumped from the swirl separator into the
240 reaction tank at a flow of 0.2 m^3/h and a retention time of 5.4 min, the remaining water was led to
241 the pump sump (Fig. 2). From the ozone reaction tank, the treated water was also transferred to the
242 pump sump with an overflow, before moving on through biofilters (excess water from the trickling
243 filter returned to the pump sump) and ultimately to the fish tank. In these units, ozone gas,
244 controlled by 6mm stainless steel needle valves (to regulate backpressure), was injected (via plastic

245 tubing) into the ozone reaction tanks (from PVC and Plexiglas), to achieve predefined ozone
246 concentrations.

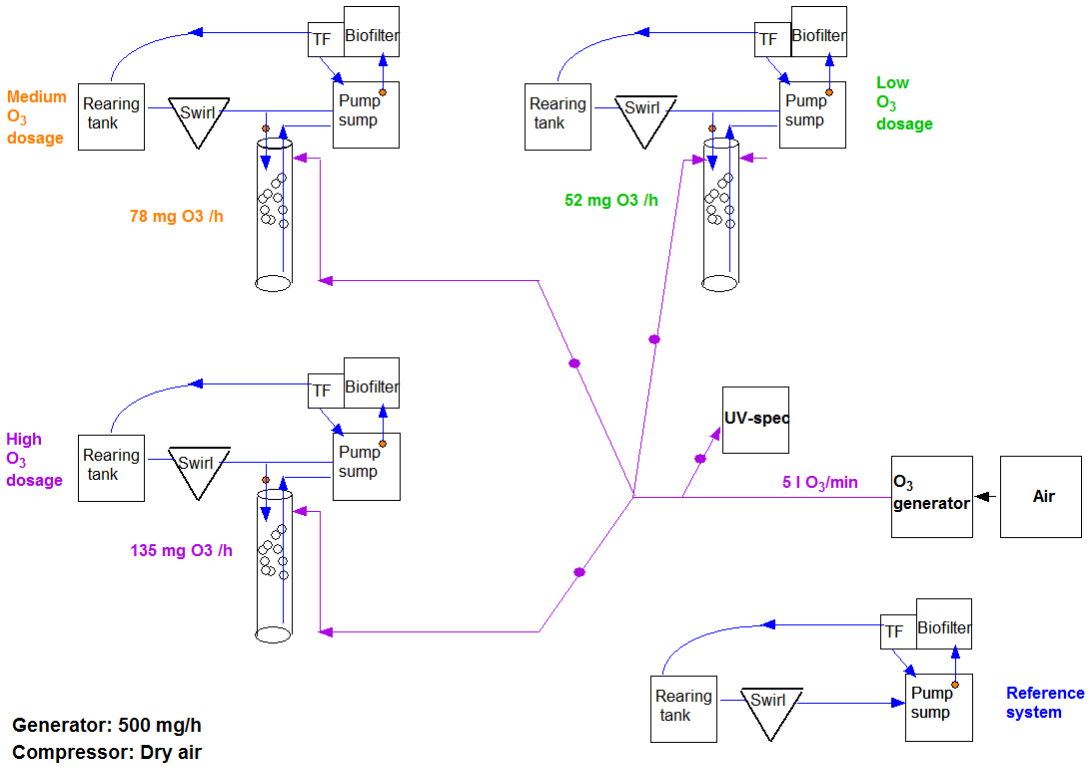


Figure 2: Schematic representation of the four different ozonation-level pilot-scale RAS.

247 Online probes in the rearing tanks continuously recorded DO and REDOX. Oxygen concentration
248 was maintained at 8-8.5 mg/L via aeration and the addition of pure oxygen with diffusers in the
249 rearing tanks. Fish were inspected and any mortality was noted daily.

250 The ozone gas was monitored daily with a spectrophotometer (Section 2.3.1.1). Dissolved ozone
251 was measured in the pump sump and rearing tank (Section 2.3.1.2) by the indigo method (Antoniou
252 et al., 2013) and the fluorescence intensity removal method (Spiliotopoulou et al., 2017). Water
253 samples were collected daily from the rearing tank and the pump sumps of the four running units
254 for further analysis.

255 After the first one-week trial (control, low, medium and high O_3), the experiment was replicated by
256 conducting a similar set of trials in four new units, identical to the previous protocol. These tanks,

257 during the first one-week-trial, were handled similarly to the reference tank. Finally, an additional
258 RAS was used to test the high O_3 x2 dosage over a period of three days.
259 To protect staff from ozone, off-gas from the ozone reaction tanks was vented (through PVC
260 piping) outside the building. Additionally, an ozone gas sensor (Water ApS, Farum, Denmark) was
261 installed in the room to detect ozone gas (<0.14 mg O_3/L) in the surrounding air.
262

263 **3. Results and discussion**

264 The present work was divided into two distinct phases, namely a laboratory and a pilot-scale study.
265 During the laboratory study, water characterisation of the RAS, the effect of ozone on water when
266 organic matter was present, the behaviour of ozone in such water over time and the effect of
267 repeated ozonation in stimulating recirculation were investigated. Analysing these data, we
268 predicted the required ozone dosage for the system and created a dosing range. In the second phase,
269 ozone exposure covering this range was applied in pilot-scale RAS for short experimental periods to
270 validate our hypothesis.
271

272 3.1 Laboratory study

273 3.1.1 Water characterisation

274 The water matrix in terms of physicochemical characteristics of the make-up water and non-
275 ozonated RAS water were analysed and are presented in SI (Table S2). The make-up water was
276 non-chlorinated groundwater distributed through the municipal system in Hirtshals, Denmark. The
277 inlet water was analysed for all parameters similar to the samples and was found to have no
278 abnormal concentrations, thereby adding no background information to our analyses. Since all RAS
279 were identical, water samples were collected randomly from one system, to determine the water
280 quality baseline during the biofilter stabilisation period. The start-up phase moved on to the

281 sampling campaign approximately 1 week after fish were placed in the tanks, while the steady state
282 phase was reached when the biofilter were stable in terms of low TAN and nitrite and elevated
283 stable nitrate (day 70). The pH during the 70-day period was relatively stable, while a significant
284 increase of 7.9 mg O_2/L for the NVOC was observed (Table S2), due to fish activity (metabolic by-
285 products, uneaten feed etc.) and high system intensity. Such high NVOC concentrations, up to 9.7
286 mg/L, are often found in commercial trout RAS (Spiliotopoulou et al., 2017).

287

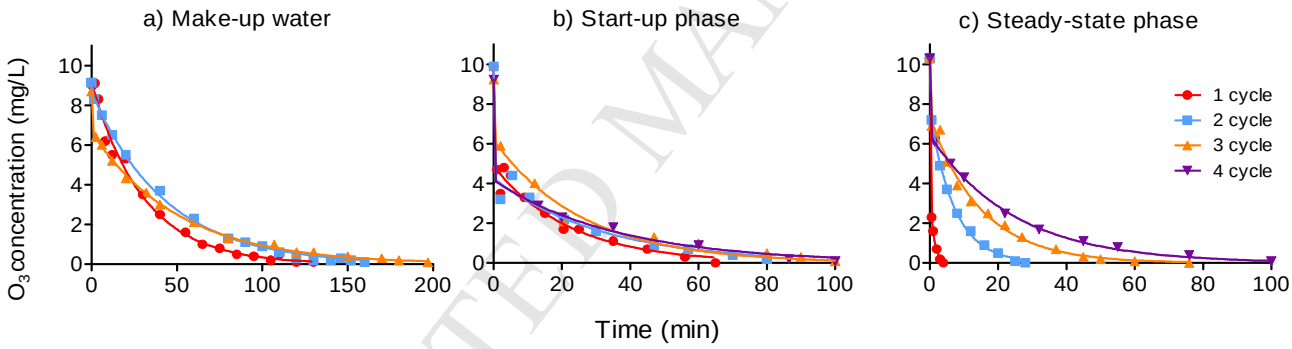
288 3.1.2 Ozone kinetics

289 Preliminary ozone experiments were conducted in the laboratory to investigate the ozone effect in
290 organic loaded RAS water. Seven different ozone dosages, ranging from 0.5-10 mg O_3/L, were
291 spiked in RAS water, which was collected over time. The methodology will be described based on
292 the dosage of 10 mg O_3/L only (Fig. 3), while the remaining results can be found in SI (Fig. S2).

293 The samples collected during the three sampling campaigns were subjected to ozonation (10 mg
294 O_3/L) and an instant ozone consumption (time = 0 min) was observed, which will be referred to
295 herein as “initial ozone demand” (Fig.3). Initial ozone demand increased in line with increasing
296 water pollution: in the make-up water, ozone consumption was 1.3 mg O_3/L (Fig. 3a), in the start-up
297 phase it was 5.3 mg O_3/L (Fig. 3b) while in the steady state it was 7.7 mg O_3/L (Fig. 3c). When
298 water quality deteriorated due to nutrient build-up/accumulation of micro particles, initial ozone
299 demand increased and the ozone lifetimes became shorter. Ozone reacts instantly with easily
300 degradable compounds, resulting in initial ozone demand. Compounds that are more recalcitrant
301 were oxidised by the subsequent ozone cycles.

302 To investigate the long-term effects of ozone on water, the samples were repeatedly ozonated upon
303 ozone depletion, to simulate recirculation. It is crucial to determine the lifetime of ozone, since it

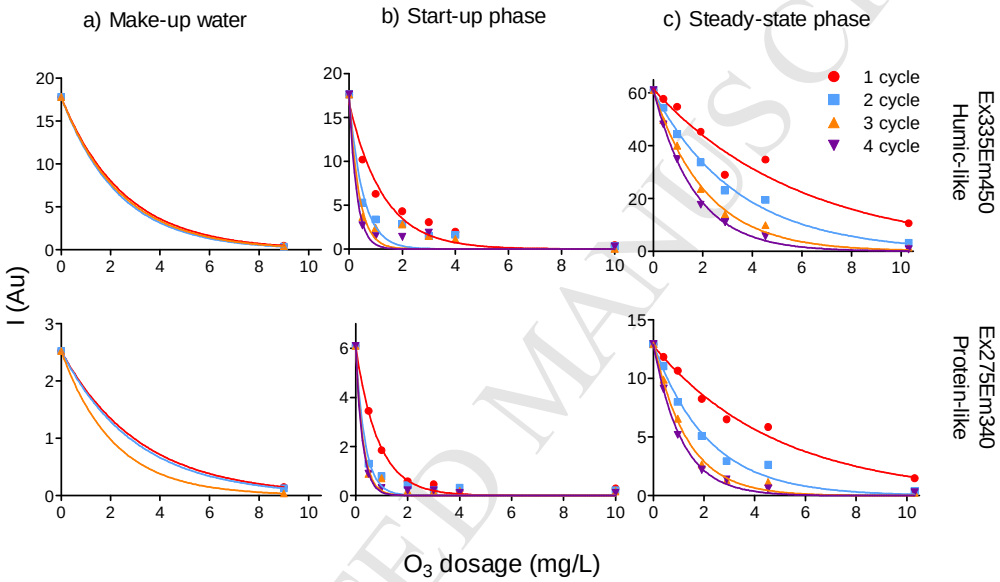
304 should not enter the culture tanks or the biofilters. Ozone lifetime in the make-up water (Fig. 3a)
305 was constant between the cycles, suggesting that the make-up water did not contain any organic
306 matter that might react with ozone, and thus its decomposition was extended to more than 130 min.
307 Water from the start-up phase responded differently, since ozone lifetimes varied among the
308 repetitions (Fig. 3b). The first applied dosage degraded fully within 65 min, followed by prolonged
309 cycles up to 100 min, since there was less organic matter present to react with the ozone. Even
310 shorter lifetimes were observed in the highly loaded water (Fig 3c), where the first 10 mg O_3/L were
311 rapidly consumed (4 min). The three following ozone spikes/cycles had considerably shorter
312 lifetimes than in the start-up phase and make-up water.



313
314 Figure 3: Ozone kinetics of RAS water during 4 consequent dosings (i.e. cycles) in a) make-up water, b) start-up phase
315 and c) steady-state phase. Expected nominal concentrations of ozone was equivalent to 10 mg O_3/l.

316 3.1.3 Fluorescence removal

317 RAS water contained naturally fluorescent compounds originating mainly from the feed, the inlet
318 water and organic matter produced by the fish and the water treatment process (Hambly et al.,
319 2015). The intensities for both humic and protein-like fluorophores increased as water quality
320 deteriorated (Fig. 4; 0 mg O_3/L). Similar high fluorescence intensities have been observed in full-
321 scale RAS where trout and eels are farmed (Spiliotopoulou et al., 2017).



322

323 Figure 4: Fluorescence degradation of humic and protein-like fluoropores in ozonated RAS water
324 exposed to different ozone levels in a) make-up water, b) start-up phase and c) steady-state phase.

325

326 Repeated ozonation did not have any effect on fluorescence, thereby suggesting that a single dosage
327 of 3-4 mg O_3/L (approx. 40.8 g O_3/kg of feed) was enough to remove the already low fluorescent
328 compounds (<5Au) from the water. During the start-up phase (prior to ozonation), the initial
329 fluorescence intensity did not change considerably compared to the make-up water (Fig. 4b; 0 mg
330 O_3/L). In terms of humic-like content, intensity was the same as in the make-up water; however, an
331 increase in protein-like fluorophores was observed, possibly due to fish excretions. After ozone
332 addition, all of the fluorophores degraded rapidly; 1-2 mg O_3/L (14-27 g O_3/kg of feed) was needed

to decrease fluorescence intensity to 5 Au (Fig. 4b). The first three ozonation cycles overlapped, so they did not have any impact on fluorescence degradation, and only the fourth cycle slightly differed. Thus, mildly polluted water could be treated with a low ozone dosage, since organic matter consists of easily degradable compounds.

In the steady-state samples (Fig. 4c), fluorescence intensity in both wavelength transitions was significantly higher compared to the start-up phase, being in agreement with the NVOC increase over time (Table S2), due to waste accumulation. Fluorescence intensity was highly affected by ozone, and even the lowest ozone dosage reduced it significantly, being in agreement with a previous study that predicted its usability for the sensitive online control of ozone treatment (Spiliotopoulou et al., 2017). Humic-like fluorophores were the most pronounced in this regard, while the protein-like counterparts were lower, albeit still present. After recurrent ozonation, distinct fluorescence removal was observed between the cycles. The higher the ozone dosage, the higher the fluorescence removal.

3.1.4 Required ozone dosage

To address ozone demand in a given system, it has been suggested to dose ozone based on feed administered (Bullock et al., 1997; Good et al., 2011) or automatically adjusted to either changes in fish feeding ratio (Summerfelt et al., 2009) or naturally fluorescent organic matter content and degradation (Spiliotopoulou et al., 2017). A wide range of ozone dosages has been reported for RAS, given as either a feed ratio (3-24 g O_3/kg of feed; Bullock et al., 1997; Summerfelt et al., 2009), or ORP not exceeding perhaps 300 mV (Bullock et al., 1997). Full-scale ozonation experiments have suggested that improved water quality occurs from 15 to 25 g O_3/kg of feed (Summerfelt & Hochheimer, 1997; Summerfelt et al., 2009; Davidson et al., 2011), though these

356 dosages were not used for disinfection but are intended primarily to ameliorate general water
357 quality (Davidson et al., 2011).

358 The ozone dosage required to satisfy ozone demand might be influenced by feed loading, feed
359 utilisation, water treatment, degree of dilution, etc. in an RAS (Summerfelt et al., 2009). The results
360 presented in Fig. 3 can also be used to interpret the ozone demand of the system based on fish waste
361 production over a 70-day-period. Since the make-up water had no background loading, ozone
362 demand was defined exclusively based on feed input and the associated metabolic excretion. Based
363 on the kinetics for the dosage of 10 mg O_3/L, for instance, we can conclude that ozone demand for
364 the start-up phase water ranges from 15 to 20 mg O_3/L. The second, third and fourth cycles
365 overlapped (Fig. 3), meaning that after the second cycle there was no effect of additional dosage; so
366 we conclude that the demand was above 10 mg O_3/L (1^{st} cycle) but less than 20 mg O_3/L (2^{nd}
367 cycle), with each cycle meaning the addition of 10 mg O_3/L. Therefore, it is estimated that ozone
368 demand would be roughly 18 mg O_3/L. For the steady state phase, there was available organic
369 matter to react with ozone until the 3^{d} cycle, and only in the 4^{th} cycle, the kinetics became slower.
370 Thus, we estimated that the ozone demand for this water was between 30 and 40 mg O_3/L.
371 Ozone demand for the start-up phase was a result of organic matter accumulating over 7 days, while
372 ozone demand for the steady state phase was due to organic matter levels stabilized over 70 days.
373 Based on the results, daily ozone demands (ozone demand/days of waste accumulation) during the
374 start-up and steady state phases were 2.6 and 0.50 mg/L/day, respectively. By converting daily
375 ozone demand to an hourly rate (0.1 and 0.02 mg/L/h) and multiplying it by the total volume of the
376 system (1.7 m^3), we calculated that 182.1 and 35.4 mg O_3/h were needed to treat water in the two
377 phases, respectively. Subtracting these, 147.7 mg O_3/h was found to be needed to treat the water,
378 corresponding to 28 g O_3/kg of feed. A similar dosage was found in Summerfelt et al. (2009). This
379 amount of ozone, if applied on a continuous basis, would purify the water to a great extent.

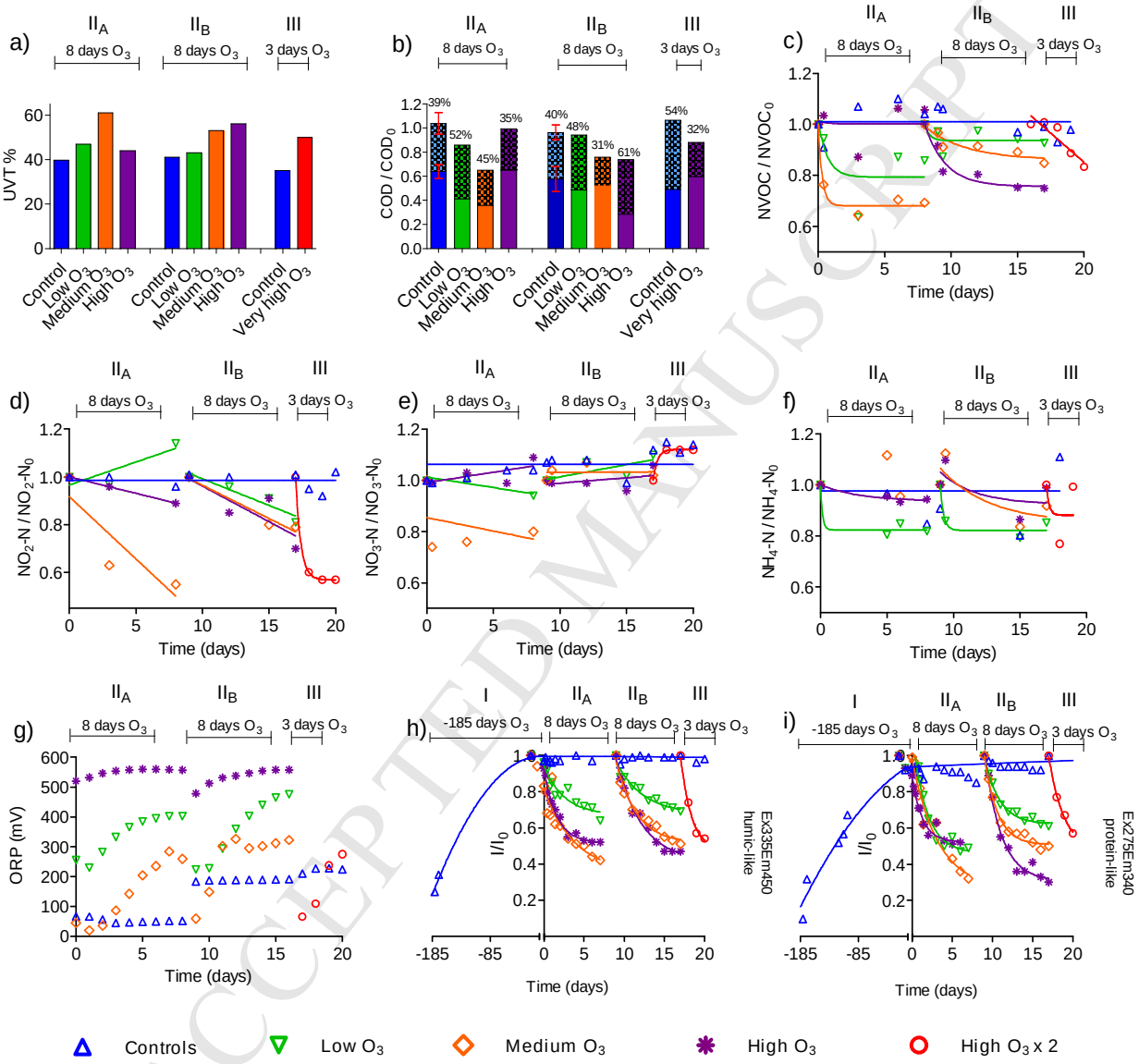
Therefore, lower ozone dosages than the calculated (28 g O_3/kg of feed) will be applied: 10, 15 and 26 g O_3/kg of feed, respectively (since, e.g., 26 g O_3/kg of feed * 0.125 Kg of feed/day = 3.3 g O_3/day = 3.3 *10^{-3} Kg O_3/day = 135 mg O_3/h, which was less than the 174.7 mg O_3/h required to purify the water). These dosages were obtained by adding 0.26, 0.39 and 0.68 mg O_3 /L into the ozone reaction tanks (e.g. 3.3 *10^{-3} Kg O_3/day *10^6 g/kg / 3.33 L water/min /1440 = 0.68 mg O_3 /L). According to previous studies (Summerfelt et al., 2009; Powell & Scolding, 2016), these concentrations were sufficient to treat the water, still ensuring that no residual ozone could reach neither the rearing tanks, nor the biofilters (Schroeder et al., 2015). The daily ozone dosages required to overcome the demand of an RAS were that low due to continuous water treatment, as the water might go through ozonation up to 50 times per day (Summerfelt et al., 2009). The sufficient retention time of ozone in the systems ensured the absence of residual ozone. A secondary barrier, to compensate for ozone excess, was the side stream design. In the pump sump, ozonated and non-ozonated water was mixed and further diluted by water coming from the trickling filter.

3.2 Pilot-scale study

Based on the initial ozone demand of the system, four different ozone dosages, including a reference system (Section 3.1), were applied to pilot-scale RAS to validate if the predicted ozone dosages would improve water in operating RAS and to determine the long-term effects of continuous dosing, if any.

During the first ozonation trial (II_A), the set-up and miscellaneous connections were tested to ensure system resilience to ozonation. Unfortunately, ozone delivered at the high dosage (an actual dose of 26 g O_3/kg of feed) caused transient leakage from the piping material, and consequently less ozone was delivered to that specific tank than originally envisaged. After this incident, the piping material was changed to PVDF tubing and ozone was delivered properly throughout the following phases

404 (II_B and III). Therefore, the discussion will be based mainly on results derived from Phases II_B and
405 III, though all of them will be presented in Fig. 5, which illustrates the sensitivity of various
406 parameters to ozonation.



407
408 Figure 5: Effect of ozone on a) UVT% after 8 days, b) COD after 8 days (the % and the dotted (upper) parts of the bars
409 represent the particulate COD, while the lower part is dissolved COD-normalised data, standard deviation only in
410 control, c) NVOC-normalised data, d) nitrite-normalised data, e) nitrate-normalised data, f) ammonium-N-normalised
411 data, g) ORP, h) protein-like fluorescence-normalised data and i) humic-like fluorescence degradation-normalised data.
412 Graphs with raw data can be found in SI.

413 3.2.1 Continuously measured water quality parameters

414 Dissolved oxygen and temperature were controlled continuously (Fig. S13). The mean water
415 temperature ranged from 18.0 - 19.4^{o}C during the 2.5-week trial, with a mean temperature of
416 18.6^{o}C. Mean dissolved oxygen was 8.38, 8.50, 9.02 and 8.46 mg/L for the low, medium, high O_3
417 and control treatments, respectively. Assuming that aeration in all RAS was identical, ozone was
418 found to enrich the water with oxygen. Oxygen probes were calibrated at the beginning of each
419 trial.

420

421 3.2.2 Ozone monitoring

422 The risk of exposing fish to ozone must be diminished, since it might damage their tissues or gills
423 or even kill them (Powell & Scolding, 2016). Changes in fish behaviour (feeding and/or swimming)
424 are indicative signs of exposure to toxic concentrations (Bullock et al., 1997). However, during this
425 study, none of these adverse effects was recorded.
426 Ozone gas was determined on a daily basis, before being injected into the reaction tank (Section
427 2.3.1.1), and the flows were regulated accordingly, when needed. Water samples were collected
428 from both the pump sump (after ozone) and from the rearing tank, to test for potential ozone
429 presence. By using the indigo method, no ozone residues were detected in any RAS at any time.
430 The absence of residual ozone in the system was expected, due to low ozone dosages and its short
431 lifetime, and ozone was consumed completely within the ozone reaction tank, thereby proving the
432 robustness of the system design. These findings were also supported by toxicity tests. Water
433 samples were collected immediately before ozonation (0h) and 6h after starting treatment, and they
434 were subjected to microtox analysis. The results indicated no toxicity (less than 20% inhibition),
435 and so no results will be presented, since it was conclusive that ozone did not have any toxic effect
436 in RAS water.

437 3.2.2.1 Reduction potential (ORP)

438 ORP measurements were made with Oxyguard REDOX probes, in order to acquire a baseline and
439 to facilitate comparison with previous studies. REDOX probes were calibrated at the beginning of
440 each trial. ORP levels can be used to monitor the baseline in a given RAS, and once established,
441 changes in ORP can also be used as an indication of residual ozone build-up (safety measure).
442 The set point in the present study was at 200 mV (Fig. 5g). Sensors were calibrated a few hours
443 after ozone injection. The REDOX potential in the different systems did not have the same starting
444 point. Essentially, the higher the ORP, the more oxidising agents in the water. Different opinions
445 regarding the ORP set point have been suggested in the literature. Bullock et al. (1997) suggested
446 300 mV was safe for rainbow trout, while Summerfelt et al. (2009) found that ORP levels up to 340
447 mV equalled 0 mg O_3/L. Davidson et al., (2011) suggested a minimum of 250 mV in culture tanks.
448 The OPR level might vary among RAS due to changes in feeding, waste production cycles, oxygen
449 levels and treatment system.
450 In the present study, the REDOX potential stabilised after 4 days (Phase II), while for the short-term
451 high ozone dosage (Phase III), more time was required to achieve a steady state (Fig. 5g). Based on
452 our findings, adding 10, 15 and 26 g O_3/kg of feed, respectively, into the water, increased the
453 REDOX by up to 475, 516 and 549 mV, respectively. Summerfelt et al. (1997) applied dosages
454 with overlapping ranges, from 24-32 g O_3/kg of feed (28 $\pm$ 4 g/kg, 29 $\pm$ 3 g/kg, 29 $\pm$ 2 g/kg),
455 achieving various REDOX levels (375, 450 and 525 mV). Comparing the highest applied dosage in
456 this study and the derived REDOX with the findings of Summerfelt et al. (1997), the values
457 reported here were higher.

458 Sensors worked satisfactorily, although ozone is a difficult oxidant to monitor. In an overview
459 study, Li et al. (2014) established that comparing ORP levels across different RAS was difficult,
460 due to different water compositions, system designs, probe specifications and calibration times.

Nonetheless, ORP probes are often used as part of feedback mechanisms to aid in adjusting ozone dosages to the ozone generator (Powell & Scolding, 2016).

3.2.2.2 Fluorescence

Fluorescence has been used widely for monitoring water quality parameters in full-scale applications, in order to optimise processes like aeration (Reynolds & Ahmad, 1997) and to identify various deteriorating agents of biological (Cumberland et al., 2012) and chemical origin (Baker and Inverarity, 2004; Hudson et al., 2007; Carstea et al., 2016). Hambly et al. (2015) introduced the fluorescence technique in RAS for dissolved organic matter determination, while Spiliotopoulou et al. (2017) suggested a method to correlate fluorescence degradation upon ozonation to the delivered ozone dosage.

Fluorescence is more sensitive to low-ozone level applications such as RAS than ORP probes, as a number of characteristic specific fluorescence compounds exist and can be detected with minimal interference. Water from the pilot RAS was analysed for fluorescent organic matter composition, before ozone implementation (Phase I; Fig. 4) as well as during ozonation (Phases II and III; Fig. 5h, i). Besides the accumulation of organic matter during the first 185 days, the effect of continuous ozonation in different dosages (Section 3.1.3) was depicted (20 days), demonstrating that fluorescence is a suitable method for monitoring organic biomarkers/waste accumulation in RAS.

Although water samples were analysed in several wavelength transitions to describe humic and protein-like fluorophores (Fig. S18), only two of them will be discussed herein. Both the humic and the protein-like fluorophores responded alike towards ozone, in that they had the same tendencies as the NVOC. The control reached steady state (day 35, or the 150th monitoring day) and remained stable until the end of the trial (day 20).

Significant fluorescent organic matter degradation, in line with increasing ozone dosage, was observed. This decay pattern is in line with the batch experiments (Fig. 4), and also with a recent bench-scale study (Spiliotopoulou et al., 2017). Humic-like compounds oxidised by up to 53% in the presence of a high dosage, with no significant difference from the medium dosage (Fig. 5h). The high O_3 2x dosage resulted in a 46% reduction during the 3-day treatment. Protein-like fluorophores were reduced by 70% with more distinct effects among treatments than the humic-like fluorophores (Fig. 5i). The high dosage resulted in the same reduction as for the humic-like fluorophores (43%). However, the water still contained some fluorophores, which presumably were not reactive to ozone.

Fluorescence was found to describe the accumulation over time reasonably well as the effect of different applied ozone dosages on water quality. During the bench-scale experiment (Fig. 4c), it was predicted that approximately 1 mg O_3/L was required to clarify the water (20 Au), which was confirmed by the pilot-scale study, since the high ozone dosage (approximately 0.7 mg O_3/L) resulted in the same fluorescence removal success (Fig. S2). Therefore, fluorescence could be used as a tool to monitor dissolved ozone in water.

The ozone levels applied in our pilot experiment improved the resulting water quality, albeit not to the extent originally expected. This study differs from other aquaculture studies by investigating mature RAS in steady state conditions (more than three months of constant rearing conditions). The high levels of organic matter were reduced by ozone but at the same time, there was a constant daily organic input via the constant feeding regime. The applied ozone dosages could neither completely eliminate all organic matter nor increase UVT to very high levels, thus leaving future studies to identify the upper threshold for safe ozone application in RAS.

3.2.3 Water transparency

Water samples collected from the pump sump were analysed further, to establish chemical water composition. In a closed system, the water quality is generally the same, independent of sampling location within the system (Rojas-Tirado et al., 2016), which was also confirmed by sampling for residual ozone in several locations throughout the system.

Transmission of UV-light at 254nm is a widely used method in both clean and wastewater applications (APHA, 2012). Water transparency is commonly used as an indicator of general water quality and is described by UVA, which represents the amount of light absorbed by particles within a sample. The removal of carbon-based compounds (e.g. NVOC, COD and fluorescent organic matter) resulted in visually cleaner water. UVT is used to define water clarity. In the present study, non-ozonated samples had a UVT of approximately 40% (Fig. 5a) in agreement with previous studies, typically having UVT values for non-ozonated RAS ranged from 30 to 60%, depending on system intensity (Davidson et al., 2011). The UVT% of the treated RAS water rose by 15% compared to the control (Fig. 5a), resulting in 50-60% UVT%. For Phases II_B and III, the same increase in UVT% was observed, suggesting a correlation between the applied ozone dosage and the treatment period; in Phase III the applied dosage was doubled and the system was ozonated 5 days fewer than in II_B. It can be concluded that the increase in UVT% was proportional to the treatment period and that ozone boosted UVT (Summerfelt et al., 1997; Christensen et al., 2000; Summerfelt et al., 2009; Davidson et al., 2011), resulting in potential benefits for the fish. Increased visibility can also improve UV treatment efficacy and several water treatment processes within the unit.

527 3.2.3.1 Organic matter

528 Ozone is a highly reactive agent rapidly oxidising organic matter and contaminants (Summerfelt et
529 al., 1997; Good et al., 2011). At the beginning of Phase I (Table S2), the NVOC was 3.1 mg/L,
530 while at the end of the phase it had been elevated to 9.7 mg/L (approximately day 70). From that
531 point onwards, and until Phase II (day 181), the NVOC was relatively stable (Table S2). However,
532 upon ozonation, NVOC concentration diminished (Fig. 5c) dosage dependently, up to 25% (high
533 dosage, II_B); while in the high O_3 x2 dosage (III) the decrease was 17%. In the latest phase, NVOC
534 reduction was proportional to ozone exposure, while in Phase II_B a rapid decrease was observed
535 within the first exposure days, following which removal intensity was smoother, albeit present
536 (first-order decay).

537 A supplementary parameter to quantify oxidisable organic matter in water is the COD. The
538 dissolved fraction was the dominant (Fig. 5b). However, due to short-term treatment, the effect of
539 ozone on COD was not clear and there was variability in the control samples (standard deviation in
540 control samples; Fig. 5b).

541

542 3.2.3.2 Nitrogen-based compounds

543 Nitrite is toxic to cultivated organisms in RAS (Kroupova et al., 2005; Svobodová et al., 2005), and
544 therefore proper nitrification is vital for oxidising nitrite into harmless nitrate. Nitrite-N
545 concentration was approximately 0.06 mg N/L (Fig. S9), which was relatively low compared to
546 previous studies (Pedersen et al., 2012). NO_2^- -N removal was approximately 20%, without a
547 significant difference between ozone dosages (Fig. 5d) – in line with previous studies (Summerfelt
548 et al., 1997; Davidson et al., 2011). The 2x high-ozone dosages removed more than 40% of the
549 system's nitrite, reacting rapidly at the beginning of the treatment and reaching a new steady state in
550 the ensuing days.

551 NO_3^- -N concentrations were unaffected by ozonation (Fig. 5e), since ozone does not react with
552 nitrate, and the filters were operating properly during the experimental period. The nitrate-N level
553 was approximately 60 mg N/L (Fig. S12), which is comparable to previous studies performing in
554 similar conditions (Pedersen et al., 2012; Fernandes et a., 2015).
555 Concentrations of total NH_4^+-N/NH_3-N (TAN) during ozonation were low (0.10 to 0.18 mg N/L)
556 and not strictly related to ozone dosage (Fig. 5f) as previously found by Davidson et al. (2011).
557 Ammonia is not readily oxidised by ozone (Timmons et al., 2002), unless pH levels are 9 or above
558 (Rice et al., 1981).
559

560 **4. Conclusions**

561 Ozonation for all test dosages generally resulted improved water quality. Compared to the
562 unexposed RAS control, treated water in RAS exposed to medium- and high-ozone dosages saw a
563 reduction in organic compounds of up to 53% and 70% reductions in humic and protein-like
564 fluorophores, respectively. During the 20 days of ozone exposure, no fish mortality occurred,
565 ozonation improved UVT by 30 % and no toxic ozonated by-products were formed or detected.
566 By testing individual water samples from a RAS, laboratory tests can be used to predict the ozone
567 demand for the specific system, thus improving treatment efficacy. The main conclusions are
568 summarised thus:

569 - Bench-scale experiments can predict the effect of continuous ozonation in pilot-scale RAS.
570 - Fluorescence describes well the build-up and removal of organic matter caused by fish in an
571 RAS.
572 - Thus, fluorescence can potentially serve as an online measurement to control water quality.

Acknowledgements

The authors wish to acknowledge Innovation Fund Denmark, OxyGuard International A/S and Water ApS for funding the PhD stipend for A. Spiliotopoulou. The authors are grateful to the laboratory staff at DTU Environment and DTU Aqua for assisting in analysing samples, with the latter also thanked for providing the facilities in Hirtshals.

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Highlights

- Bench-scale experiments predicted continuous ozonation in pilot-scale RAS
- Fluorescence describes the waste build-up caused by fish and the removal by ozone
- Ozone lifetime decreases with organic loading and increases with continued ozonation
- Ozone degraded organic matter and thus improved ORP and transparency
- Ozone by-products and toxicity are not found in freshwater RASs.