General introduction

The need for aquaculture to supply the still growing global demand for fish is undisputed. Aquaculture takes place in several production systems, including net/pen systems, pond aquaculture, raceway systems and recirculating aquaculture systems (RAS). The major difference between RAS and other production systems is the isolation of the culture water from the surface water. By recirculating and treating the culture water, water can be re-used several times, depending on the quality and intensity of water purification. Due to this isolation of the culture water from the surface water, RAS offer several important benefits such as: better control over a-biotic parameters, reduced water consumption, concentration and separation of waste streams and better disease control (Martins et al., 2010). As a result of costs for the treatment of incoming water, such as temperature control, disinfection and costs for waste water discharge there is a trend to reduce water consumption (Blancheton et al., 2007). A reduced water consumption by an equal feed load leads to an intensification of water utilization. Consequently several substances, originating (in)-directly from the feed, accumulate in the culture water. Several studies for both marine and fresh water fish species have shown that intensification of water use, could negatively affect fish performance (Davidson et al., 2009, 2011, Deviller et al., 2005, Matins et al., 2009, 2011). Due to the experimental design used in these studies, different water exchange rates by equal feed loads, observed effects are multi-causative. It is suggested that these effects are caused by accumulating substances, e.g. nitrate (Davidson et al., 2011, Martins et al., 2009), ortho-phosphate (Martins et al., 2009) or metals (Davidson et al., 2009, Deviller et al., 2005, Martins et al., 2011). Monofactorial studies on the effects of accumulating substances, especially for marine fish species are lacking. Consequently such substances are not treated by specific technologies in RAS. Standard water treatment in marine RAS consists of mechanical filtration (e.g. drum-filtration and foam fractionating) and nitrification process (e.g. biological nitrification and ozonation). However by increased intensification of marine RAS other water treatment processes, accumulation of additional substances might become relevant. Thus by knowing the fish tolerant limits for nitrate, phosphate and metals new water treatment techniques should be focused.

Electrochemical water treatment using graphite and metal electrodes to remove accumulating substances and to disinfect aquaculture water has been studied in the past (Jorquera et al., 2002, Lin and Wu, 1996). Recent developments in waste and ballast water treatment have made electrochemical water treatment with artificial diamond electrodes commercially available. Artificial diamonds can be produced with a technique called chemical vapor deposition (CVD). Using this technique diamond can be deposited on non-diamond surfaces, but moreover impurities such as Boron can be controlled incorporated in the crystalline diamond structure. Boron Doped Diamond (BDD) electrodes are conductive carrier materials whereon a thin film of carbon and boron, in 100:1 ratio, is deposited. As a result of the boron incorporation this new material is semi-conductive but still has the unique properties of diamond, such as hardness, thermal conductivity and chemical inertness (Tribidasari et

al. 2005). Furthermore the surface of a BDD electrode is hydrophobic, and therefore when water is electrolyzed with BDD electrodes, the rate of water electrolysis is negligible and the rate of oxidant production is maximal. Thus instead of the evolution of H_2 and O_2 as in normal electrolysis, hydroxyl radicals ($HO^{\bullet}$) and hydrogen (H^+) are formed according to the formula:

$H_2O \rightarrow OH^{\bullet} + H^+ + e^-$

Due to the high oxidation potential of hydroxyl radicals, waste water treatment with BDD electrodes is studied intensively. Since early 1990 the use of BDD-electrodes for waste water treatment was studied and first results showed potential for electrochemical cathodic nitrate reduction (Tenne et al., 1993) anodic organic pollutant oxidation (Carey, 1995), phosphorus removal (Feng et al., 2003) and trace metal removal (Comnellis et al., 2005). However BDD electrodes nowadays are mostly applied to degrade non-biodegradable substances such as aromatic compounds, dyes, pharmaceutical compounds, and pesticides into biodegradable fractions, which can then be treated using common biological methods (Wang & Xu, 2012). Another field of application of BDD electrodes is the treatment of ballast water in order to produce disinfectants in the water itself without the need for external chemicals or processes (Echardt & Kornmueller, 2009). Due to these both applications in other fields, (in)-organic removal and disinfection BDD-electrolysis might be a suitable water treatment technique for RAS aquaculture but until now, application of BDD electrodes for aquaculture purposes is limited to only one laboratory study (Diaz et al., 2012). In this study BDD electrodes were tested as alternative to biofiltration, in order to reduce total ammonia nitrogen, nitrite and chemical oxygen demand in marine aquaculture water. However results were obtained in a laboratory environment with a volume of ca. 1 liter, clean electrodes and without fish stocks. But more important effects on other accumulating substances, long term effects, interaction with fish and the biofiltration process and practical handling couldn't be assessed.

Therefore in this thesis two main research topics were targeted. First the effect of the accumulation of in-organics on health and production performance of fish was studied. Therefore in the first part of the present thesis the maximal allowable water quality concentrations for the major pollutants nitrate (chapter 1), orthophosphate (chapter 2) and the trace minerals iron (Fe), zinc (Zn), copper (Cu), cobalt (Co) and manganese (Mn) (chapter 3) for the culture of marine fish were determined. In these three chapters the turbot (*Psetta maxima*) was used as model species. By knowing these limits minimal water exchange rates can be calculated and bottlenecks in water treatment can be found.
Secondly the use BDD-electrodes to improve water quality in RAS was explored, by the use of replicated small scaled RAS. In chapter 4, commercial farming in marine RAS was simulated, using white leg shrimp (*Litopenaeus vannamei*) as model species because susceptibility of turbot and shrimp to disinfection by-products is comparable (Reiser et al., 2010, Schroeder et al., 2010). Effects of

additional water treatment with ozonation or BDD electrolysation on water quality, disinfection and shrimp performance were studied. In chapter 5 the previous experiment was scaled up to a commercial scaled RAS size culturing turbot (*Psetta maxima*) and effects of additional ozonation or additional BDD electrolysation on water quality, disinfection and practical handling of the electrocell was studied. Finally in chapter 6 the possibilities for the use of BDD electrodes in fresh water RAS was evaluated. Fresh water has a different ionic water matrix and therefore other effects of BDD treatment on water quality can be expected. Here the European eel (*Anguilla anguilla*) was used as model species and again effects on water quality, disinfection, fish health and practical handling were evaluated.

Thus by knowing the tolerable water quality limits for marine fish and opportunities for BDD electrolysation in RAS new water treatment strategies can be determined.

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Chapter 1:

The chronic effect of nitrate on production performance and health status of juvenile turbot (*Psetta maxima*).

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Abstract

The chronic effect of nitrate on the production performance and health of marine cultured fish species is still unknown. Thus, the aim of the present research was to evaluate the chronic effect of nitrate on production parameters and health status of turbot (*Psetta maxima*). Juvenile turbot were exposed to 0 mg/l NO_3-N (control C), 125 mg/l NO_3-N (low nitrate LN), 250 mg/l NO_3-N (medium nitrate MN) and 500 mg/l NO_3-N (high nitrate HN) for 6 weeks in small-scaled recirculating aquaculture systems (RAS). After 42 d, biomass yield, length, weight and specific growth rate (SGR) were significantly ($p<0.05$) lower for LN, MN and HN compared to C. Mortality, food conversion ratio (FCR), condition factor (CF) and splenic index (SI) did not differ significantly ($p>0.05$) between C and LN but were significantly ($p<0.05$) higher in the MN and HN groups. Hepatosomatic index (HSI), total hemoglobin (Hb) and methemoglobin (MetHb) did not show significant differences ($p>0.05$) between treatments. Thus, nitrate negatively influences the production performance of turbot even at low concentrations, and nitrate management is therefore a key-factor in turbot RAS production.

1. Introduction

Over the last decades, fish production in land based recirculating aquaculture systems (RAS) has become increasingly important in the aquaculture industry. Due to high levels of water re-use, water renewal is less than 5 % per day in modern RAS. As a result, waste is accumulating in these systems making water treatment and waste management key factors in RAS technology. The principle excretory metabolite of fish is ammonia, which is highly toxic and, therefore, needs to be eliminated from the water. Biological nitrification has been established in RAS as the most common method for the removal of ammonia, involving a two-step oxidation of ammonia to nitrite and finally to nitrate by aerobic chemoautotrophic bacteria (Eding et al., 2006). As a consequence, nitrate accumulates to high levels in modern RAS with low water exchange and high hydraulic retention times. Concentrations of 100-1000 mg/l NO_3-N are not uncommon and nitrate management becomes increasingly important (van Rijn, 2010). Nitrate can be removed from the rearing water by denitrification or, less desirable in terms of sustainable production, by water exchange. To manage and optimize these processes, maximal allowable concentrations of nitrate in RAS have to be determined.

Acute and chronic effects of nitrate have been reported in several fresh water fish species (Camargo et al., 2005; Kamstra and van der Heul, 1998; Hamlin, 2006) and marine invertebrates (Hirayama, 1974; Camargo et al., 2005; Romano and Zeng 2007; Kuhn et al. 2010). In contrast, data on nitrate toxicity in marine fish are limited. Acute nitrate toxicity has only been studied in 8 marine species (Brownell, 1980; Pierce et al., 1993) and 2 anadromous species reared in saline water (Westin, 1974) revealing LC-50 concentrations between 573 and 5050 mg/l NO_3-N. Although these values are in fact very high, one has to consider that nitrate is usually added as sodium nitrate ($NaNO_3$) in these experiments. Hence, addition of sodium-nitrate in high quantities results in extreme salinities, which are more likely to cause mortality than the nitrate itself (Brownell, 1980). Furthermore, addition of sodium nitrate causes a Na^+/K^+ imbalance in water, which contributes to adverse effects and thereby underestimates the nitrate tolerance of marine animals (Romano and Zeng 2007; 2009). Chronic toxicity has only been studied in one juvenile tropical marine species. In this study 100 mg/l NO_3-N affected color and growth of *Amphiprion occelaris* compared to a control group reared at 16 mg/l NO_3-N (Frankes and Hoff, 1982).

The mechanisms of nitrate toxicity to aquatic animals are due mainly to methemoglobinemia, caused by the oxidation of hemoglobin (Hb) to methemoglobin (MetHb) in blood (Camargo et al., 2005). In fish the pathways of nitrate uptake are not clear. Nitrate is taken up via the branchial system, but due to the low permeability of the gills to nitrate, uptake is limited and other mechanisms are suggested (Stormer et al., 1996). Another possible pathway might be trans-dermal uptake of nitrate in the gastro intestinal tract, as was reported for nitrite in European flounder (*Platichthys flesus*) (Grosell and Jensen, 2000). Nevertheless, so far this has not been examined for nitrate uptake in fish. Because the exact pathways of nitrate uptake are not known, the relation between salinity of the water and nitrate

uptake is unclear. Although several studies showed that nitrate toxicity decreases with increasing salt concentrations, no causal explanation has been found yet (Tsai and Cheng, 2002; Romano and Zeng, 2007; 2009; Kuhn et al., 2010).

Due to the lack of experimental studies on chronic effects of nitrate on marine fish species, literature safety levels are theoretical values calculated from acute toxicity data (LC-50 values) or based on experience in routine aquaculture. As a consequence, safety levels in the literature vary between 20 mg/l NO_3-N (Spotte, 1970) and 500 mg/l NO_3-N (Pierce et al., 1993; Timmons and Ebeling, 2010), or nitrate is considered to be not significantly toxic (Russo and Thurston, 1991). Data on the chronic impact of realistic nitrate levels, which are actually met in RAS, on production performance and health of marine cultured fish species are lacking. Thus, the aim of the present study was to evaluate the impact of nitrate concentrations commonly observed in RAS on mortality, growth performance, feed utilization and health status of juvenile turbot (*Psetta maxima*).

2. Material & Methods

2.1 Experimental setup

Juvenile turbot were obtained from Maximus A/S (Bedsted Thy, Denmark) and acclimatized to culturing systems at the facilities of the GMA (Büsum, Germany) for one week prior to the six week experimental exposure. A total of 168 turbot with a mean average weight (±SD) of 18.6 ±1.1g (range: 17.0-21.0 g) were randomly distributed to twelve separate RAS, which provided a control at 0 mg/l NO_3-N (control C) and three treatments at 125 mg/l NO_3-N (low nitrate LN), 250 mg/l NO_3-N (medium nitrate MN) and 500 mg/l NO_3-N (high nitrate HN), with three replicates each. Average weight per system was 261.0± 0.4 g resulting in an initial density of 373 g/m². Each RAS (total volume of 300 l) consisted of a rectangular rearing tank (275 l, 0.7 m² footprint), a moving bed biofilter (Kunstoff-Spranger, Plauen, Germany) and a protein skimmer (Model 1 AH 1100, Erwin Sander, Uetze-Eltze, Germany) using compressed air. Flow rate through the moving bed biofilter was 700 l/h, flow rate through the protein-skimmer was 800 l/h, making a total flow rate of 1500 l/h, equal to 5 tank volumes per hour. Temperature was automatically adjusted to 19°C with a temperature-controlled 500 W heater (Aqua Medic, Bissendorf, Germany). A photoperiod of 14 hours light (06:00-20:00), 10 hours dark was provided. Fish were fed *ad libitum* twice a day, 6 days per week with a commercial turbot feed (Aller 488 BM, Aller Aqua, Christiansfeld, Denmark). Uneaten pellets were netted out and counted one hour after feeding.

Prior to the exposure, RAS were filled with UV disinfected seawater (22 ppt salinity) and nominal concentrations of 125, 250 and 500 mg/l NO_3-N were obtained by the addition of $NaNO_3$ and KCl solution, providing a Na^+/K^+ weight-ratio of 1:46 to avoid imbalances of the cellular homeostasis. Weekly, fresh water was added in equal amounts to all RAS to compensate evaporation losses. Every

second week, 200 l water per RAS were exchanged to minimize accumulation of metabolic NO_3-N. During exposure, water parameters were kept in the optimum range for turbot, summarized in table 1.

Table 1. Water quality ranges for optimal growth of juvenile turbot (*Psetta maxima*).

Parameter	Range	Reference
Temperature	19-22 °C	Imsland et al., 1996; Imsland et al., 2000; Imsland et al., 2001
Dissolved Oxygen	> 5 mg/l	Pichavant et al., 2000
pH	6.5-8	Poxton and Allouse, 1982
Salinity	15-25 ppt	Imsland et al., 2001
NH_3-N	< 0.1 mg/l	Hamspon et al., 1976; Person-Le Ruyet, 1997
NO_2-N	< 2 mg/l*	Bianchini et al., 1995
Flow rate	> 4.7 (tank vol./h)	Schram et al., 2009

*Values for European and Japanese flounder

2.2 Water quality analysis

Temperature, total ammonia nitrogen (TAN), NO_2-N, NO_3-N, pH, salinity, flow rate and dissolved oxygen (DO) were measured twice a week. TAN, NO_2-N and NO_3-N were determined spectrophotometrically using a HACH-DR 2800 spectrophotometer (Hach-Lange GmbH, Berlin, Germany) and powder pillow detection kits based on the ammonia salicylate method, diazotization method and the cadmium reduction method, respectively (Hach-Lange GmbH, Berlin, Germany). Temperature and pH were measured with a WTW 340i portable multiparameter instrument (WTW GmbH, Weilheim, Germany). Salinity was determined with a digital refractometer HI 96822 (HANNA Instruments, Woonsocket, USA), dissolved oxygen with a Handy Polaris oxygen probe (Oxygard International A/S, Birkenrød, Denmark).

2.3 Sampling

During the experimental period feed intake and mortality were recorded daily, group weight weekly. After six weeks of exposure group weight as well as individual length and wet weight of fish were determined. Weight was recorded to the nearest 0.1 g, length was recorded to the nearest mm. Upon sampling three fish per tank were killed, liver and spleen were removed and weighed to the nearest 0.001 g. Blood samples were drawn from the caudal vein. For methemoglobin (MetHb) analysis a subsample was immediately frozen on dry-ice and stored at -80° C. For total hemoglobin (total-Hb) a subsample was cooled on crushed ice and analyzed within 3 hours after sampling.

2.4 Analysis of blood parameters (total Hb, MetHb)

Total-Hb was determined by the cyanmethemoglobin method using a Hemoglobin FS reagent kit (DiaSys, International, Holzheim, Germany) in the whole blood within 3 h after sampling. All samples were measured in duplicates with a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland) at 540 nm and calculated from a standard hemoglobin dilution series (HEM QS, Diaglobal GmbH, Germany) using 5µl blood and 1 ml reagent.

Methemoglobin was determined as the ratio of MetHb to total-Hb using the cyan ferrocyancomplex method (Hegesh et al., 1970). In brief, hemolysis of 20 µl sample was carried out in 1 ml MilliQ (hypotonic media) for 15 min at room temperature, followed by the addition of 600 µl saponin solution (1% in phosphate buffer [9 g Na_2HPO_4 * 12 H_2O, 5.7 g KH_2PO_4, pH 6.6 to 1 L MilliQ]), vortexing and centrifugation (10 min, 3000 g) to separate cell debris. From the supernatant, the ratio of MetHb to total Hb was determined in duplicates at 633 nm, measuring the absorption in: 250 µl supernatant (A1), followed by the addition of 5 µl 1% KCN and incubation for 10 min (A2) and in 250 µl supernatant after addition of 5 µl $K_4[Fe(CN)_6]$ (A3), followed by an addition of 5 µl 1% KCN and incubation for 10 min (A4). Total Hb:MetHb was calculated as (A1-A2)/(A3-A4).

2.5 Data analysis

Data are presented as mean ± standard deviation (SD) of n samples. Statistical analysis was performed using SPSS 17.0 (IBM inc, Armonk, USA). Data were tested for normality and for equal variances ($p<0.05$) using Levene's test and the Kolgomorov-Smirnov test, respectively. Multiple comparisons were carried out by the parametric Tukey's HSD or the non-parametric Dunnet's T3 test. Differences were considered significant at $p<0.05$.

3. Results

All water quality parameters measured (table 2) (with the exception of nitrate) were in the optimum range for turbot (table 1) at all sampling times. Average nitrate levels for LN, MN and HN were comparable to the nominal concentrations, deviating within 2 % of the nominal value (tab. 2). Average pH and nitrite nitrogen levels differed significantly between treatments, ranging from 7.82 to 7.97 and 0.07 to 0.27 mg/l, respectively (table 2). Temperature, dissolved oxygen, salinity and total ammonia nitrogen did not significantly differ between treatments.

Table 2. Mean values (± SD) of water quality parameters. Letters indicate significant differences (ANOVA with Tukey HSD or Dunnet's T3 post hoc test, $p<0.05$, n= 36).

Treatment	DO (mg/l)	TAN (mg/l)	NO_2-N (mg/l)	NO_3-N (mg/l)	pH	Salinity (ppt)	Temp. (°C)
Control	8.31 ± 0.208	0.10 ± 0.16	0.07^{a} ± 0.06	3.9^{a} ± 2.9	7.82^{a} ± 0.18	23.5 ± 1.5	19.2 ± 0.5
Low Nitrate	8.33 ± 0.229	0.10 ± 0.09	0.13^{b} ± 0.08	126.1^{b} ± 1.8	7.91^{bc} ± 0.08	23.9 ± 1.5	19.2 ± 0.4
Medium Nitrate	8.33 ± 0.281	0.08 ± 0.04	0.18^{bcd} ± 0.10	250.1^{c} ± 3.7	7.91^{bc} ± 0.06	24.0 ± 1.6	19.3 ± 0.5
High Nitrate	8.36 ± 0.230	0.06 ± 0.03	0.27^{d} ± 0.18	504.7^{d} ± 4.4	7.97^{d} ± 0.06	24.4 ± 1.2	19.1 ± 0.5

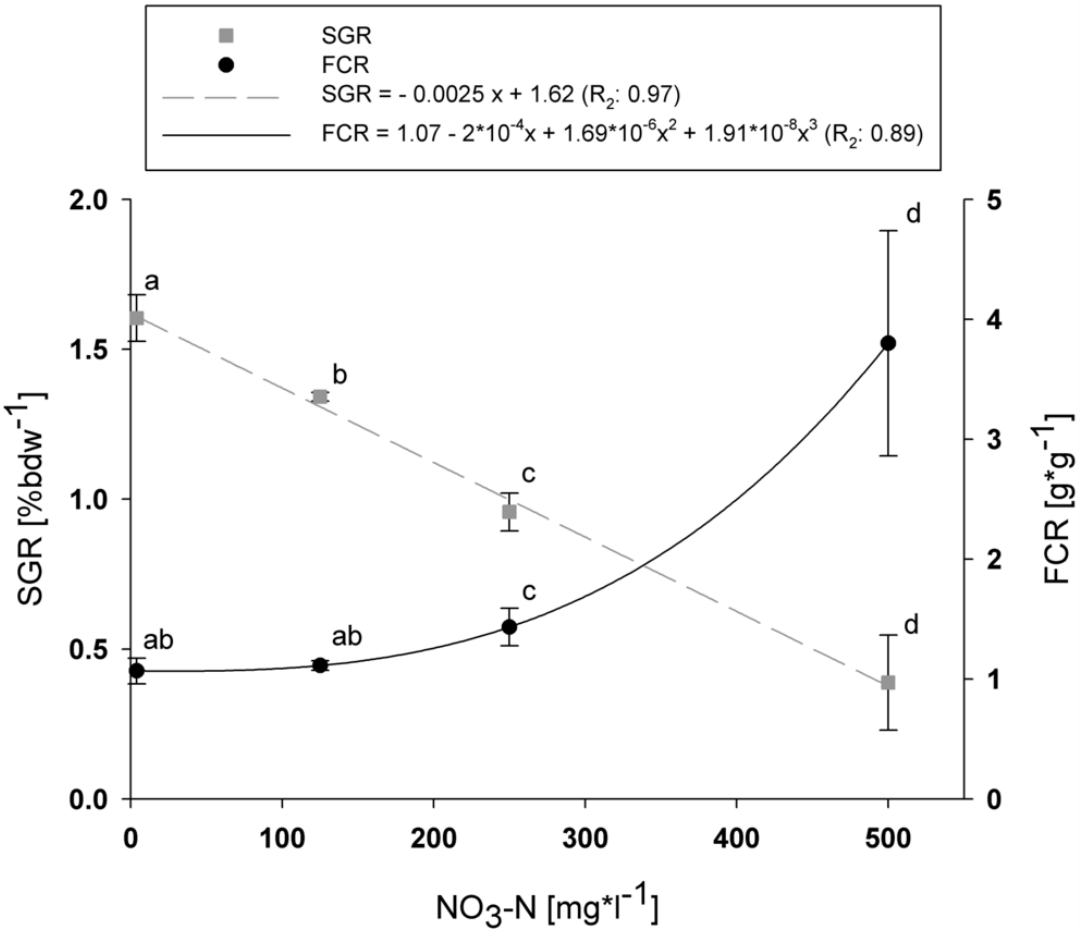
Mortality in the control group was zero, but increased with increasing nitrate concentration to 9.5 % in MN and HN, both revealed significantly (n=3, Tukey, $p<0.05$) higher values. At the end of the exposure, mortality rates were 0, 2.4 ± 4.1, 9.5 ± 4.1 and 9.5 ± 10.9% in the C, LN, MN and HN treatments, respectively.

After 42 d, final length and weight decreased with increasing nitrate concentration and were significantly different (C: n=42; LN: n=41; MN: n=38; HN: n=38, Tukey, $p<0.05$) between all groups (table 3). Condition factor (CF) decreased with increasing nitrate concentration. Mean CF of C, LN, MN and HN was 1.85 ± 0.11, 1.83 ± 0.11, 1.77 ± 0.12 and 1.62 ± 0.22, respectively, revealing significant differences (C: n=42; LN: n=41; MN: n=38; HN: n=38, Dunett T3, $p<0.05$) in MN and HN compared to C. Biomass yield as a result of increased mortality and decreased growth, was significantly different between all groups (n=3, Tukey, $p<0.05$) and decreased with increasing nitrate concentration. Mean biomass yield was 512.9 ± 16.8, 450.5 ± 2.9, 363.0 ± 10.3 and 286.0 ± 20.9 g in C, LN, MN and HN, respectively.

Over the 42 day experimental period, specific growth rates (SGR) were significantly different (n=3, Tukey, $p<0.05$) between all treatments and negatively correlated with nitrate concentrations. Individual growth rates were linear related with nitrate concentration according to the formula:

$$SGR = SGR_{control} - 0.0025(NO_3\text{-}N) \quad \text{(figure 1).}$$

Figure 1. Specific Growth Rate (SGR) (% bodyweight^{-1}) and Feed Conversion Ratio (FCR) (g/g) against average nitrate-N concentration (mg/l).



Total feed intake (TFI) was negatively correlated with nitrate concentrations. Significant differences ($p<0.05$) in feed intake were notated in all treatment groups (LN: 219.9 ± 5.3 g, MN: 185.5 ± 29.6 g, and HN: 164.2 ± 25.4 g) compared to C at 267.8 ± 21.3 g. Daily feed intake (DFI) based on the actual number of feeding days was significantly lower in LN, MN and HN compared to control.

FCR increased with nitrate concentration, revealing 1.07 ± 0.11, 1.11 ± 0.04, 1.43 ± 0.16, 3.8 ± 0.94 in the C, LN, MN and HN groups, respectively.

HSI did not differ significantly (n=9, Tukey, $p>0.05$) between treatments. In contrast SI increased with increasing nitrate concentration. SI of the control was significantly lower (n=9, Dunett T3, $p<0.05$) compared to the SI of MN and HN groups. SI values ranged from 0.053 ± 0.013 (C), 0.059 ± 0.019 (LN), 0.094 ± 0.039 (MN) to 0.101 ± 0.027 (HN).

Total-Hb was comparable between treatments, ranging from 3.9 ± 0.5 (HN) to 4.6 ± 0.6 (LN). Equally, the percentage of Met-Hb did not vary significantly between treatments. Both, total-Hb as well as MetHb revealed high individual variability as indicated by a high standard deviation.

Table 3. Mean values (± SD) of fish growth and health parameters. Different letters indicate significant differences (ANOVA with Tukey HSD or Dunnet T3post hoc test, $p<0.05$).Yield = life biomass - start biomass; FCR = (final total biomass - start biomass)/ total feed intake; SGR = (ln final weight – ln start weight) / 42*100; CF = (weight / length^3)*100; HSI = (liver weight/ final weight) *100; SI = (spleen weight / final weight) *100.

Parameter	N per treatment	Control C (0 mg/l NO_3-N)	Low nitrate LN (125 mg/l NO_3-N)	Medium nitrate MN (250 mg/l NO_3-N)	High nitrate HN (500 mg/l NO_3-N)
Ind. start weight (g)	42	18.7 ± 1.1	18.7 ± 1.1	18.6 ± 1.1	18.6 ± 1.1
Ind. end weight (g)	38-42	36.6^{a} ± 6.0	33.0^{b} ± 5.7	28.7^{c} ± 6.0	22.6^{d} ± 4.6
Start biomass (g)	3	261.3 ± 0.1	261.4 ± 0.2	260.7 ± 0.2	260.9 ± 0.6
End life biomass (g)	3	512.9^{a} ± 16.8	450.5^{b} ± 14.2	363.0^{c} ± 15.2	286.0^{d} ± 42.8
End total biomass (g)	3	512.9^{a} ± 16.8	458.8^{b} ± 3.2	389.8^{c} ± 10.3	307.4^{d} ± 21.3
Total feed intake (g)	3	267.8^{a} ± 21.3	219.9^{bc} ± 5.3	185.5^{bcd} ± 29.6	164.2^{d} ± 4.0
Daily feed intake (%bdw)*	3	2.14 ± 0.18^{a}	1.83 ± 0.08^{b}	1.70 ± 0.12^{cd}	1.67 ± 0.06^{cd}
Final morality (%)	42	0.0^{a} ± 0.0	2.4 ± 4.1	9.5^{bc} ± 4.1	9.5^{bc} ± 10.9
Yield (g)	3	512.9^{a} ± 16.8	450.5^{b} ± 2.9	363.0^{c} ±10.3	286.0^{d}± 20.9
FCR (g/g)	3	1.07^{ab} ± 0.11	1.11^{ab} ± 0.04	1.43^{c} ± 0.16	3.80^{d} ± 0.94
SGR (%/d)	3	1.60^{a} ± 0.08	1.36^{b} ± 0.03	1.03^{c} ± 0.09	0.45^{d} ± 0.12
Length (cm)	38-42	12.5^{a} ± 0.6	12.1^{b} ± 0.6	11.7^{c} ± 0.6	11.1^{d} ± 0.6
CF	38-42	1.85^{a} ± 0.11	1.83^{abc} ± 0.11	1.77^{c} ± 0.12	1.62^{d} ± 0.22
HSI (%)	9	1.432 ± 0.265	1.660 ± 0.277	1.495 ±0.204	1.401 ± 0.267
SI (%)	9	0.053^{a} ± 0.013	0.059^{abc} ± 0.019	0.094^{bcd}± 0.039	0.101^{d} ± 0.027
Total HB (g/dl)	9	4.5 ± 0.4	4.6 ± 0.6	4.5 ± 0.8	3.9 ± 0.5
Met-HB (%)	9	17.6± 11.1	19.0 ±11.0	17.8 ± 10.3	19.6 ± 3.6

4. Discussion

Throughout the experiment all relevant water quality parameters, with the exception of nitrate, were in the optimum range for maximal growth performance, including temperature, oxygen, total ammonia nitrogen and salinity. Nitrite and pH were correlated to the nitrate concentrations and were significantly different between treatments. Still, both parameters were within the optimum range for maximum growth performance of turbot. In particular nitrite was < 0.3 mg/l NO_2-N in average and maximum nitrite concentration over the entire experimental period was 0.56 mg/l NO_2-N. Since lowest observed effect concentration (LOEC) of nitrite in Brazilian flounder (*Paralichthys orbignyanus)* has been reported 3 mg/l (Bianchini et al., 1996) and acute toxic levels in turbot are 67 mg/l NO_2-N (Boeuf et al., 1999), nitrite is considered well below concentrations accounting for effects as the ones reported here. Differences in growth performance and production as well as health parameters between treatments are, therefore, thought to be monofactorial, resulting from nitrate concentrations during exposure. The nitrate concentrations were achieved by addition of $NaNO_3$, meeting nominal concentrations of 125, 250 and 500 mg/l with deviations < 2%. In RAS, nitrate concentrations accumulate as a consequence of the nitrification process and concentrations of 100-1,000 mg/l NO_3-N have frequently been reported (van Rijn, 2010). Romano and Zeng (2007; 2009) reported higher susceptibility to nitrate in shrimp when nitrate was purely added as $NaNO_3$, probably due to ionic imbalances if Na^+ increased substantially compared to K^+. As a consequence KCl was added to assure a Na:K mole ratio of 46:1, modified to the protocol of Romano and Zeng (2009).

Overall growth performance of the control groups during the experimental period was low with a SGR ranging between 1.54 and 1.69 for the replicates. In previous studies SGR reported in turbot of comparable size was generally between 2.0 and 3.5 (Arnason et al., 2009; Imsland et al., 2002; Irwin et al., 1999). During the study presented here, fish were fed twice a day for 6 days and group weighing was carried out at day 7. The 6 days feeding regime and the handling-related stress might thus explain lower SGR values. Still, here, FCR of the control group varied between 0.99 and 1.19 and is comparable with previously reported values in turbot (Arnason et al., 2009; Foss et al., 2009; Imsland et al., 2002). Thus, acceptable growth rate of the control with regard to the feeding regime, a normal FCR and the fact that fish biomass almost doubled during the experimental period of 42 days reveals good rearing conditions, representative for turbot production and, therefore, allows evaluation of nitrate exposure on growth performance and health.

In the present study, health status of turbot was roughly assessed as mortality, HSI, SI, total-Hb and Met-Hb. Mortality was positively correlated with nitrate concentrations, with zero mortality in the control group, increasing to 9.5% in MN and HN and is the most crucial indicator of nitrate impact on the health of turbot. In Pacific white shrimp (*Litopenaeus vannamei*), nitrate concentrations of > 220 mg/l NO_3-N resulted in increased mortality (Kuhn et al., 2010). In contrast, Kamstra et al. (1998) reported a decrease in mortality of eel (*Anguilla anguilla*) at high nitrate levels and suggested that fish

are inactive and therefore less aggressive at high nitrate levels. In anemone fish (*Amphiprion occelaris*) Frankes and Hoff (1982) reported zero mortality in both control and 100 mg/l NO_3-N treatment. The hepatosomatic index (HSI) indicates the nutritional state, commonly increasing with improving nutritional state (Arfsten et al., 2010). Still, increased HSI may also be observed if inadequate feeding results in fat accumulation in the liver (Tusche et al., 2011) and has to be evaluated carefully. Here, HSI fluctuated between 1.4 and 1.7, which is comparable to HSI frequently reported in healthy, well-nourished turbot (Fournier et al., 2002; Abele et al., 2007; Peres and Oliva-Teles, 2008). Splenic index (SI) showed a positive correlation with nitrate concentration, increasing from 0.053 in the control to 0.059, 0.094 and 0.101 in the LN, MN and HN group, respectively. Since 0.05 is considered a normal SI for farmed turbot (Quentel and Obach, 1992) a twofold increase might be considered pathological. Furthermore, as the spleen is the principal organ of blood cell storage and production in fish, increased SI is indicative of respiro-circulatory impact such as methemoglobinemia. Here, MetHb was comparable between treatments and thus did not indicate impeded respiro-circulatory function. Nevertheless, increased SI can be an indication of diseases or immunological problems (Goede and Barton, 1990) or the impact of toxicants (Hutchinson and Manning, 1996). Although mortalities and SI suggest a severe health impact during nitrate exposure, total-Hb does not confirm a respiro-circulatory dysfunction as values are within a comparable range as previously reported by Person le Ruyet et al. (2002), Pichavant et al. (2003) and Waring et al. (1996). Nitrate is known to transform Hb into MetHb in aquatic animals (Camargo et al., 2005), consequently reducing oxygen binding capacity and ultimately resulting in respiro-circulatory constrains. In fish a MetHb reductase system compensates for MetHb formation by conversion of MetHb to Hb (Freeman et al., 1983). This acclimatization is an energy consuming activity (Urrutia and Tomasso, 1987). Consequently, conversion of MetHb into Hb may not just explain constant MetHb values observed here but also provide an explanation for reduced growth observed here, as already suggested by Colt and Armstrong (1981). Values for metHb formation in marine fish are scarce and highly species-specific. In Chinook salmon (*Oncorhynchus tshawytscha*) and sea bass (*Dicentrarchus labrax*) MetHb was not detectable below a species-specific nitrite threshold (Crawford and Allen, 1977; Scarano et al., 1984). Still, European flounder (*Platichthys flesus*) exhibited 4-6% (Grosell and Jensen, 2000) and Atlantic cod (*Gadus morhua*) 27% MetHb (Graham and Fletcher, 1986) in unexposed fish. Here, MetHb was between 17.6 and 19.6 %, which is considered basal value in turbot with regard to the control fish assessed.

All growth parameters (SGR, final weight, final length, biomass yield) are negatively linearly correlated with nitrate concentration and all these parameters showed significant differences between control and nitrate treatments. These results are in line with results from Frankes and Hoff (1982) who reported reduced growth and color in anemone fish at 100 mg/l NO_3-N compared to a control group raised at 16 mg/l NO_3-N, and Kamstra et al. (1998) who observed growth depression in eel at 250 mg/l NO_3-N compared to control group at 50 mg/l NO_3-N. Here, reduced growth performance can be

explained by energetic costs to maintain homeostasis, in particular with regard to the potential conversion of MetHb by the nitrite reductase as discussed above. The increased energy demand is thereby not compensated by increased food intake. This resulted in a reduced CF in the nitrate exposed groups, which is another indicator for the increased energy demand due to nitrate exposure. Furthermore, feed utilization was less efficient in terms of feed conversion ratio (FCR) at increasing nitrate concentrations. Again, this suggests an energy mobilization for detoxification, which is consequently not available for body growth. Reduced growth performance due to nitrate exposition is therefore a factor that impairs production in RAS-based turbot aquaculture.

5. Conclusions

Nitrate negatively influences growth and health status of turbot, even at concentrations far below most safety levels suggested in literature. In the present experiment significant negative impact of nitrate on growth performance were observed at ≥125 mg/l NO_3-N, and on health and feed efficiency at ≥ 250 mg/l NO_3-N. Thus, in view of an economic turbot production nitrate management in RAS-based aquaculture needs to be optimized between taking into account increased production costs as a consequence of lower growth, less efficient feed utilization and higher mortality, and the actual costs for nitrate removal or water exchange.

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Chapter 2:

The effect of high ortho-phosphate water levels on growth, feed intake, nutrient utilization and health status of juvenile turbot (*Psetta maxima*) reared in intensive RAS.

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Abstract

In intensive recirculating aquaculture systems (RAS) ortho-phosphate (ortho-P) is one of the main accumulating substances, but effects of chronically elevated concentrations on fish health and production performance are still unknown. Therefore 120 juvenile turbot (*Psetta maxima*) were exposed to ortho-P concentrations of 3 mg/L (control - C), 26 mg/L (low - LP), 52 mg/L (medium - MP) and 82 mg/L (high - HP) for 56 days and fed until satiation with a commercial diet. Health status and feed conversion ratio (FCR) were not significantly affected by treatment ($p>0.05$). Specific growth rates (SGR) and daily feed intake (DFI) of C were not considered significantly different from LP, MP and HP treatments, however LP showed significant higher DFI and SGR than HP ($p<0.05$). Using non-linear regression between SGR and ortho-P concentrations, 27 mg/L ortho-P was found as the optimum for turbot growth. Although not reflected in blood plasma P levels ($p>0.05$) a potential aqueous P uptake might result in metabolic benefits leading to the observed growth enhancement in the LP treatment.

In a second experiment 114 juvenile turbot were exposed to ortho-P concentrations of 2 mg/L (C2) and 25 mg/L (LP2) for 63 days and fed until satiation with a low P diet (4.6 g digestible-P / kg diet). Overall production performance was low due to low voluntary feed intake. Whereas the FCR was unaffected by treatment ($p>0.05$), significantly higher feed intake and biomass gain were observed for LP2 compared to C2 ($p<0.05$). LP2 treatment showed a trend for higher protein retention efficiency and lower whole body lipid content ($p<0.1$). The dry matter, ash, Phosphorus, Calcium and protein content in whole body did not significantly vary between treatments ($p>0.05$).

In conclusion the accumulation of ortho-P in RAS does not negatively affect health of turbot. Elevated ortho-P seems to have slight positive effects on production performance of juvenile turbot. Further research to quantify dietary P requirements for turbot in general, as well as for turbot raised under elevated ortho-P conditions in RAS is strongly required.

1. Introduction

In intensive recirculating aquaculture systems (RAS) the use of make-up water is tried to minimize in order to reduce water consumption and costs for energy due to heating or cooling. As a result 'inert' substances such as nitrate, ortho-phosphate and metals originating from make-up water and feed accumulate in the culture water (Davidson et al. 2009). Applied feed is the main source of ortho-phosphate phosphorus (ortho-P) and the fraction of dietary phosphorus (P) that can accumulate is dependent on the amount of dietary-P, the retention efficiency of P and leaching of particulate-P from faeces.

In fish the fraction of digested P excreted as ortho-P is dependent on the excess amount of available P (Sarker et al., 2009, Coloso et al., 2003) and the fraction of total-P as available-P is mostly depending on the amount and source of P (Hua & Bureau 2006, Pimentel-Rodrigues & Oliva-Teles, 2006). The amount of ortho-P that leaches from faeces is dependent on the retention time in the water and stability of the faeces (Brinker et al., 2005). Therefore accumulation of ortho-P is strongly dependent on formulation and physical properties of formulated diets. In commercial aquaculture turbot are fed with diets high in total P content, e.g. 14-16 g total-P/kg diet (Efico sigma 570 Biomar, Aller 505 ex Aller aqua) and a high available P content (table 2). Furthermore turbot are mostly cultured in shallow tanks causing a relative high stocking density per water volume, resulting in a high potential for ortho-P accumulation when cultured in RAS.

In intensive RAS ortho-P concentrations of 25-45 mg /l are not uncommon (Rishel & Ebeling, 2006, Schneider et al., 2006, Tal et al., 2009, Martins et al., 2009a,). In RAS Ortho-P can be reduced by water exchange but this is undesired in terms of water consumption and nutrient discharge. Methods that are developed and used in experimental RAS are biological removal, chemical flocculation or integrated multitrophic aquaculture (IMTA) (Barak et al, 2003, Rishel & Ebeling, 2006,Metaxa et al., 2006). However this is not common praxis as limitations in removal efficiency and cost effectiveness restrict a commercial application until now (Martins et al., 2010). The use of hydroxyl radicals to reduce ortho-P in pond effluent water (Feng et al, 2003) or in marine RAS (van Bussel et al., 2012a) might be a promising future technique. To determine if in-system treatment is necessary and to optimize efficiency of either in-system or end-of-the-pipe treatment maximal allowable concentrations of ortho-P for fish production have to be determined.

Several authors have reported negative effects of increased water re-use on growth and health of both fresh water and marine fish species (Davidson et al 2011, Deviller et al., 2005, Good et al. 2009, Martins et al. 2009b). Ortho-P is suggested as a possible cause, but due to multiple variables in these studies there is no causal conclusion possible. Acute toxicity of ortho-P has only been studied for two fresh water fish species revealing LC-50 values between 28 and 186 mg/l ortho-P (Ufodike & Onusiriuka, 1990, Wallen et al., 1957) but chronic toxicity to carp (*Cyprinus carpio*) larvae is much higher revealing toxicity effects at concentrations as low as 0.14 mg/l ortho-P (Toor et al., 1983). For

marine fish species data are lacking completely, however some data for marine bivalves exist. At levels as low as 0.04 mg/l ortho-P affects shell formation (Bernhardt et al., 1985), increased mortality in oyster larvae (*Crassostrea gigas*) is observed at 0.27 mg/l ortho-P (Kunigelisa & Wilbura, 1987). However 5 month exposure of abalone (*Haliotis iris*) to ortho-P concentrations of 0.3-2 mg/l ortho-P resulted in significant lower growth (James & Barr, 2012) in contrast juvenile oyster (*C. gigas*) exposed to levels up to 19 mg/l ortho-P for 96 hours did not show any effect on mortality or feed intake (Epifanio &Srna, 1975).

In fish ortho-phosphate can be uptake from the water by the gastro-intestinal tract, and the nephron has the capacity for both net renal P secretion and net P reabsorption in both marine and fresh water species (Hickman and Trump 1969). Although fish are thus able to excrete P, increased water ortho-phosphate levels leads to hyperphosphatemia (Urasa and Wendelaar Bonga, 1987) that generally causes hypocalcemia in mammals due to excessive precipitation of calcium phosphate in soft tissues which result in organ dysfunction and eventually in death (Suki et al. 1973).

Thus, the aim of the present study was to evaluate the chronic effect of ortho-P concentrations, common in commercial RAS or possible in future low-discharge RAS, on health and production performance of juvenile turbot (*P. maxima*). Results obtained from the toxicity study indicated an optimum for feed intake and growth at around 25 mg/l. To reveal these differences turbot were raised under control and optimum ortho-P concentration and fed a low-P diet. In addition to health and production performance, proximate body composition and nutrient retention was evaluated.

2. Material & Methods

2.1 System set up

Each of the 12 experimental RAS (total volume of 300 l) consisted of a rectangular rearing tank (275 l, 0.7 m² footprint), a moving bed biofilter (30 l, Kunstoff-Spranger, Plauen, Germany) and a protein skimmer (Model 1 AH 1100, Erwin Sander, Uetze-Eltze, Germany) using compressed air. Flow rate through the moving bed biofilter and protein skimmer was 700 l/h and 800 l/h respectively, resulting in a total flow rate of 1500 l/h, equal to 5 tank volumes per hour. Water temperature was not adjusted and thus dependent on room temperature which was kept constant at 18 ºC. A photoperiod of 14 hours light (06:00-20:00), 10 hours dark was provided. Prior to the exposure, RAS were filled with UV disinfected seawater (24 ppt salinity) and nominal concentrations of ortho-P were obtained by the addition of Na_2HPO_4 * 2 H_2O and KCl solution, providing a Na^+/K^+ weight-ratio of 46:1 to avoid imbalances of the cellular homeostasis. Weekly, fresh water was added in equal amounts to all RAS to compensate evaporation losses. Every second week, 200 l water per RAS were exchanged to minimize accumulation of metabolic NO_3-N and PO_4-P. During exposure, salinity, pH, temperature, dissolved

oxygen, total ammonia nitrogen, nitrite and nitrate were kept in the optimum range for turbot, for details see van Bussel et al. (2012b).

2.2 Experimental set-up

a) toxicity study

Juvenile turbot were obtained from Maximus A/S (Bedsted Thy, Denmark) and acclimatized to culturing systems at the facilities of the GMA (Büsum, Germany) for one week. A total of 120 turbot with an average wet weight (±SD) of 32 ± 3 g (range: 30-40 g) were randomly distributed to twelve separate RAS, providing a control at 0 mg/l ortho-P (control C) and three treatments at 25 mg/l ortho-P (low ortho-phosphate LP), 50 mg/l ortho-P (medium ortho-phosphate MP) and 75 mg/l ortho-P (high ortho-phosphate HP) with three replicates each. Average total fish weight per system (±SD) was 344 ± 7 g resulting in an initial density of 492 g/m². Fish were cultured for 56 days and fed *ad libitum* twice a day, 7 days per week with a commercial turbot feed (Aller 505 ex, Aller Aqua, Christiansfeld, Denmark). Uneaten pellets were netted out, counted and replaced half an hour after feeding in order to determine daily feed intake. During the experimental period groups were changed randomly between experimental units every second week to minimize tank effects.

b) growth study

A total of 120 turbot with an average wet weight (±SD) of 17 ± 0 g (range 16.5-17.5 g) were randomly distributed to six separate experimental RAS, providing a control at 0 mg/l ortho-P (control C2) and treatment at 25 mg/l ortho-P (low ortho-phosphate LP2) with three replicates each. Fish were cultured for 63 days and fed twice a day until apparent satiation. The diet was formulated to be low in total-P content and not limiting in essential amino acids (table 1) and was manufactured using a pellet press at a temperature of 60 °C to 4 mm pellets (L 14–175, AMANDUS KAHL, Reinbeck, Germany). Uneaten pellets were netted out, counted and replaced half an hour after feeding in order to determine daily feed intake. During the experimental period groups were changed every third week between tanks to minimize tank effects.

Table 1. Formulation of the experimental diets (g/kg dry matter) used in the growth experiment

Herring meal 68% CP [1]	110
Casein[2]	200
Free amino acid mix[3]	200
Wheat gluten [4]	153
Wheat starch [4]	140
Fish oil [1]	160
Vitamin / mineral mixture [5]	7
Titianium dioxide [6]	10
Calcium Carbonate[7]	20

[1] VFC GmbH, Cuxhaven, Germany
[2] Molkerei MEGGLE GmbH, Wasserburg, Germany
[3] Evonik industries, Hanau, Germany. Free amino acid content in g/200 g AS mix; L-lysine HCL 8.0, DL-methionin 11.5, L-cystin 2.6, L-threonine 14.4, L-glycin 74.5, L-valin 6.7, L-isoleucin 11.7, L-leucin 15.5, thyrosine 0.0, Phenylalanine 6.8, L-histidine HCL*H_2O 2.9, L-arginine HCL 43.2, L-tryptophan 2.5
[4] Kröner Stärke GmbH, Ibbenbüren, Germany
[5] AA-Mix 517158 & 508240, Vitfoss, Gråsten, Denmark
[6] Kronos Titan GmbH, Leverkusen, Germany
[7] Lehmann & Voss & Co, Hamburg, Germany

2.3 Water quality analysis

Ortho-P was measured spectrophotometrically using a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland) according to the protocols of Grasshoff et al (1983) and adapted to microplate format using the ascorbic acid method. Temperature, total ammonia nitrogen (TAN), NO_2-N, NO_3-N, pH, salinity, flow rate and dissolved oxygen (DO) were measured weekly. TAN, NO_2-N and NO_3-N were determined spectrophotometrically using a HACH-DR 2800 spectrophotometer and powder pillow detection kits (Hach-Lange GmbH, Berlin, Germany) based on the ammonia salicylate method, diazotization method and the cadmium reduction method, respectively. Temperature and pH were measured with a WTW 340i portable multiparameter instrument (WTW GmbH, Weilheim, Germany). Salinity was determined with a digital refractometer HI 96822 (HANNA Instruments, Woonsocket, USA) and dissolved oxygen with a Handy Polaris oxygen probe (Oxygard International A/S, Birkenrød, Denmark).

2.4 Sampling

During the toxicity experiment feed intake and mortality were recorded daily and group weight of fish was recorded every second week. Before final sampling fish were starved for 48 hours, at sampling

group weight as well as individual length and wet weight of fish were determined. Weight was recorded to the nearest 0.1 g, length was recorded to the nearest mm. Upon sampling three fish per tank were killed, liver and spleen were removed and weighed to the nearest 0.001 g. From three other fish blood samples were drawn from the caudal vein. For blood plasma-P analysis a subsample was immediately frozen on dry-ice and stored at -80° C. For total hemoglobin (total-Hb) a subsample was cooled on crushed ice and analyzed within 3 hours after sampling.
For the growth experiment the sampling protocol was equal, except that 5 fish were sampled for intestinal analysis and five fish for blood analysis.

2.5 Blood, fish and feed nutrient analysis

Total-Hb was determined by the cyanmethemoglobin method using a Hemoglobin FS reagent kit (DiaSys, International, Holzheim, Germany). Blood plasma-P was determined according to the protocol of Fiske and Subbarow (1925) and adapted to microplate format. All samples were measured in triplicates with a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland). Diets and homogenized fish body samples were analyzed for dry matter (DM), ash, crude protein, crude fat and energy ash, calcium, total phosphorus and phytic acid (diets only) according to EU guideline (EC/152/2009) (European Union, 2009). DM was determined after drying at 105 °C until weight remained stable and ash content after 4 h incineration at 550 °C with a combustion oven (P300; Nabertherm, Lilienthal, Germany). Crude protein content (N×6.25) was detected by the Kjeldahl method (InKjel 1225 M, WD 30; Behr, Düsseldorf, Germany), crude fat content after hydrolysis with hydrochloric acid followed by a petroleum ether extraction with a Soxleth extraction system (R 106 S; Behr). Gross energy was measured in a bomb calorimeter (C 200; IKA, Staufen, Germany). Nitrogen free extracts (NfE) were calculated by 100−(% crude protein+ % crude fat+ % ash).Total phosphorus, calcium was and phytic acid phosphorus was analyzed by ÖHMI Analytik GmbH (Magdeburg, Germany) according to guidelines of ISO 11885.

Table 2. Proximate composition (g/kg dry matter) of diets used in the toxicity study (Aller Aqua 505 EX) or growth study (experimental diet).

	Aller aqua 505 ex	Experimental diet
Moisture	100.0	81.7
Crude protein	555.5	627.2
Crude lipid	177.8	181.2
NFE + CF	177.8	133.4
Energy (MJ/kg DM)	22.87	24.55
Ash	100.0	58.2
Total P[1]	16.2	5.94
Phytic acid P[1]	1.66	0.44
Bone-P[2]		0.22
Organic-P[3]		5.27
Digestible-P [4]		4.58

1) Analysed by ÖHMI, Berlin, Germany
2) Bone-P= FM inclusion * ash content FM * bone-P content FM with bone-P =Ash (%) * 0.188 − 0.852 after Hua et al.,(2005)
3) Organic-P = TP –bone-P – phytic acid-P
4) digestible P = 0.68 bone-P + 0 phytate-P + 0.84 organic P − 0.03 $(\text{bone-P})^2$ after Hua & Bureau (2006)

2.6 Data analysis

Data are presented as mean ± standard deviation (SD) of n samples. Statistical analysis was performed using SPSS 17.0 (IBM inc, Armonk, USA). Data were tested for equal variances and for normality ($p<0.05$) using Levene's test and the Shapiro-Wilk test, respectively. Multiple comparisons, for the toxicity experiment, were carried out by the parametric Tukey's HSD, non-normality was only observed for splenic index (SI) so here or the non-parametric Dunnet's T3 test was used. Single comparisons, for the growth experiment, were carried using the students T-test, in case of non-normal distribution, which was observed for SI, GPRE and PRE, the Mann-Whitney test was used. Differences were considered significant at $p<0.05$.

3. Results

3.1 Toxicity experiment

All water quality parameters measured were in the range that we evaluated as optimum range for turbot, at all sampling times and did not vary between treatments (table 3). Average ortho-P level in C was 3.0±0.2 mg/l, for LP, MP and HP measured ortho-P concentrations (26.2±4.3, 51.8±2.1 and 81.8±6.2 mg/l) were slightly higher than the nominal concentrations (25, 50 and 75 mg /l) (table 3).

Table 3. Mean values (± SD) of water quality parameters. Letters indicate significant differences

Treatment	Ortho-P (mg/l)	DO (mg/l)	TAN (mg/l)	NO_2-N (mg/l)	NO_3-N (mg/l)	pH	Salinity (ppt)	Temp. (°C)
Control 1	3.0 ± 0.2a	8.4 ± 0.1	0.12 ± 0.17	0.18 ± 0.15	>10	7.56 ± 0.43	25 ± 0	18.2 ± 0.0
Low ortho-P	26.2 ± 4.3b	8.5 ± 0.1	0.15 ± 0.14	0.15 ± 0.10	>10	7.53 ± 0.47	25 ± 1	18.1 ± 0.0
Medium ortho-P	51.8 ± 2.1c	8.5 ± 0.1	0.14 ± 0.14	0.20 ± 0.18	>10	7.61 ± 0.44	25 ± 1	18.2 ± 0.1
High ortho-P	81.8 ± 6.2 d	8.4 ± 0.2	0.13 ± 0.10	0.19 ± 0.12	>10	7.49 ± 0.39	25 ± 1	18.2 ± 0.1
Control 2	1.6 ± 0.6a	7.7 ± 0.2	0.24 ± 0.21	0.27 ± 0.25	>10	8.02 ± 0.26	26 ± 1	17.8 ± 0.5
Low ortho-P 2	24.7 ± 4.0b	7.7 ± 0.2	0.21 ± 0.17	0.24 ± 0.21	>10	8.04 ± 0.26	26 ± 1	17.8 ± 0.5

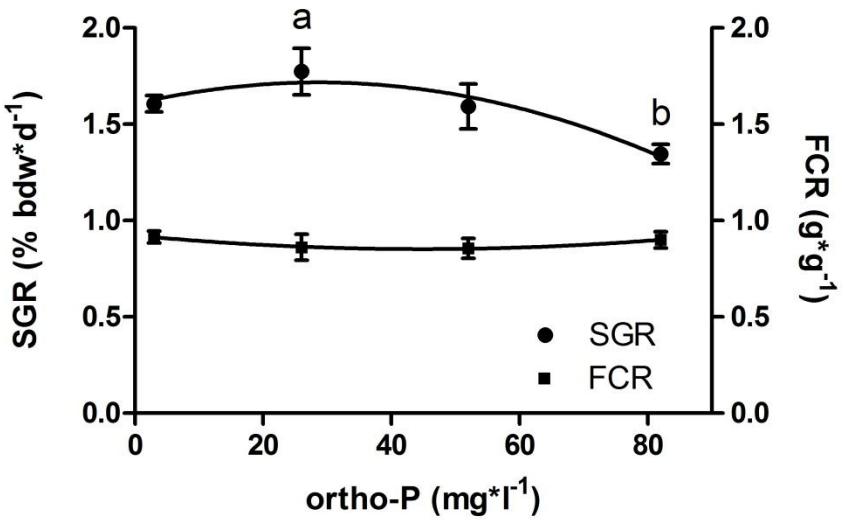
(ANOVA with Tukey HSD or Dunnet T3post hoc test, $p<0.05$, n= 24).

No significant effect of any of the ortho-P levels tested on health parameters was observed. Mortality was low with 1 dead fish per treatment in C, MP and HP and no mortality in LP treatment ($p>0.05$). Average relative spleen size (SI) varied between 0.09 and 0.11 and did not show significant differences between treatments ($p>0.05$) neither did average heptosomatic index (HSI) which varied between 1.3 and 1.4 ($p>0.05$). Average condition factor (CF) varied between 2.1 and 2.2 ($p>0.05$) and did not show significant differences. Average total hemoglobin levels in blood ranged between 3.2 and 3.5 g/dl were lowest for HP and highest for LP and showed significant differences between treatments ($p>0.05$). Blood plasma phosphorus levels varied between 6.7 mg/dl for MP treatment and 7.6 mg/dl for HP treatment but did not significantly vary (>0.05) (table 5).

Significant effects of ortho-phosphate on production performance were found. Ortho-phosphate significantly affected daily feed intake (DFI) ($p<0.01$). DFI was lowest for the HP treatment (1.15±0.02) and highest for the LP treatment (1.42±0.03), differences were found significant ($p<0.05$) between C (1.35± 0.02) and HP and between LP and HP. Ortho-P did not show an overall effect on feed conversion ratio (FCR) ($p>0.05$). Specific growth rate (SGR) showed an overall trend to dependent on ortho-P ($p=0.058$). SGR for the HP treatment (1.35±0.09) was lowest and SGR of the LP

treatment (1.77±0.21) was highest, SGR of C and MP were 1.61±0.07 respectively 1.59±0.21, but differences were only considered significant between LP and HP ($p<0.05$).

Figure 1. Mean values (± SD) of SGR and FCR of juvenile turbot after 58 days of exposure to different ortho-P concentrations. Different letters indicate significant differences (ANOVA with Tukey HSD, $p<0.05$).



3.2 Growth experiment using low-P diets

All water quality parameters measured were in the optimum range for turbot at all sampling times and did not vary between treatments (table 3). Ortho-P concentration in control was 1.6±0.6 mg/l and in the LP2 treatment 24.7±4.0 mg/l.

During the 63 days experimental period no mortalities occurred. HSI and SI of control groups were lower compared to LP2 treatments and did significantly vary (HSI: C2=1.41±0.31 LP=1.83±0.34, $p=0.002$ and SI: C2=0.08± 0.03, LP2=0.13 ± 0.06, $p= 0.044$). No significant effect of ortho-P was observed on condition factor ($p=0.922$), total-HB ($p=0.781$) or blood plasma P levels ($p=0.205$). DFI was higher for LP2 treatment compared to C2 but differences were not significant (LP2=0.95 ± 0.04, C2=1.02±0.05, $p=0.100$), however total feed intake was significantly higher for LP2 ($p=0.047$). FCR was comparable between treatments (C2=1.17±0.08, LP2=1.15±0.03, $p=0.671$). As a result of equal FCR and higher feed intake biomass gain was also significantly higher in the LP2 treatment ($p=0.022$). Ortho-P increased therefore the SGR with approximately 10%, resulting in significant higher growth rates (LP2= 0.92 ± 0.03, C2=0.83 ± 0.03, $p=0.014$).

Table 4. Mean values (± SD) of fish performance and health parameters. Different letters indicate significant differences (ANOVA with Tukey HSD or Dunnet T3post hoc test, $p<0.05$).Biomass gain = life biomass - start biomass; Daily feed intake = ((total feed intake / average biomass) / feeding days) * 100, FCR = (final total biomass - start biomass)/ total feed intake; SGR = (ln final weight – ln start weight) / 42*100; CF = (weight / length^3)*100; HSI = (liver weight/ final weight) *100; SI = (spleen weight / final weight) *100.

Parameter	N	Control (0 mg/l ortho-P)	Low ortho-P (25 mg/l ortho-P)	Medium ortho-P (50 mg/l ortho-P)	High ortho-P (75 mg/l ortho-P)
Initial weight (g)	30	32 ± 4	32 ± 4	32 ± 3	32 ± 3
Final weight (g)	29-30	75^{ab} ± 14	82^{a} ± 18	75^{ab} ± 17	65^{b} ± 14
Final length (cm)	29-30	15.2^{a} ± 1.0	15.5^{a} ± 1.1	15.1^{ab} ± 1.0	14.4^{b} ± 0.9
Initial biomass (g)	3	321 ± 1	320 ± 2	320 ± 1	321 ± 1
Biomass gain (g)	3	417^{ab} ± 33	503^{a} ± 89	422^{ab} ± 78	333^{b} ± 30
Total feed intake (g)	3	381^{ab} ± 17	430^{a} ± 44	360^{ab} ± 54	299^{b} ± 14
Daily feed intake (% day^{-1})	3	1.35^{a} ± 0.02	1.42^{a} ± 0.03	1.27^{ab} ± 0.10	1.15^{b} ± 0.02
Final mortality (%)	30	3.3 ± 4.7	0.0 ± 0.0	3.3 ± 4.7	3.3 ± 4.7
FCR (g*g^{-1})	3	0.92 ± 0.03	0.86 ± 0.07	0.86 ± 0.05	0.90 ± 0.04
SGR (%*d^{-1})	3	1.61^{ab} ± 0.07	1.77^{a} ± 0.21	1.59^{ab} ± 0.20	1.35^{b} ± 0.09
CF	29-30	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.2	2.1 ± 0.1
HSI (%)	9	1.3 ± 0.2	1.3 ± 0.1	1.4 ± 0.3	1.3 ± 0.3
SI (%)	9	0.10 ± 0.05	0.11 ± 0.05	0.09 ± 0.05	0.10 ± 0.05
Total HB (g*dl^{-1})	9	3.4 ± 0.3	3.5 ± 0.5	3.3 ± 0.5	3.2 ± 0.3
Blood plasma-P (mg*dl^{-1})	9	7.5 ± 1.6	7.6 ± 1.7	6.7 ± 1.5	7.6 ± 1.1

*Based on number of feeding days.

Table 5. Mean values (± SD) of fish performance and health parameters. Different letters indicate significant differences (independent T-test, $p<0.05$). Biomass gain = life biomass - start biomass; Daily feed intake = ((total feed intake / average biomass) / feeding days) * 100, FCR = (final total biomass - start biomass)/ total feed intake; SGR = (ln final weight – ln start weight) / 42*100; CF = (weight / length3)*100; HSI = (liver weight/ final weight) *100; SI = (spleen weight / final weight) *100.

Parameter	N per treatment	Control 2 (0 mg/l ortho-P)	Low ortho-P 2 (25 mg/l ortho-P)	p-value (2-tailed)
Initial weight (g)	60	17.0 ± 0.0	17.0 ± 0.0	0.981
Final weight (g)	60	27.6 ± 4.4	29.3 ± 4.7	0.050
Final length (cm)	60	11.3 ± 0.5	11.5 ± 0.5	0.017
Biomass gain (g)	3	213 ± 10	245 ± 12	0.022
Total feed intake (g)	3	248 ± 6	281 ± 27	0.047
Daily feed intake (% day^{-1})	3	0.95 ± 0.04	1.02 ± 0.05	0.100
FCR ($g*g^{-1}$)	3	1.17 ± 0.08	1.15 ± 0.03	0.671
SGR ($\%*d^{-1}$)	3	0.83 ± 0.03	0.92 ± 0.03	0.014
CF	60	1.91 ± 0.13	1.91 ± 0.15	0.922
HSI (%)	15	1.41 ± 0.31	1.83 ± 0.34	0.002
SI (%)	15	0.08 ±0.03	0.13 ± 0.06	0.044
Total HB ($g*dl^{-1}$)	15	3.0 ± 0.4	3.0 ± 0.5	0.781
Blood plasma-P ($g*dl^{-1}$)	15	5.1 ± 0.7	5.5 ± 0.9	0.205

*Based on number of feeding days.

Whole body dry matter content, crude protein content, crude ash content, calcium content and phosphorus content did not show significant differences between treatments ($p>0.05$) but crude lipid showed a trend ($p<0.1$) to be higher in the control group (table 6).

Table 6. Body composition (mean ± sd) of original substance in g/kg or in MJ/kg for gross energy of turbot fed with the experimental diet and raised in C2 or LP2 treatment.

Proximate body composition	Initial	Control 2 (0 mg/l ortho-P)	Low ortho-P 2 (25 mg/l ortho-P)	p-value (2-tailed)
Dry matter	231	220 ± 5	218 ± 3	0.458
Crude protein	139	146 ± 1	149 ± 4	0.262
Crude lipid	40	34 ± 5	27 ± 2	0.088
Energy	4.7	4.7 ± 0.2	4.5 ± 0.1	0.234
Ash	38	33 ± 2	34 ± 1	0.503
P	6.4	5.7 ± 0.4	5.9 ± 0.1	0.827
Ca	9.0	7.6 ± 0.7	8.3 ± 0.5	0.248
Ca/P	1.41	1.35 ± 0.03	1.41 ± 0.11	0.507

Available phosphorus retention was higher for LP compared to control but differences were not considered significant (C2=82.7±18.0, LP2=97.7±4.2, $p=0.252$). Crude protein retention showed a trend to increase with high ortho-P (C2=23.5± 0.7, LP2=24.7 ± 1.6, $p=0.099$), ash retention and energy retention did not significantly vary ($p>0.05$) (table 7).

Table 7. Nutrient retention efficiency (mean ± sd) in % of nutrient or energy intake of turbot fed the experimental diet and raised in C2 or LP2 treatment.

Retention efficiency	Control 2 (0 mg/l ortho-P)	Low ortho-P 2 (25 mg/l ortho-P)	p-value (2-tailed)
GPRE [1] (%)	23.5± 0.7	24.7 ± 1.6	0.099
GERE [2] (%)	17.6 ± 2.2	16.2 ± 1.5	0.369
ARE [3] (%)	47.6 ± 5.9	52.3 ± 3.3	0.293
PRE [4] (%)	69.2 ± 15.2	81.6 ± 5.9	0.268
aPRE [5] (%)	82.7 ± 18.0	97.7 ± 4.2	0.252

1 Gross protein retention efficiency ((%final body protein content × final body weight) − (%initial body protein content × initial body weight) / crude protein intake × 100).
2 Gross energy retention efficiency ((%final body energy × final body weight) − (%initial body energy × initial body weight) / gross energy intake × 100).
3 Crude ash retention efficiency ((%final body crude ash × final body weight) − (%initial body crude ash × initial body weight) / crude ash intake × 100).
4 Total phosphorus retention efficiency ((%final body phosphorus × final body weight) − (%initial body phosphorus × initial body weight) / total phosphorus intake × 100).
5 Available phosphorus retention efficiency ((%final body phosphorus × final body weight) − (%initial body phosphorus × initial body weight) / available phosphorus intake × 100).

4. Discussion

During both experimental periods all measured water quality parameters were in the optimal range as described in table 3. No significant differences within experimental treatments, with the exception of ortho-P, were observed for both experiments. Therefore effects observed are thought to be monofactorial, resulting from inorganic phosphorus treatments. Average ortho-P levels for control treatments C was 3.0 mg/l C and for C2 1.6 mg/l, these levels are comparable to average ortho-phosphate concentrations in commercial partial RAS systems with daily exchange rates of 50% (≈ 4000 l/kg feed) (Aubin et al., 2006). Ortho-P concentrations for LP1 and LP2 treatments were 25 respectively 26 mg/l and are comparable to ortho-P levels reached in experimental RAS with water exchange rates of 30-120 l/kg feed (Martins et al. 2009a, Schneider et al. 2006). Concentrations of ortho-P obtained in MP and HP treatments (52 mg/l respectively 82 mg/l) are not likely to occur in commercial turbot RAS yet, but concentrations above 40 mg/l are observed in fresh water RAS (Schneider et al. 2006), and can become realistic in turbot RAS when water exchange rates are further minimized.

4.1 Toxicity of ortho-phoshate

To our knowledge this is the first research studying the chronic effect of elevated ortho-phosphate concentrations on health and or production performance of marine fish species, therefore literature that is directly comparable is lacking. Epifanio and Srna (1975) determined LC-50 for two oyster species (*Mercenaria mercenaria* and *Crassostrea virginica*), however exposure to 3-19 mg/l ortho-P, added as sodium-phosphate, did not result in any mortality and concentrations of 3-25 mg/l ortho-P did not show any effect on feed intake A 5 month exposure of abalone *(Haliotis iris*) to 0.3-3.5 mg/l ortho-P did not results in higher mortalities compared to a control, but concentrations above 0.3 mg/l significantly affected shell growth and weight gain (James and Barr, 2012). For fresh water fish species LC-50 values for sodium phosphate and tri-sodium phosphate exists. Reported 96 h LC-50 values of tri-sodium phosphate for African catfish (*Clarias garipinus*) is 61 mg/l ortho-P (Ufodike & Onusiriuka, 1990), 96 h LC-50 values for the western mosquito fish (*Gambusia affinis*) is 186 mg P/l added as sodium phosphate and 28.5 mg P/l as tri-sodium phosphate (Wallen et al., 1957). The higher toxicity of tri-sodium phosphate compared to sodium phosphate is probably the result of sodium presence and not of phosphate toxicity. In contrast to marine species, which are hypotonic compared to their environment and therefore constantly drinking water and enriching itself with ions, fresh water species drink occasionally and have to absorb the majority of ions through the skin or the gills (Kaushik, 2001). Comparison between marine and fresh water species might therefore be inappropriate and reported LC-50 values are underestimated due to sodium toxicity.

After mortality being the most severe toxicity indicator, physiological changes might be an indicator for sub-lethal exposure of fish to toxins. Changes in the HSI of flatfish are an indicator of chronic exposure to water borne toxins (Lye et al., 1997, Pereira et al., 1993). During the present toxicity study the average HSI varied between 1.3 and 1.4 but no significant differences between treatments were found. An increased SI in flatfish is another indicator of exposure to toxins (Hutchinson and Manning, 1996, van Bussel et al., 2012b) but during the present toxicity study no significant increase in SI was observed. In addition average plasma-P levels ranged between 6.7 and 7.6 mg P/dl and did not vary between treatments. Values are comparable to plasma P values of 8.1 mg P/dl observed for turbot (Payan et al. 1997) and 6.4-8.2 mg P/dl observed for Japanese flounder (*Paralichthys olivaceus*) (Kikuchi, 1999) suggesting that values measured during our study are within the normal range. Urasa and Wendelaar Bonga (1987) measured blood plasma phosphate values of tilapia (*Oreochromis mossambicus)* by elevated orthophosphate concentrations and found only significant elevations of P in blood when water P concentrations exceeded plasma P concentrations. During the present toxicity study average water ortho-phosphate levels for C, LP and MP treatments were below average blood plasma phosphate concentrations, plasma-P was thus hypertonic compared to the environment. In the HP treatment average water ortho-P concentration was 81.8 ± 6.2 mg/l, thus slightly exceeding blood plasma ortho-P concentrations, but this was not reflected in blood-P levels.

In the present toxicity study the daily feed intake of the HP treatment was significantly lower compared to the daily feed intake of C but feed conversion ratio was not significantly affected by ortho-P concentrations. This is in line with studies for chronic effects of ammonia and nitrate to turbot were feed intake was the most sensitive parameter leading to reduced growth, whereas feed conversion ratio was unchanged at the same concentrations (Person-Le Ruyet et al., 1997, van Bussel et al., 2012b). The reduced feed intake might be a result of osmotic stress (Boeuf & Payan 2001) caused by hypertonic ortho-P concentrations in the water. Specific growth rates of C1 did not significantly vary compared to other treatments, however SGR of LP was found significantly higher compared to HP.

We conclude that ortho-P does not affect health of turbot, even at concentrations as high as 82 mg/l, normal growth and feed intake was observed between 3 and 52 mg/l and concentrations should not exceed this range. To obtain maximal feed intake phosphate concentrations managed at concentrations of ± 25 mg/l.

4.2 Effect of ortho-phosphate at optimum concentrations

Using non-linear regression the ortho-P concentration for highest feed intake and growth during the toxicity experiment was found at around 27 mg/l (figure 1). The higher feed intake, by equal feed efficiency resulting thus in higher growth was also observed during the second experiments were turbot were fed the low-P diets, and results of both experiments were thus in line. However in the growth experiment differences were found in liver and spleen size. As discussed before an increased

HSI and SI might be indications of water borne toxicity but these effects were not observed during the toxicity study with much higher concentrations. The HSI can also indicate the nutritional state of turbot, commonly increasing with improving nutritional state (Arfsten et al., 2010). Thus the increased HSI and SI are thus rather an indirect effect of higher feed intake, than a direct effect of ortho-P.

Improved water quality can lead to increased feed intake and might be a possible explanation for the increased feed intake and higher growth performance. Addition of ortho-phosphate is increases pH and total alkalinity but during the present study no significant differences in pH were observed. Ortho-phosphate is also known to reduce nitrite toxicity in fresh water (Russo et al, 1981), however considering the high salinity and low nitrite concentrations this is not likely to explain differences in feed intake. Earl & Whiteman (2010) reported that frog tadpoles raised for 15 days in control treatment (0 mg/l ortho-P) and a high phosphate treatment (200 mg/l ortho-P) had significant lower condition factor and bodymass compared to a treatment of 100 mg/l ortho-P. Furthermore, the presence of fungus (*Saprolegnia sp*.) was also lower in the 10-200 mg/l ortho-P treatments compared to the control but in contrast the high salinity and the absence of any external health differences in feed intake during the present study cannot be explained with the previous study. Schram et al. (2009) reported a significant higher motivation to eat in medium sized turbot (500-700 g) raised in RAS (1.4 and 5.0 m^3/kg feed) compared to flow through systems (70 m^3/kg feed), but no significant effect on growth was found. Martins et al. (2010) reported that small tilapia were significantly more motivated to eat and grew significantly faster in RAS with water renewal rates of 70 l/kg feed, compared to RAS with water exchange rates of 1500 l/kg feed. Increased water re-use, and thus a higher accumulation of in-organics including ortho-P, is thus known to positively affect the feeding motivation of fish. This enhanced motivation to eat might be an explanation for the higher feed intake observed in the present study.

Another mechanism that might affect feed intake and growth of turbot is the uptake and utilization of ortho-P from water. Uptake of the minerals Ca^+, Mg^+, Na^+ and Cl^- by rainbow trout (*Oncorhynchus mykiss*) from saline water, and its contribution to nutrition is reported by several authors and is reviewed by Timmons and Ebeling (2010). In fresh water tilapia species (*Oreochromis. sp*.) can uptake of ortho-phosphate from the water through the gills and the gastro intestinal tract (Al-Kholy et al., 1970, Urasa and Wendelaar Bonga 1987) and can utilize this for growth when fed high-P and low-P diets in restricted amounts, leading to higher feed utilization efficiencies (Eding et al., 2012). Uptake and utilization of ortho-phosphate seems to be less in marine species compared to fresh water species. For example Lall & Bishop (1979) cultured rainbow trout (*O. mykiss*) in both freshwater and seawater for 12 weeks fed with the same diet. Fish raised in freshwater had higher Ca and P content in the body than those reared in seawater. Similar observations have been made for Atlantic salmon (*Salmo salar*) fed with same diets but raised in fresh or seawater (Shearer, 1993). Indirect evidence of ortho-P uptake by marine fish using retentions experiments fed deficient P diets is reported by several authors and some fish react by increasing their feed intake, however this seem to be species dependent (Cowey,

1995, Dias, 2005, Pimentel-Rodrigues & Oliva-Teles, 2001, Laining, et al. 2012, Oliva-Teles & Pimentel-Rodrigues, 2004, Roy & Lall, 2003, Uyan et al., 2007). In all studies an increased FCR by phosphorus deficient diets was observed. During the growth study the FCR for both treatments was around 1.16 and is comparable to FCR of 1.0-1.2 reported in studies using low fishmeal (80-150 g / kg feed) with a phosphorus content of 7.0- 18.8 g P /kg diet (Kroeckel et al., 2012, Nagel et al, 2012, Slawski et al., 2011). During the toxicity study available phosphorus content of the diet was 14.5 g/kg, during the growth trial using low-P diets available phosphorus content was 5.5 g/kg diet and estimated digestible P was 4.6 g/kg diet. Dietary requirements for turbot (*P. maxima*) are unknown, but dietary requirement for juvenile Japanese flounder (*P. olivaceus*) range between 4.5 and 5.1 g TP / kg diet (Wang et al., 2005, Choi et al., 2005). During the present study available phosphorus retention was above 80% in the C2 treatment and almost reached 100% in the LP2 treatment. Typical phosphorus deficiency symptoms such as deformities of vertebrae and skull and decrease of ash content were not observed, but turbot raised in the control group showed a trend to have a higher whole body crude lipid content which is also considered to be a typical phosphorus deficiency symptom (Kaushik, 2001). This might be an indication that turbot can prevail dietary phosphorus deficiency effects by uptake and utilization of water borne P, and regulate feed intake accordingly. Although ortho-P did not improve FCR, crude protein retention showed a trend to increase with increased ortho-P, an effect which was also observed for tilapia (Eding et al., 2012), which can be another indication that turbot are able to use ortho-P for metabolism and regulate feed intake accordingly.

Considering the absence of negative effects of ortho-P on the health of turbot, the accumulation of ortho-P is not a risk for optimal production in intensive turbot RAS. The positive effects of ortho-P levels at around 25 mg/l on feed intake, protein utilization and growth of turbot during the present study, make the accumulation in RAS even desirable. Therefore, if denitrification is used, water exchange should be managed at rates that maintain ortho-P levels constant at 25 mg/l in order to improve production performance of turbot. Studies on phosphorus requirements of turbot in flow through systems and intensive RAS are recommended to determine phosphorus requirements of turbot in order to reduce eutrophication effects caused by turbot RAS production.

5. Conclusions

Based on the given results we can conclude that ortho-P does not negatively affect health of turbot in the tested range of 3-82 mg/l and concentrations between 3 and 52 mg/l do not negatively affect production performance. The accumulation of ortho-phosphate is thus not likely to affect health of turbot in RAS. Concentrations of around 25 mg/l do increase feed intake and growth of turbot and water exchange rates or phosphate removal should be managed to keep ortho-P levels around 25 mg/l.

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Chapter 3:

Feed induced aquatic accumulation of metals (Fe, Zn, Cu, Co, Mn) in RAS changes body composition but not growth performance or health of juvenile turbot (*Psetta maxima*).

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Abstract

Developments in RAS production are characterized by intensification of production, resulting in decreased water use per volume of fish produced. As a result metabolites, including the main dietary metals Fe, Zn, Cu, Co and Mn, accumulate in the culture water. Effect of such sub-lethal concentrations of these metals on growth and health of marine fish species is still unknown

Therefore lab scale RAS were filled with seawater and a mixture of Fe, Zn, Cu, Co and Mn, representing different culture intensities. The amounts added simulated water exchange rates of 1000, 330, 100, 33 and 10 l/kg feed applied. Metal concentrations in water were monitored over time. During exposure 90 juvenile turbot (*Psetta maxima*) (16.8±0.1 g) were randomly divided over the 5 RAS, fed *ad libitum* with a commercial diet and exposed for 63 days.

Water concentrations of Zn, Cu and Co were stable over time. Fe showed a quick precipitation in RAS water, $t_{1/2}$ was 1.4±0.7 d. Mn showed a slower breakdown in RAS, $t_{1/2}$ was 6.3±0.4 d.

Production performance, assed as growth, feed intake and feed conversion was not significantly affected by treatment ($p>0.05$). Health status assessed as mortality, relative liver size, relative spleen size and total hemoglobin content was neither significant affected by treatment ($p>0.05$).

Amounts of metals in RAS water were reflected in turbot body composition. Correlations between exposure and body composition were found significant ($p<0.05$) for Zn, Mn and Co but not for Fe and Cu. Dry matter content significantly decreased with increased metal content ($p<0.05$), in contrast crude ash content not.

Although body composition is slightly changed by metal exposure, the absence of effects on health and production performance suggests that metal accumulation is not a limiting factor for turbot grow-out in RAS yet. However by intensification of marine RAS production zinc accumulation can become potentially toxic and should be monitored carefully.

1. Introduction

Fish production in land based recirculating aquaculture systems (RAS) is steadily growing since decades. Due to increased costs for make-up water and waste-water discharge and separation of waste streams there is a trend in RAS towards intensification of water re-use by technical improvements of water treatment (Blancheton et al., 2007). Especially in culture systems using high density of fish per water volume, such as race-way systems for shrimp or flatfish culture, feed originating substances do accumulate to high levels.

In the nutrition of turbot (*Psetta maxima*) much effort was given over the last years to reduce the inclusion of fishmeal protein by alternative animal or plant based proteins (Burel et al., 2000, Nagel et al., 2012, Kroeckel et al., 2012). The ash fraction of these alternative protein sources is considerably lower in available trace-minerals like iron, zinc, copper, cobalt, manganese, nickel, chromium, selenium (Hertrampf and Piedad-Pascual, 2000). All these trace minerals are essential chemical elements, involved in the normal metabolism of fish (Wanatabe et al., 1997). Thus by replacing fish proteins sources by plant based protein sources, mineral deficiencies might occur resulting in decreased growth and poorer health status (Kaushik, 1999). To overcome these deficiencies, trace-elements are added to commercial diets in the form of pre-mixes. Premixes for marine fish species included zinc sulfate, copper sulfate, cobalt sulfate and manganese sulfate (Kaushik, 1999). Furthermore contamination of raw materials or make up water can be a source of minerals. Therefore increasing re-use of water leads to increased accumulation of like Arsenic, Barium, Boron, Copper, Iron, Lithium, Manganese, Nickel, Strontium and Zinc in fresh water RAS (Martins et al., 2009, Martins et al., 2011, Davidson et al., 2009, Davidson et al. 2011).

Several studies have shown that increased re-use of water in RAS leads to elevated concentrations of metals in liver or muscle tissue of fish. Increased levels of Arsenic, Lead, Chromium and Manganese were found in muscle or liver of Nile tilapia (*Oreochromis niloticus*) raised in fresh water (Martins et al. 2011) and increased levels of chromium, manganese, cobalt, nickel, copper, arsenic, thallium, cadmium were observed in European seabass (*Dicentrarchus labrax*) raised marine water (Deviller et al., 2005). It was suggested that the accumulation of metals was a likely cause of higher mortality, deformation and lower hatching success for carp (*Cyprinus carpio*) larvae (Martins et al., 2009), increased mortality and higher occurrence of deformities in rainbow trout (*Oncorhynchus mykiss*) (Davidson et al., 2009) and 15 % growth reduction of European seabass (*D. labrax*) (Deviller et al., 2005).

Although all these authors suggest a linkage between metal concentrations and fish health or growth no causal conclusion can be drawn from these studies because intensification of water re-use changes multiple parameters, but sub-lethal effects of these metals, relevant for concentrations observed in RAS, are unknown. Therefore maximal allowable concentrations of metals in the culture water have to be determined to manage water renewal rates and to optimize treatment techniques.

Thus in the present study, metal concentrations were artificially increased by adding the above mentioned metals in the sulphate form, to obtain a mono-variate study. This study was limited to the 5 main essential dietary metals; iron, zinc, copper, cobalt and manganese. Furthermore metal concentrations in a 45 m³ turbot RAS and in a commercial turbot diet were measured to link experimental concentrations with praxis data.

Table 1. Nutrient requirement of marine fish species (in mg/kg diet) and standard used to simulate accumulation by different water exchange rates.

Fish species	Iron	Zinc	Copper	Cobalt	Manganese
Atlantic salmon [abe]	30 – 100	37 – 67	5 – 6		10-20
Red drum [ab]		20 - 25			
Red sea bream [d]	150 – 199				
Grouper [e]			4 -6		
Miscellaneous [c]	30 – 170	15 – 40	1 – 5	0.05 – 1	2 – 20
Used as standard	**100**	**40**	**5**	**1**	**20**

[a] Kaushik 1999

[b] Lall 2002

[c] Wannatabe et al., 1997

[d] Sakamoto and Yone 1978

[e] NRC, 1993

2. Material & Methods

2.1 Experimental setup

The experimental set-up and data analysis were carried out in accordance to OECD TG215; Fish, Juvenile growth test (OECD/OCDE, 2000). 5 different metal concentrations, in a log-linear design were randomly divided over the 5 RAS. Because fish size was very homogeneous (16.8 ± 0.1 gram) pseudo-specific growth rates could be calculated, additionally data were analyzed by regression (concentration-response modeling) (OECD/OCDE, 2000). Juvenile turbot were obtained from Maximus A/S (Bedsted Thy, Denmark) and acclimatized to culturing systems at the facilities of the GMA (Büsum, Germany) for one week prior to the nine week experimental exposure. For a detailed system and management description see van Bussel et al. (2012). Briefly 5 RAS, consisting of a mechanical particle filter, moving bed biofilm reactor and a protein skimmer using compressed air, were stocked with 18 juvenile turbot each. At the start and after every three weeks, RAS were filled with 300 l disinfected sea water (26 ppt) and a mixture of $ZnSO_4$*7 H_2O, $MnSO_4$* H_2O, $CoSO_4$*7 H_2O, $CuSO_4$* 5 H_2O and $FeSO_4$* 7 H_2O was added to obtain desired metal concentrations. Sodium

sulfate was added to all tanks to obtain equal amounts of sulfate in all tanks. Test solution was exchanged every three weeks to minimize accumulation of metabolics. Amounts of metals added were based on assumptions of commercial production system:

- amount of minerals in a standard marine diet (table 1),
- a stocking density of 50 kg/m^3
- an average feed intake (over the whole production cycle) of 1% bdw per day,
- cumulative feed burden (CFB) by water exchange rates of 1000, 330, 100, 33 and 10 l/kg feed.

$$CFB = \text{Feed added (g)} / V_{total}\ (l) \wedge (-V/V_{total}) \quad (1)$$

where "feed added" is the simulated amount of feed added per tank in g, "V_{total}" is the total culture volume and "–V" is the amount of water replaced.

With the standard mineral content (table 1) and the calculated CFB the potential amount of metals that accumulate can be calculated and is given in table 2. Treatments were named after exchange rates; 1000 liters per kg feed applied (ER-1000), 330 liters per kg feed applied (ER-330), 100 liters per kg feed applied (ER-100), 33 liters per kg feed applied (ER-33) and 10 liter per kg feed applied (ER-10). Temperature was not controlled internally and thus controlled by room temperature. Fish were fed *ad libitum* twice a day, 7 days per week with a commercial turbot feed (56% CP, 18% CL, 1.6% TP, on dry weight basis). Uneaten pellets were netted out and counted within one hour after feeding.
To study the risk of accumulation concentrations were added in a log-linear design and metal concentrations in the water were measured regularly to estimate half-life times, and thus asses the risk of accumulation. Growth, health parameters and body composition were evaluated after 9 weeks of exposure.

Table 2. Nominal concentrations of iron, zinc, copper, cobalt and manganese, all added in sulfate form to obtain different treatment. Treatments are named after simulated water exchange rates (ER) in l make up water/kg feed applied. Metals are expresses in mg/l.

Treatment	ER-1000	ER-330	ER-100	ER-33	ER-10
CFB	0.5	1.0	4.5	15.1	49.5
Fe	0.05	0.10	0.45	1.51	4.95
Zn	0.02	0.04	0.18	0.58	1.98
Cu	0.003	0.005	0.023	0.073	0.248
Co	0.0005	0.0010	0.0045	0.0145	0.0495
Mn	0.01	0.02	0.09	0.29	0.99

2.2 Water quality, fish and feed nutrient content analysis

Temperature, total ammonia nitrogen (TAN), NO_2-N, NO_3-N, pH, salinity, flow rate and dissolved oxygen (DO) were measured twice a week. TAN, NO_2-N and NO_3-N were determined spectrophotometrically using a HACH-DR 2800 spectrophotometer (Hach-Lange GmbH, Berlin, Germany) and powder pillow detection kits based on the ammonia salicylate method, diazotization method and the cadmium reduction method, respectively (Hach-Lange GmbH, Berlin, Germany). Temperature and pH were measured with a WTW 340i portable multiparameter instrument (WTW GmbH, Weilheim, Germany). Salinity was determined with a digital refractometer HI 96822 (HANNA Instruments, Woonsocket, USA), dissolved oxygen with a Handy Polaris oxygen probe (Oxygard International A/S, Birkenrød, Denmark). Dissolved metals were analyzed at nine sampling dates in duplicate using inductively coupled plasma mass spectrometry (ICP-MS) method (TeLa, Technische Lebensmittel- und Umweltanalytik GmbH, Bremerhafen, Germany) in 100 ml samples. Detection limits were <0.01 mg/l for Fe, <0.01 mg/l for Zn, <0.01 mg/l for Cu, < 0.002 mg/l for Co and <0,004 mg/l for Mn. Alkalinity was determined according to Grashoff et al. (1999).
From each tank 9 fish were freeze dried and grinded. Diets and homogenized fish body samples were analyzed for dry matter (DM), ash and metals. DM was determined after drying at 105 °C until weight remained stable and ash content after 4 h incineration at 550 °C with a combustion oven (P300; Nabertherm, Lilienthal, Germany). For metal analysis 50 gram of homogenized freeze dried sample were digested using hydrofluoric acid, nitric acid and hydrogen peroxide catalyzed by microwave heating. Digested samples were than analyzed using ICP-MSmethod (TeLa, Technische Lebensmittel- und Umweltanalytik GmbH, Bremerhafen, Germany). Metal content was expressed as mg metal per kg dry matter.

2.3 Sampling

During the experimental period feed intake and mortality were recorded daily, group weight weekly. After 63 days of exposure group weight as well as individual length and wet weight of fish were determined. Weight was recorded to the nearest 0.1 g, length was recorded to the nearest mm. Upon sampling nine fish per tank were killed, liver and spleen were removed and weighed to the nearest 0.001 g. The other nine fish were killed and used for proximate body composition analysis.

2.5 Data analysis

Toxicity experiments and data analysis were carried out in accordance to OECD TG215; Fish, Juvenile growth test (OECD/OCDE, 2000) with the exception that turbot (*P. maxima*) was used instead of one of the recommended fresh water species and that the experiment lasted 63 days instead of 28 days. Data are presented as mean ± standard deviation (SD) of n samples. Half life time of metal in water was calculated as:

$$T_{1/2} = (t*\log(2)) / \log(C_t / C_{t+x}) \quad (2)$$

Were C_t is initial concentration, C_{t+x} is concentration at time=x and t is time between t and tx (d). Exposure was calculated as the sum of the daily average concentrations of metals. Fish date were analyzed in accordance to 'OECD TG215; Fish, Juvenile growth test' (OECD/OCDE, 2000) using an ANOVA and ordinary linear regression. Statistical analysis was performed using SPSS 17.0 (IBM inc, Armonk, USA). Data were tested for normality and for equal variances ($p<0.05$) using Levene's test and the Kolgomorov-Smirnov test, respectively. Multiple comparisons were carried out by the parametric Tukey's HSD or the non-parametric Dunnet's T3 test. Differences were considered significant at $p<0.05$. For growth parameters, feed utilization and body metals concentration data was analyzed using or Grahpad Prism 5.00 (GraphPad Software, San Diego, USA). Data were analyzed using ordinary linear regression and slopes were compared using an F-test. Differences were considered significant at $p<0.05$.

3. Results

3.1 Water quality parameters

Water temperature, salinity, oxygen, pH, dissolved nitrogen (TAN, NO_2, NO_3) and ortho-phosphate did not show significant differences between treatments (table 3). For metals significant differences between treatments were only observed for Zn, Cu and Co (table 3). Due to high Zn and Co levels in the initial seawater, Zn and Co concentrations in ER-1000, ER-333 and ER-100 treatments were higher than nominal values, however increase of Zn and Co were comparable to nominal added amounts. Cu levels in the ER-1000 and ER-330 were higher as nominal values due to the initial seawater matrix, whereas ER-33 and ER-10 levels were less than 10 % of the nominal concentration.
For Fe and Mn no significant differences between averages were observed because the breakdown over time resulted in high standard deviation of the average concentration (table 3 and figure 1).Fe could only be detected in the first two days after addition for the two highest treatments (ER10 and ER 33) thereafter values were at or below detection limit (0.01 mg/l). Observed half-life time of Fe for measured concentration in ER10 was 1.4 ± 0.7 days. Mn was above detection limits at all sampling

events for ER10 but not for other treatments. Observed half-life time for Mn in ER10 treatment was 6.3±0.4 days.

Table 3. Water quality parameters during the 63 day experimental period. Different superscripts in the same row denote significant differences between treatments (Dunnet-T3, n=9-15, p<0.05).

Parameter	N	ER-1000	ER-330	ER-100	ER-33	ER-10
Temp. (C)	10	17.8 ± 0.5	17.8 ± 0.4	17.7 ± 0.5	17.9 ± 0.5	17.8 ± 0.4
Sal. (ppt)	16	26 ± 1	26 ± 1	26 ± 1	26 ± 1	26 ± 1
O_2 (mg/l)	9	8.1 ± 0.6	8.1 ± 0.6	8.2 ± 0.6	8.1 ± 0.6	8.1 ± 0.6
pH	9	7.2 ± 0.3	7.2 ± 0.3	7.2 ± 0.3	7.2 ± 0.3	7.2 ± 0.3
TAN (mg/l)	9	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Alkal. (mM/l)	9	1.6 ± 0.3	1.8 ± 0.2	1.7 ± 0.2	1.5 ± 0.3	1.5 ± 0.2
NO_2-N (mg/l)	10	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2
NO_3-N (mg/l)	9	<10	<10	<10	<10	<10
PO_4-P (mg/l)	9	<1	<1	<1	<1	<1
Fe (mg/l)	15	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.02	0.01 ± 0.02	0.02 ± 0.02
Zn (mg/l)	12	0.23 ± 0.05^{a}	0.28 ± 0.06^{ab}	0.48 ± 0.17^{c}	0.72 ± 0.26^{cd}	1.26 ± 0.45^{d}
Cu (mg/l)	9	0.01 ± 0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}	0.05 ± 0.00^{b}	0.09 ± 0.02^{c}
Co (µg/l)	9	6 ± 3^{a}	5 ± 4^{a}	15 ± 5^{b}	22 ± 17^{abc}	53 ± 29^{c}
Mn (µg/l)	9	0 ± 0	3 ± 3	9 ± 7	40 ± 47	422 ± 331

Metal concentration in disinfected seawater; Fe < 0.01 mg/l, Zn 0.20 mg/l, Cu = 0.01 mg/l, Co 4 µg/l, Mn < 2 µg/l.

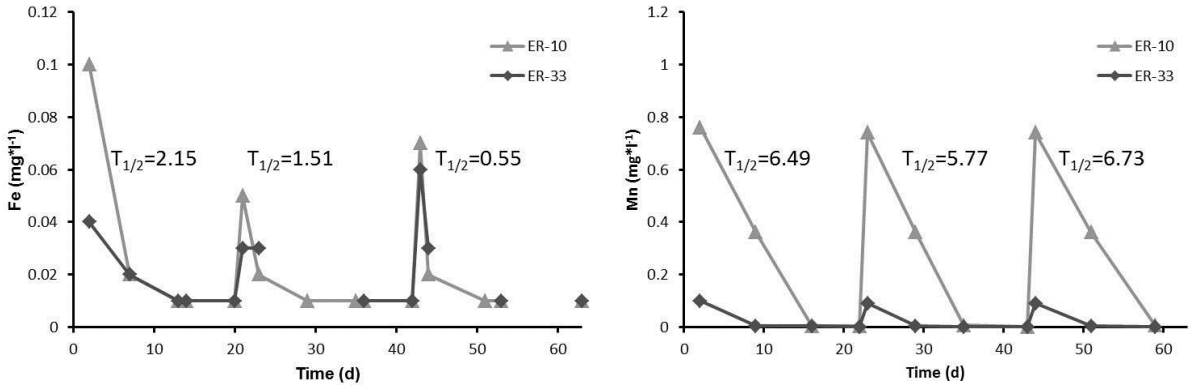

Figure 1. Iron and manganese concentrations of ER-10 and ER-33 during the experimental period and half life time $T_{1/2}$ (d) in the period between first and second measurement after metal addition.

3.2 Fish performance and health status

Fish performance and health status were compared using an ANOVA in the case of multiple observations per treatment. No significant differences ($p>0.05$) in any of the measured parameters were observed. All health and production performance parameters were linear regressed with the CFB. The low observed correlation ($R^2=0.00$-0.38) suggested that there was no correlation at al between metal concentration and health or production performance and therefore no significant effect ($p>0.05$) of CFB on health or production performance was observed (table 4).

Table 4. Growth and health parameters. No significant differences between treatments were observed (Tukey-b, n=8-18, $p>0.05$). R^2 values are the correlation coefficient between simulated cumulative feed burden (table 1) and the parameter measured.

Parameter	N	ER-1000	ER-330	ER-100	ER-33	ER-10	R^2
Wi (g)	18	16.7 ± 0.2	16.8 ± 0.2	16.9 ± 0.1	16.8 ± 0.1	16.6 ± 0.2	0.00
Wf (g)	17-18	33.6 ± 5.0	36.6 ± 8.6	33.5 ± 5.5	31.7 ± 8.1	35.5 ± 10.6	0.00
DFI (g/d)	1	1.16	1.14	1.11	1.10	1.16	0.09
SGR (%/d)	17-18	1.18 ± 0.28	1.32 ± 0.41	1.16 ± 0.26	1.08 ± 0.41	1.29 ± 0.48	0.38
FCR (g/g)	1	0.98	1.10	1.01	0.95	1.07	0.06
Mortality (%/d)	18	0.01	0.00	0.00	0.00	0.00	0.01
CF	17-18	1.74±0.11	1.79±0.17	1.76±0.08	1.75±0.10	1.72±0.11	0.02
HSI (%)	9	1.00±0.15	1.29±0.17	1.29±0.23	1.23±0.16	1.12 ±0.23	0.01
SI (%)	9	0.13±0.07	0.13±0.05	0.10±0.05	0.10±0.03	0.11±0.04	0.02
Hb (g/dl)	8-9	2.9 ± 0.4	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	0.01

3.3 Fish body composition

There was no relation between ash content in whole body and simulated CFB ($R^2=0.03$) and therefore no effect of metal accumulation on crude ash content. In contrast dry matter content in fish whole body showed a negative relation with simulated CFB, this relation fitted well ($R^2=0.83$) and was found significant ($p<0.05$) different from 0. Metal content in whole body dry matter showed a positive relation with metal exposure for all metals. A good correlation ($R^2>0.8$) was only found for Zn, Co and Mn, and these correlations were also considered significant ($p<0.05$) (figure 2).

Table 5. Proximate feed and body composition in g/kg for dry matter and crude ash or in µg/kg for metals.

	Feed	Initial	ER-1000	ER-330	ER-100	ER-33	ER-10
Dry matter	940	231	249	246	251	244	238
Crude ash	85	38	40	36	37	39	37
Fe	359.59	14.80	2.92	2.58	2.93	2.30	4.53
Zn	340.12	17.36	16.74	13.70	17.02	16.78	20.25
Cu	7.06	0.77	0.54	0.50	0.57	0.47	0.72
Co	0.14	0.011	0.012	0.010	0.007	0.009	0.021
Mn	13.33	5.73	5.65	5.57	5.59	5.28	6.42

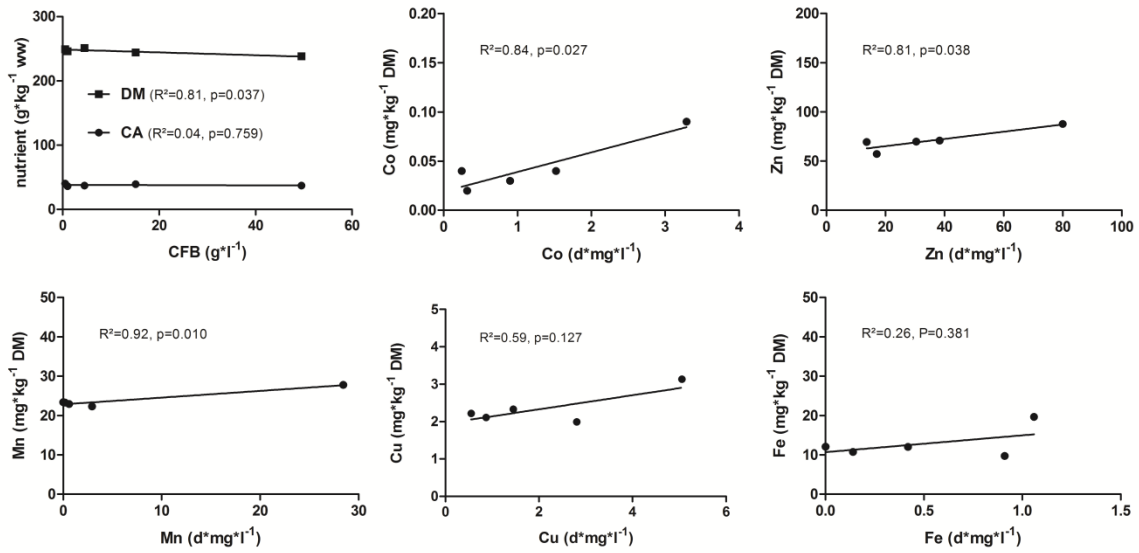

Figure 2. Relation between dry matter (DM) or crude ash (CA) and the cumulative feed burden (CFB) in whole body of turbot, and relations between metal exposure and metal content in dry matter of whole body of turbot, after 9 weeks of exposure. All samples are mixed whole body sample of 9 turbot.

4. Discussion

4.1 General water quality parameters

During the 63 day exposure time all measured water quality parameters, with the exception of metals, were all in the optimum range for turbot (Person-Le Ruyet et al., 1997, Bianchini et al., 1996, Boeuf et al., 1999, van Bussel et al. 2012, Pichevant et al. 2000, Person-Le Ruyet et al., 2002, Imsland et al.,

1996, Schram et al., 2009, Imsland et al. 2001, Imsland et al., 2002). Differences observed between treatments are, therefore, thought to be monofactorial, resulting from differences in metal concentrations during exposure.

4.2 Metal behavior in marine RAS water

Mn concentrations during the first sampling event after mineral mixture addition were comparable to added nominal concentrations, but showed a clear breakdown over time with an estimated half-life time of ca. 6 days. Fe showed also a clear breakdown however concentrations during the first sampling event, were less than 2 % of the nominal values. Estimated half life time of the residual Fe was ca. 1.5 days.

The behavior of these metals in marine RAS environment can be explained by their chemical characteristics. Zinc and Cobalt are highly soluble in seawater and for both Zn^{2+} and Co^{2+} are the main and stable forms present in natural seawater (Long and Angino, 1977, Ellwood and van den Berg, 2001) and formation of complexes with humic acids are low in seawater (Mantoura et al., 1978). Copper is also highly soluble in (saline) water and Cu^{2+} is the main form (Long and Angino, 1977) but in natural marine water, which contains Ca and organics, Cu^{2+} is known to form complexes with humic acids or other ligands (Herring and Morel, 1988). In intensive RAS, humic-acid bound copper is thus the main form present but when humic acid is oxidized copper will bind to other organics. In marine environment Iron exists in the Fe^{2+} form, which is highly soluble in water, and Fe^{3+} form, with an extreme low solubility of 0.2-0.6 nM (Millero, 1998). Oxidation of Fe^{2+} to Fe^{3+} in marine water is fast, the half-life time is <3 hours (Roekens and van Grieken 1983). As a result Iron will quickly precipitate in marine RAS. During the present study decrease in dissolved manganese levels was observed in the two lowest exchange rates. Manganese is highly soluble in seawater, however precipitation, absorption on detritus and interactions with iron, the formation of ferromanganese nodules, will reduce free Mn in RAS water (Bender et al., 1977).

Concentrations of metals during the present experiment for the ER-1000 and ER-330 treatment do reflect praxis concentrations for a typical turbot RAS with exchange rates of 455 l/kg feed, with the exception that Cu concentrations were below 10 µg /l in the 45 m^3 RAS. In freshwater RAS (table 6) concentrations of Fe and Cu are generally higher in fresh water, were as Zn concentrations are lower, due to different behavior of metals in marine waters.

4.4 Metal uptake, bioaccumulation and effects on marine fish.

Effects of metals on fresh water fish are well studied, and toxicity models are formulated all based on the hardness of the water (and on other metals do compete for binding at the gill membrane, affecting the toxicity of a specific metal due to competition (Diamond et al., 1992, Marr et al., 1998, McGeer

2000, Pagenkopf, 1983, Playle, 1998, Stubblefield et al., 1997). In marine water this is not so clear and toxicity is more dependent on salinity and thus osmoregulation, e.g. by drinking water (Grosell et al., 2004, Grosell et al., 2007, Partridge and Lymbery, 2009). During the present study no significant differences in growth performance or health parameters were observed. More distinct, correlation coefficients between simulated water exchange and growth or health parameters were extremely low, indicating very clear that in the tested range there was no effect of metals on production performance or health (table 4). Although production performance and health parameters were not affected, an effect on dry weight content of the body and metal bioaccumulation were observed. Differences in metal content in the flesh of farmed turbot compared to caught turbot are observed for Cobalt, Copper, Iron, Manganese and Zinc (Martinez et al., 2010), but this can be a (combined) effect of diet or environment. For seabass (*D. labrax*) it was observed that at least differences in bioaccumulation of Mn, Co and Cu are caused by water exchange rates, e.g. matrix (Deviller et al., 2005). Therefore uptake and bioaccumulation of individual metals by marine fish is discussed.

Iron uptake. Marine fish species take up ions mainly through the intestine, but in seawater Fe (III) is the main form present, and has a low bio-availability to fish (Bury and Grosel, 2003). Despite the lower bioavailability some uptake of Fe^{3+} in the posterior part of the intestine of European flounder does takes place (Bury et al., 2001). However considering the low solubility of Fe in marine water and the low drinking rate of 1 ml/kg/h for turbot (Caroll et al., 1994) it can be estimated aquous uptake is only a fraction of the daily dietary Fe intake. This is in line with the low correlation between Fe exposure and Fe body content observed during the present study. Therefore we consider aqueous Fe accumulation in marine RAS not a relevant health or growth affecting issue.

Zinc uptake. In marine fish uptake of zinc takes place in the anterior region of the digestive tract as it was demonstrated for winter flounder (Shears and Fletcher, 1983) and aqueous Zn can contributes for 20-40 % of the total zinc uptake (Hoss, 1964, Renfro et al., 1975, Willis and Sunda, 1984). Zinc homeostasis is maintained in fish by regulating the excretory mechanisms and controlling the gastrointestinal uptake (Wannatabe et al., 1997), preventing dietary toxicity as is reported for turbot exposed to high (1100 mg Zn/kg) dietary zinc (Overnell et al, 1988). Aqueous zinc seems much more toxic with reported 96h LC-50 values for marine fish species range between 8 and 21.5 mg/l (Taylor et al., 1985, Mohapatra and Rengarajan 1997, Rajkumar et al., 2011). However it can be questioned if LC-50 values are a relevant value for safe culture of fish in RAS. Reported 96 h LC-50 values for red sea bream (*Pagrus major*) larvae are as high as 10.1 mg/l, but increased deformities and increased mortality of larvae are already observed at 0.3 mg/l after 10 days of exposure (Huang et al., 2010). During the present experiment exposure to sublethal concentrations of aqueous zinc did not influence health or production performance of turbot, but led to bioaccumulation of zinc. This is in line with observation of McGeer et al. (2000) who exposed rainbow trout to sub-lethal zinc concentrations and did not find differences in feed intake, growth or FCR, although significant ionic imbalances in the blood as well as Zn accumulation in the gills were observed. The absence of

negative effects of sublethal aquous zinc on growth, feed intake or feed utilization is also reported for parr (*Salmo salar*) (Farmer and Ashfield, 1970). Furthermore these authors reported bioaccumulation of zinc combined with a decreased caloric content of the whole body. Although a proximate body analysis was not done, it is very likely that the reduced caloric content was caused by an increased incorporation of water in the tissue, as is also reported for clam exposed to aqueous zinc (Belanger et al., 1986). These observations might explain the significant negative relation between dry matter content of the turbot and water exchange rates observed during the present study.

Copper uptake. Fish appear to be more tolerant of copper in the diet than of dissolved copper in the water. Acute lethal concentrations (LC-50) of copper sulfate vary between >0.1 to 1.5 mg /l copper, for both marine and fresh water fish species (Friedman and Shibko, 1972, Taylor et al, 1985, Grosell et al., 2007) although mainly osmolality physiology can reduce LC-50 values for Cu a 50-fold (Grosell et al., 2007). Due to complex formation of Cu in seawater, the bio-availability of Cu is low. Furthermore it is known that dissolved organic carbon reduces Cu toxicity even more. For the sensitive mussel embryos (*Mytilus trossolus*) LC-50 values were increased a 4 fold (Nadella et al., 2009). In marine fish it is observed that sub-lethal exposure to copper leads to an initial bioaccumulation of Cu in gills and intestine, however after an 8 day period stable levels of Cu were maintained suggesting excretion or impairment of Cu (Grosell et al., 2004). Although this effect is not studied for turbot it might be an explanation for the low correlation between Cu bioaccumulation and exposure in the present study.

Manganese uptake. The uptake of manganese by fish from water has been demonstrated (Miller et al., 1980; Srivastava and Agarwal,1983) but the mechanisms of manganese uptake are not clear and is low, dietary uptake is the main source of Mn. For marine fish, data available are limited to a study comparing effects on juvenile mulloway (*Argyrosomus japonicus*) exposed to 0 or 5 mg/l Mn at 3 different salinities for two weeks. At all 3 different salinities mullets showed decreased feed intake, lower feed efficiency and as a result lower or even negative growth, as well as Mn bioaccumulation in all tissues sampled (Partridge and Lymbery, 2009). Deviller et al. (2005) found that bioaccumulation of Mn in seabass (*D. labrax*) was dependent on water exchange rates. Results of these studies are thus in line with present findings, were bioaccumulation of Mn was found significant.

Cobalt uptake. Both dietary and aqueous cobalt can function as cobalt source, aqueous uptake is dependent on water temperature and alkalinity (Phillips et al., 1957). For fresh water fishes toxicity of cobalt increases with decreased water hardness and is species dependent, no observed concentrations vary between 0.3 and 4 mg/l although inhibition of feeding is reported at concentrations of 4.8 mg/l (Diamond et al., 1992, Hassan et al., 1992 Marr et al., 1998). For marine water no data exist but the accumulation of Co in tissue of seabass (*D. labrax*) is reported (Deviller et al., 2005). Present findings also show that bioaccumulation of Co trough water takes place.

4.5 Potential risks of metal accumulation in marine RAS on fish production

Both feed and make up-water are potential sources of metal accumulation in RAS (Davidson et al., 2009). Based on dietary requirements of metals by marine fish (table 1) and the metal composition in the commercial diet used (table 5) it can be concluded differences between requirements and dietary content of zinc shows a large discrepancy. Based on the seawater analysis (table 3), seawater is a potential source for both Zinc and Co.

Metal behavior and concentrations during the present experiment and observed in the 45 m^3 RAS indicate that accumulation of Fe and Cu is neglectable, however for Zn, Co and Mn accumulation in marine RAS can be expected. This is also reflected in the bioaccumulation of metals during the present study. Based on literature data, differences between lowest observed LC-50 values for zinc and concentrations observed in RAS only vary a factor 40, whereas differences in LOEC and in the RAS for both Mn and Co vary a factor 1000. Concentrations in the feed are a factor 100 higher for Mn compared to Co. Therefore we estimate the potential risk for negative effects on fish production in marine RAS, and is ranked as Zn>Mn>Co>Cu>Fe.

Table 6. Concentrations of various metals (µg/l) observed in experimental RAS. All RAS had biological nitrification and mechanical filtration. Additional treatment using biological denitrification is indicated by D, additional ozonation is indicated with O_3.

Water	Treatment	l/kg	Fe	Zn	Cu	Co	Mn	Ref
SW		455	13	196	n.d.	7	7	1
	O_3	455	16	139	n.d.	n.d.	5	
FW		2564	--	12	5	1	--	2
		244	--	27	45	1	--	
		18	41	128	119	--	8	3
	O_3	18	n.d.	78	41	--	n.d.	
FW		100	17	20	13	--	n.d.	4
	D	100	22	26	13	--	6	
	D	50	22	33	19	--	n.d.	
	D	25	34	39	19	--	n.d.	
FW		1500	n.d.	85	14	--	2	5
	D	30	n.d.	146	59	--	5	
		1500	2	5	10	--	1	6
	D	70	25	73	13	--	6	
	D	30	35	53	14	--	4	

-- not measured

1 Present study, detection limit Cu = 10 µg/l, Co = 4 µg/l

2 Davidson et al. 2009

3 Davidson et al. 2011, detection limit Mn =2 µg/l

4 Kamstra et al 1998, detection limit Mn = 6 µg/l

5 Martins et al. 2009, detection limit Fe = 100 µg/l

6 Martins et al. 2011

5. Conclusion

Although the present experiment lasted only nine weeks, and the full grow out period for turbot is 2 years we did not see any negative effects on growth performance or health. Significant bioaccumulation of Zn, Mn and Co and significantly decreasing dry matter content of whole fish took place during these 9 weeks of exposure. Concentrations of Zinc in the culture water may become problematic as a result of excess levels of zinc in the diet and zinc concentrations in makeup water. However based on the absence of growth and health effects under extreme low water exchange rates, we don't consider metal accumulation as a factor limiting intensification of RAS turbot production.

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Chapter 4:

Safe oxidation of feed originated accumulating substances and bacteria in marine recirculating aquaculture systems (RAS) using Boron Doped Diamond (BDD)-electrodes - a comparison with conventional ozonation.

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Abstract

Hydroxyl radical production with Boron doped diamond (BDD) electrodes might be a promising alternative to ozonation of the culture water in marine recirculating aquaculture systems (RAS) but the formation of toxic total residual oxidants (TRO) might limit applications and doses. Data available for marine (aquaculture) water are scarce and are limited to laboratory studies, exceeding TRO safety levels for fish and shrimp in magnitudes.

To evaluate the effect of BDD electrodes on water quality and bacterial load, a BDD electrolyzation treatment, an ozone treatment (injection of air/ozone mixture) and a control treatment were performed in 12 identical 300l marine RAS, arranged in triplicate experimental design. These RAS were stocked with juvenile white leg shrimp (*Litopenaeus vannamei*) and cultured for 30 days fed restricted amounts of feed. Dosage of tested oxidation processes were standardized at a TRO safety level of ≤ 0.06 mg/l Cl_2.

After 30 days no significant differences in mortality, growth and feed efficiencies between treatments were found. Accumulation of total organic carbon (TOC), ortho-P and colored dissolved organic matter (CDOM) were significantly lower in the BDD-treated RAS compared to control RAS, but no significant effects on ammonia or nitrate levels were observed. Nitrite oxidation followed a first-order kinetic but overall effect was neglectable compared to biofiltration. Bacterial disinfection was low and no changes in bacterial density in the culture tanks were observed. Rapid build-up of lime-stone was observed at the cathodic side of the electrodes, changing the actual current density of the electrodes. Ozone oxidized significantly more TOC compared to BDD treatment, but did significantly increase ortho-P levels but decreased bacterial counts in the culture tank.

We conclude that safe removal of TOC, CDOM and ortho-P in marine RAS using BDD-electrodes is possible to maintain system water quality and to decrease nutrient emissions. The formation of limestone has to be avoided to maintain current densities. For disinfection purposes the present BDD-treatment is less effective than ozone, probably caused by the low contact area between water and oxidant due to short half-life time of hydroxyl radicals.

1. Introduction

Over the last decades, fish and shrimp production in land based recirculating aquaculture systems (RAS) has become increasingly important in the aquaculture industry. To reduce water consumption in RAS, water re-use and stocking density are intensified (Martins et al. 2010). This leads to an increase in the applied feed load per water volume. Consequently waste products originated from feed accumulate in the recirculating water requiring technical improvements of water treatment (Blancheton et al, 2007). Water treatment in RAS consists in general of mechanical (e.g. filtration, sedimentation, foam fractionation) and biological (e.g. nitrification, denitrification) treatment processes. Because biological denitrification and phosphate removal are not common praxis in commercial RAS and several dissolved organics are non-biodegradable, nitrate, phosphate and total organic carbon accumulate in RAS with high levels of water re-use (Barak and van Rijn, 2000, Martins et al, 2010). Furthermore particles, originating from feed or faeces, are broken down in size due to pump sharing making effective mechanical removal using sedimentation or filtration difficult. Therefore, physic-chemical treatment processes such as a combination of UV-light and ozonation are widely used not only for disinfection purposes but also to improve water quality by removing feed originated accumulating substances from the culture water (Summerfelt and Hochheimer, 1997) . In marine RAS ozonation is widely used and has been proven effective in removal of small particles, yellow substances, and nitrite as well as in reducing bacterial numbers (Schroeder et al., 2011). Oxidation of ammonia in seawater by ozone via breakpoint bromination is also possible. However this process requires the previous formation of toxic brominated ozone-produced oxidants (OPO) as intermediates and is thus considered as unsafe and not practical for aquaculture purposes (Schroeder et al., 2011). Elevated OPO levels in the culture water cause stress, diseases and increased mortality to shrimp (*Litopenaeus vannamei*), and first signs of histological alterations could have been already observed at levels as low as > 0.06 mg/l chlorine (Cl_2) equivalent (Schroeder et al., 2010). Therefore, dosage of ozone is limited to formation and degradation of these OPO. Applied doses in aquaculture praxis are commonly between 13-25 g O_3 per kg feed applied (Summerfelt and Hochheimer, 1997, Timmons and Ebeling, 2010).

Due to economic constraints and the risk of forming toxic residual oxidants, alternatives for ozonation are searched, and electrolysis of seawater with electrodes producing hydroxyl radicals is recently getting more attention. The use of hydroxyl radicals might have economic benefits over ozone, because no additional oxygen is needed to produce hydroxyl radicals, while water is still enriched with oxygen (Mook et al., 2010). Secondly hydroxyl radicals are produced *in situ* while ozone is produced outside the system water and has to be dissolved, resulting in losses (Timmons and Ebeling, 2010). Another benefit of hydroxyl radicals is their higher ability to oxidize persistent organic pollutants (Mehmet and Brillas, 2007) which might be a way to reduce off-flavour problems in RAS. A new method to produce hydroxyl radicals is electrolysation of water with BDD-electrodes (Marselli et al.,

2003). Boron Doped Diamond (BDD)-electrodes are metallic carriers whereon a thin polycrystalline layer of diamonds is deposited by using a technique called chemical vapor deposition. A controlled incorporation of impurities, in this case Boron, leads to a highly conductive Boron doped diamond (BDD) -electrode with the unique properties of diamond, such as hardness, thermal conductivity and chemical inertness (Ivandini et al, 2005). The surface of the electrodes is hydrophobic and not attractive for absorption. Due to these characteristics, over a very wide potential range little electrochemical activity is observed at the diamond electrode surface. One result is that, when a current is applied on a BDD-electrode in an aqueous medium, the rate of water electrolysis generating O_2 is negligible and a very high overvoltage can be applied. Due to this high overvoltage, hydroxyl radicals can be electro-generated (Martin et al., 2005). The formation of hydroxyl radicals can be described according to the equation:

$$H_2O \rightarrow HO^{\bullet} + H^+ + e^-$$

With an oxidation potential of 2.8 V, the produced hydroxyl radicals have the highest oxidation potential compared to other known oxidants used for aquaculture purposes. In laboratory scale BDD-electrodes have been used to remove total ammonia nitrogen, nitrite and chemical oxygen demand from saline aquaculture water, and BDD-electrodes have been proposed as alternative to biofiltration (Diaz et al., 2010). However flow rates used by Diaz et al. (2010) were 100-200 times higher than optimal flow rates suggested for RAS (Timmons and Ebeling, 2010) and concentration of toxic free chlorine exceeded safety levels for fish and shrimp health over 100 times (Schroeder et al, 2010, Reiser et al., 2010) making this system design practically impossible as alternative to biofiltration.

The formation of toxic TRO is the main problem when oxidation processes are used in aquaculture system water. Due to the high reactivity of hydroxyl radicals different secondary oxidants might be formed, depending on the characteristics and composition of the treated water. In pure water, only reactive oxygen species consisting of hydrogen or oxygen atoms are formed such as $O^{\bullet}$, O_3 and H_2O_2. These secondary oxidants are also highly reactive and therefore contribute effectively to the water treatment. In water containing other ions different intermediates and by-products are formed. Especially in saltwater, halogen-ions are oxidized to secondary oxidants. Some examples of BDD-produced oxidants in marine and brackish waters are hypohalides (e.g. hypobromite and hypochlorite), haloamines (e.g. bromamines and chloramines), trihalomethanes such as chloroform and bromoform, halo-acetic acids and oxoanions such as bromate, chlorate and perchlorate (Eckhardt et al. 2010, Diaz et al. 2011). Due to their stability some of these oxidants might accumulate in the culture water to high concentrations and represent a high risk for health and welfare of cultivated species.

Until now all previous studies conducted with BDD-electrodes are short-term laboratory studies, with only very small water volumes mostly around one liter, optimal treatment conditions such as new and perfectly clean electrodes, and most important, not biological dynamic systems, including fish, bacteria and daily feed inputs, but static chemical test solutions. Furthermore, in previous studies the effectivity of BDD-electrodes for water treatment has been rarely set into relation to the formation of

toxic total residual oxidants (TRO). But particularly with regard to aquaculture production TRO formation is highly relevant and has to be minimized to avoid deleterious impacts on animal health.
The aim of the present study was to evaluate electrochemical water treatment with hydroxyl-radicals based on BDD-electrodes for its suitability in marine recirculation aquaculture in comparison with the conventional ozonation. Therefore, potential for minimization of TRO was investigated in preliminary experiments and the effect of BDD-produced hydroxyl-radicals on feed originated accumulating substances as well as on bacterial load was studied in a long-term experiment, culturing Pacific white shrimp (*L. vannamei*) over a period of 30 days. Performance of BDD-electrodes were directly compared with conventional ozonation, based on a set safety level of TRO $\leq$ 0.06 mg/l Cl_2 equivalent, considering water treatment efficiency, shrimp performance and economical aspects.

2. Material & Methods

The whole experiment consisted of three experiments. The first series of experiments (flow through experiments) were carried out to find relations between charge input and the formation of residual oxidants and conversion of nitrogen species. The second series of experiments (accumulation experiments) were carried out to see accumulation characteristics of residual oxidants. With knowledge obtained from the first two series of experiments a long term study (shrimp culture experiment) was carried out to safely culture shrimp for 30 days and treat the water using BDD-electrodes or conventional ozone.

2.1 System setup

2.1.1 Electrodes and electro cell set up

The electro cell used in this experiment was type CONDIACELL® CP450 salt water (CONDIAS GmbH, Itzehoe, Germany) equipped with 4 pair of electrodes consisting of a Niobium carrier material and a 20 µm thick Boron doped diamond layer, type DIACHEM® 24*50 mm (CONDIAS GmbH, Itzehoe, Germany). Electrodes were once weekly cleaned with water and acid (1 molar HCL).

2.1.2 RAS set up

The experiments were conducted in 12 small scaled experimental RAS, each with a system set-up as shown in figure 1: With a total water volume of 300 l, RAS consisted of a 275 l rectangular rearing tank (1) with a footprint of 0.7 m² (Spranger Kunststoff, Plauen, Germany). Water from the tank was mechanically filtered by filter mats (2). After mechanical filtration water went through the pump (3)

and the water flow of 1500 l/h was then divided into three flows, with a flow rate of 500 l/h each. The first flow went into a protein skimmer (4) (Model 1 AH 1100, Erwin Sander, Uetze-Eltze, Germany) using compressed air. Water level in the protein skimmer was lowered to avoid foam removal. Therefore, the protein skimmer functioned only as degassing and aeration tower. The second flow went into a moving bed biofilm reactor (MBBR) (5) with a total volume of 30 l, filled with 5 l of 15 months old biocarriers originating from a MBBR of a pilot RAS (water exchange rate 800 l/kg feed) culturing European seabass (*Dicentrarchus labrax*). The third flow went through the electro cell (6) and directly back into the culture tank. All flow rates were set equal in all RAS. These flow rates were chosen because 500 l/h was the minimum flow rate for the BDD electro-cell used, suggested by the producer (Condias instruction manual). The flow rate of 500 l/h provided exactly a hydraulic retention time (HRT) of 1 min in the aeration tower which is an adequate contact time for disinfection (Timmons and Ebeling, 2010) and a HRT of 3.6 min in the MBBR.

Optionally ozone was produced in an ozone-generator (Aquamedic 200 mg/h, Germany) using compressed air and was injected at the bottom of the aeration tower (fig. 1.4) by a wooden air-stone diffuser in counter current.

Optional addition of hydroxyl radicals took place in the reaction chamber (fig.1.6) by electrolyzing the passing water with BDD-electrodes as described in *2.1.1*. The electric current required for electrolyzation was generated by a laboratory power supply (model EA-PS 3032-05 B, HEIDE power GmbH, Pürgen, Germany). In order to measure and compare the energy consumption (kWh) of electrochemical treatment and ozonation, an electricity meter was mounted prior to the laboratory power supply and the ozone generator, respectively. The applied potential (V) and current (A) on the BDD-electrodes were recorded by hand.

2.2 Experimental set up and procedure

2.2.1 Flow through experiment

During the flow through experiments only one of the 12 experimental RAS was used. RAS was filled with bulk water (table 1). RAS was operated as described above except that the flow through the electro-cell (6) was not send back into the rearing tank but was discarded. TAN, NO_2-N, TRO and oxidation reduction potential (ORP) were sampled in the discarded water. Applied potential and current were measured by the laboratory power supply and recorded by hand. All measurements were done in triplicate with replicates in time.

2.2.2 TRO accumulation experiments

For TRO accumulation experiments 3 RAS were filled with bulk water (table 1) and stocked with hybrid tilapia (*O. nilotus* x *O. mozambicus*), no feed was supplied. All flow rates were set to 500 l/h as described above (system set up 2.1). Applied current on the BDD-electrodes were 0.02, 0.05, 0.10 and 0.20 A (4.7, 11.6, 23.3 and 46.5 A/m²). Accumulation of TRO was measured over a period of 24-30 hours. After each run, water was renewed with bulk water.

Table 1. Bulk water matrix taken from 45 m³ seabass RAS. ORP = oxidation reduction potential, TRO = total residual oxidants, TOC = total organic carbon, TAN = total ammonia-nitrogen, T-CFU = colony forming units on marine agar, V-CFU = colony forming units on *Vibrio sp.* specific agar.

Parameter	Value
Temperature (C)	24
Salinity (ppt)	26
pH	7.2
ORP (mV)	200
TRO (mg Cl_2 l^{-1})	0.03
TOC (mg l^{-1})	8.30
TAN (mg l^{-1})	0.62
NO2-N (mg l^{-1})	0.65
NO_3-N (mg l^{-1})	105
PO_4-P (mg l^{-1})	9.1
T-CFU (ml^{-1})	2.8 10^5
V-CFU (ml^{-1})	6.5 10^3

2.2.3 *Shrimp culture experiment*

The 12 small-scaled RAS (figure 1) were divided in 4 system designs with 3 replicates each, providing 3 different treatments by chemical oxidation and a control without oxidation process:

- Control (I): filter mats, MBBR, aeration tower.
- OH (II): equal to control + continuously applied current on BDD-electrodes.
- O_3 (III): equal to control + continues ozone addition
- OH batch (IV):equal to control+ intermittently applied current on BDD-electrodes

In order to ensure comparability of treatment (II) and (III), dosages of hydroxyl radicals and ozone were standardized with regard to residual oxidant concentration in the tanks. Therefore, the TRO concentration was measured at the tank inflow (fig 1., (7)), in regular time intervals and adjusted to a

value of ≤ 0.06 mg Cl_2 l^{-1} by regulating the current applied for the generation of OH-radicals and ozone, respectively. To enable comparison of treatment (II) and (IV) continuous and intermitted electrolysation were standardized on the same amount of charge (Ah) used. Therefore, in treatment (IV) BDD-electrodes were switched on only twice a day for a period of 1 HRT each. The experimental period consisted of 30 days. Treatment IV was terminated after 14 days because shrimp reacted stress-full on movement and stopped feeding.

The performance of BDD-electrodes applied in two different treatments as well as ozonation were evaluated by determining the accumulation of inorganic (TAN, NO_2-N, NO_3-N, ortho-P) and organic (TOC and CDOM) substances, the disinfection potential as well as energy consumption. Furthermore, bacterial pressure of *Vibrio sp.* and total bacteria was estimated in the culture tanks and shrimp performance was assessed based on growth rate, feed utilization and mortality.

RAS were filled with a mixture of water from the seabass pilot-RAS and tab water (detailed description of the water matrix is given in table 4). A total of 1200 juvenile Pacific white shrimp (*Litopenaeus vannamei*) with a mean average weight (±SD) of 2.5 ± 1.3g (range: 1.0-5.0 g) were randomly divided in groups of 100 individuals and distributed to the 12 RAS. Average total wet weight per system was 253.3 ± 22.2 g resulting in an initial density of 362 g/m². Temperature was automatically adjusted to 28°C with a temperature-controlled 500 W heater (Aqua Medic, Bissendorf, Germany). A photoperiod of 14 h light (06:00-20:00)- 10 h dark was provided. Shrimp were fed by hand with a commercial shrimp feed (Penaeus grower 3 mm, Le gouessant, France). Feed load was restricted, resulting in equal amounts of feed for every tank. Feeding regime was 3 times a day, 7 days per week. Feed load within day 1-14 was 15 g per day, within day 15-28 25 g per day. No feed was fed at day 0 and 29. Tab water was added to compensate for evaporation losses and sand-filtered, ozonized and UV-treated seawater was used to compensate for cleaning losses.

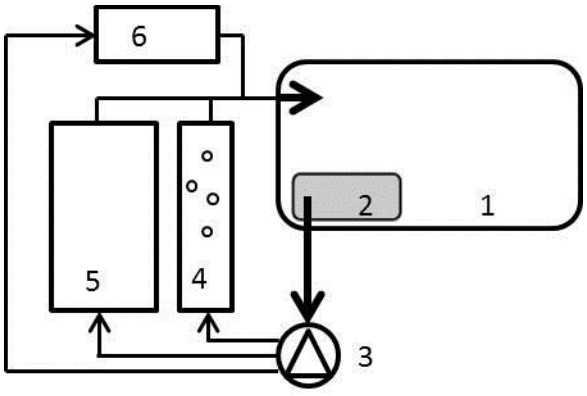

Figure 1. Sketch of system design. 1) rearing tank, 2) filter mats, 3) pump, 4) aeration tower optional with ozone addition, 5) moving bed biofilm reactor, 6) electro-cell optional with hydroxyl radical addition, 7) total residual oxidants (TRO) sampling point.

2.3 Water quality analysis

TP and O-P were measured spectrophotometrically with the ascorbic acid method using a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland) according to the protocols of Grasshoff et al. (1999) and adapted to microplate format. TAN, NO_2-N, NO_3-N, Fe and TRO were determined spectrophotometrically using a HACH-DR 2800 spectrophotometer (Hach-Lange GmbH, Berlin, Germany) and powder pillow detection kits (Hach-Lange GmbH, Berlin, Germany) based on the ammonia salicylate method, diazotization method, the cadmium reduction method, 1,10 Phenantrolin method and the N,N-diethyl-p-phenylenediamine (DPD) method, respectively. TRO is measured in mmol/l but notated during the present study as chlorine (Cl_2) equivalents in mg/l. TOC was analyzed as non purgable organic carbon using a total organic carbon analyzer TOC-V CPH/CPN (Shimadzu corp., Kyoto, Japan). Total alkalinity was calculated by pH change using titration method with hypochloric acid adapted from Grasshoff et al. (1983). Temperature and pH were measured with a WTW 340i portable multiparameter instrument (WTW GmbH, Weilheim, Germany), salinity with a digital refractometer HI 96822 (HANNA Instruments, Woonsocket, USA) and dissolved oxygen with a Handy Polaris oxygen probe (Oxygard International A/S, Birkenrød, Denmark). Dissolved colored organic matter (CDOM) was determined by direct absorption at 436 nm of pre-filtered (0,22 µm) culture water and compared against a blank of artificial sea water (ASW) at 20 g/l Cl^-. Absorption was measured with a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland). A BMT ozone analyser 963 (BMT Messtechnik GmbH, Berlin, Germany) was used to determine ozone addition by measuring flow rate and ozone concentration in the gas stream.

2.4 Microbial analysis

Within the shrimp culture experiment water samples were taken from the tanks (fig.1, (1)) of all treatments and the control for the estimation of bacterial density. Furthermore, water samples at the inflow and the outflow of both tested chemical water treatment processes, ozonation (treatment 2) (fig. 1 (4)) and BDD electrolysation (treatment 3 and 4) (fig. (6)) were taken to assess bactericidal efficiencies. Samples were taken one by one. Directly after each sample was taken, the respective sample was diluted and analyzed by the spread plate method using an unspecific marine agar (Difco Marine Agar, BD, Sparks, USA) for the detection of total viable cell counts, as well as a *Vibrio sp.* specific agar (Difco TCBS Agar, BD, Sparks, USA) for the detection of Vibrios. Colony forming units per plate were recorded after 24 and 96 h of incubation at 28° C on marine agar (T-CFU/ml) and on *Vibrio sp*. specific agar (V-CFU/ml).

2.5 Assumptions and formulas

Current density (CD) was calculated as follows:

CD = Applied current (A) / working surface electrode (1)

Working surface electrode (WSE) was calculated as follows

WSE = (length (m) * width electrode (m)) * active area electrode * surface factor (2)

where the perpendicular cross section of 1 electrode is 75*32 mm. Active area is the fraction of the electrodes functioning as cathode (or anode), depending on the three dimensional arrangement of the electrodes pair which determines if an electrode is active on one or both sides. Number of active area in our case was 7. The surface factor is a correction for the roughness of the diamond surface and a correction for the fact that a mesh is used, not a plate. The surface factor used here is 1.2. Thus value used for WSE is 75*32*7*1.2= 20160 mm^2 or 0.020160 m^2.
For the ease of understanding and calculations we assume that only hydroxyl radicals are generated at the electrodes as primary product according to equation (3), resulting in equation (4).

$H_2O \rightarrow HO^{\bullet} + H^+ + e^-$ (3)

1 mole e^- = 1 mole $OH^{\bullet}$ (4)
1 Faraday = charge of one mole e^- (5)
1 Faraday = 96485.3399 C (6)
1 C = 1 A*s (7)

When oxidized or produced substances are plotted against Faraday, the mole $OH^{\bullet}$ equivalent, the current efficiency can be estimated.

Current efficiency = substance X (mole) / $OH^{\bullet}$ (mole) or Faraday (8)

For ozone, molar efficiencies can be calculated in the same way

Molar efficiency = substance X (mole) / O_3 (mole) (9)

To compare the accumulation of substances in the culture water the function cumulative feed burden (CFB) is used. This function is combining the amount of feed added per volume of water, corrected for water exchange and is calculated as follow:

$$CFB = \text{Feed added (g)} / V_{total}\ (l)\ \wedge\ (-V/V_{total}) \quad (10)$$

where "feed added" is the amount of feed added per tank in g, "V_{total}" is the total culture volume and "–V" is the amount of water replaced.

As CFB was set to 0 at the start of the experiment although the initial process water matrix was not free of accumulating substances, accumulating water parameters (NO_3-N, OP, TP, TOC and dissolved matter) are both presented as absolute value and as increase of the parameter:

$$F(x) = mx + b \quad (11)$$

where x is the cumulative feed burden, F(x) is the accumulation of the water parameter and b is the consumption or production of the parameter at a CFB = 0.

Reduction efficiency for disinfection was calculated as the difference in CFU between inflow and outflow

$$RE = (CFU_{inflow} - CFU_{outflow} / CFU_{inflow})*100 \quad (12)$$

Reduction factor was calculated as the logarithmic difference between CFU in inflow and outflow

$$RF = \log(CFU_{inflow}) - \log(CFU_{outflow}) \quad (13)$$

2.6 Data analysis

Data are presented as mean ± standard deviation (SD) of n samples. Statistical analysis of variances was performed using SPSS 17.0 (IBM inc, Armonk, USA). Linear regression was conducted using Graphpad (Graphpad software inc., La Jolla, USA). Data were tested for normality and for equal variances ($p<0.05$) using Levene's test and the Kolgomorov-Smirnov test, respectively. Multiple comparisons were carried out by the parametric Tukey's HSD or the non-parametric Dunnet's T3 test. Single comparisons were carried out by paired or unpaired t-tests. Differences were considered as significant at $p<0.05$.

3. Results

3.1 Flow through experiments

The minimum potential needed to produce hydroxyl radicals was 2.4 V. The applied current density (A/m^2) showed a positive logarithmic relation with potential needed (V) according to the formula:

$$\text{Potential needed (V)} = 0.399 \ln(\text{CD}) + 2.1906 \quad (r^2 = 0.972) \qquad (14)$$

where CD is current density in A/m^2 and minimum potential needed is 2.4 V.

ORP decreased with increasing current density (figure 2b), an increased change of ORP was observed between 12 and 32 A/m^2 with a maximum change at 22 A/m^2 when the first derivate of ORP was plotted (figure 2d).

TRO production showed a positive relation with the applied current density (figure 2), and thus with the amount of hydroxyl radicals produced, if linear regressed according to the formula:

$$\text{TRO (mg } Cl_2/l) = 5.46 * 10^{-3} * \text{CD} \quad (r^2 = 0.970) \qquad (15)$$

$$\text{TRO (mole)} = 0.044 * OH^{\bullet} \text{ (mole)} \quad (r^2 = 0.970) \qquad (16)$$

The residual plot showed a non-normal distribution, where negative values (residuals) were found at a current density of <27 A/m^2 with lowest residuals for TRO production at 17 A/m^2 (figure 2 c).

Maximum current efficiency for TRO production was 0.05 observed at a current density of 42 A/m^2 (figure 3).

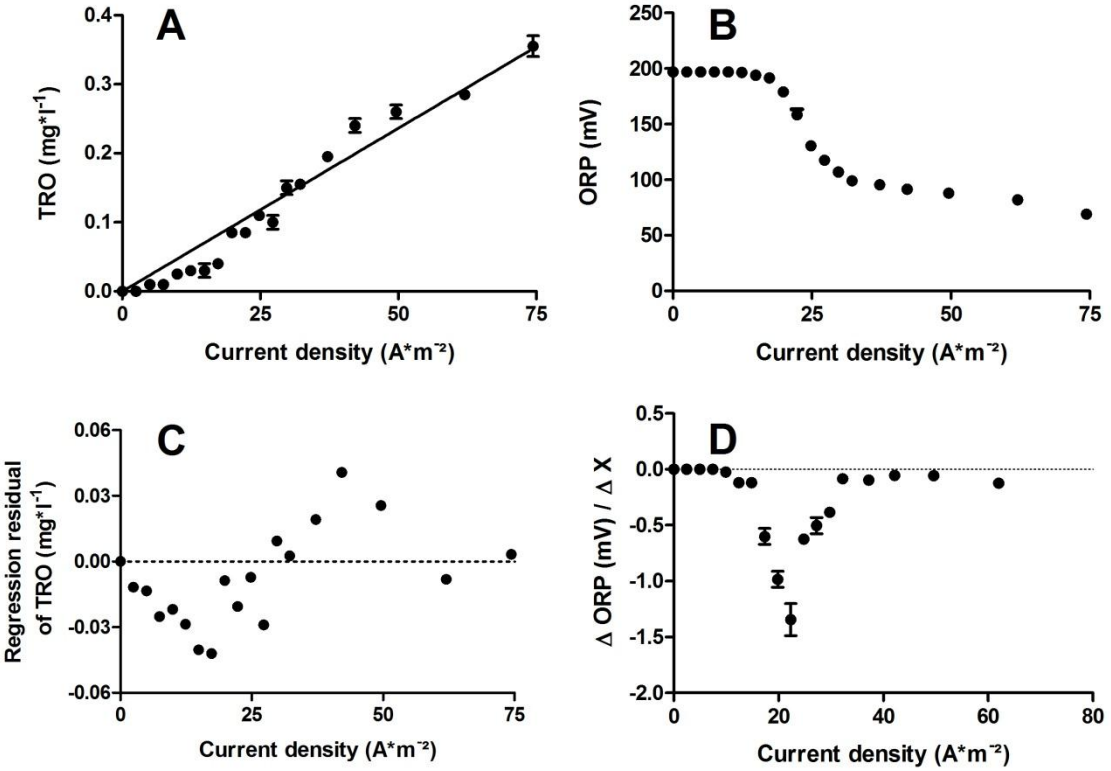

Figure 2. A) Linear Regression between applied current density and TRO (Cl_2 eq.). B) residual plot of linear regression. C) Relation between ORP. D) current density and its first derivate. Flow through experiments, perpendicularly cross-section of BDD-electrodes 24 cm², flow rate 500 l/h, n=3.

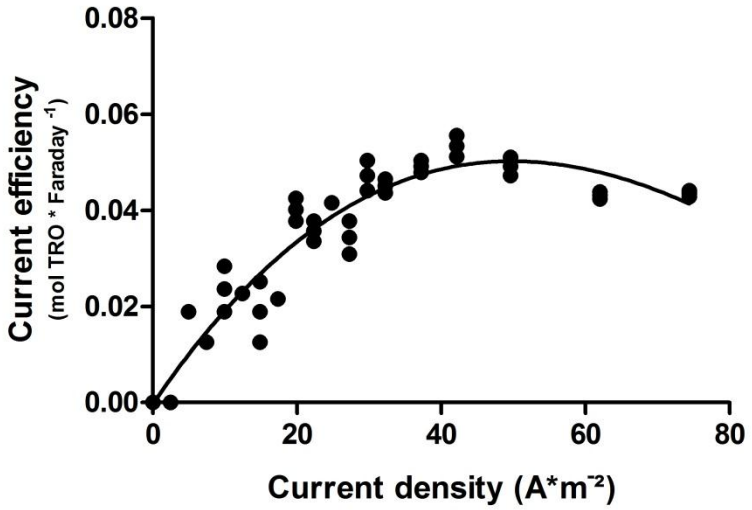

Figure 3. Current efficiency of TRO production against current density. $CE = -3.5*10^{-4} + 2.2*10^{3}$ CD $- 2.8*10^{-5}$ $CD^2 + 7.7*10^{-8}$ CD^3 $r^2 = 0.905$. Flow through experiments, perpendicularly cross-section of BDD-electrodes 202 cm², flow rate 500 l/h, n=57,

Total ammonia nitrogen (TAN) was not affected by current density in the tested range. Nitrite reduction showed a positive linear relation with current density. Nitrite concentration, respectively nitrite oxidation could be described according to the formulas:

NO_2-N (mg/l) = $-9.53\ 10^{-4}$ * CD + 0.65 (r^2=0.987) (17)

NO_2-N oxidized (mole) = 0.0639 * OH (mole) (r^2=0.987) (18)

Where CD is current density in A/m² and initial NO_2-N concentration was 0.65 mg/l.

3.2 TRO accumulation experiments

Results of the flow through experiments showed that a current density of 10.0 A/m² yielded a TRO concentration of 0.06 mg/l Cl_2 (figure 2). Therefore different current densities (1.8, 2.5, 5.0 and 10.0 A/m²) were applied and TRO ± SE was measured over a time period of 24-30 hand reached a plateau at 0.42 ± 0.02 (n = 15, r^2 = 0.96), 0.20 ± 0.02 (n = 21, r^2 = 0.94), 0.12 ± 0.00 (n = 42, r^2 = 0.98) and 0.06 ± 0.00 (n = 18, r^2 = 0.94) respectively (figure 4).

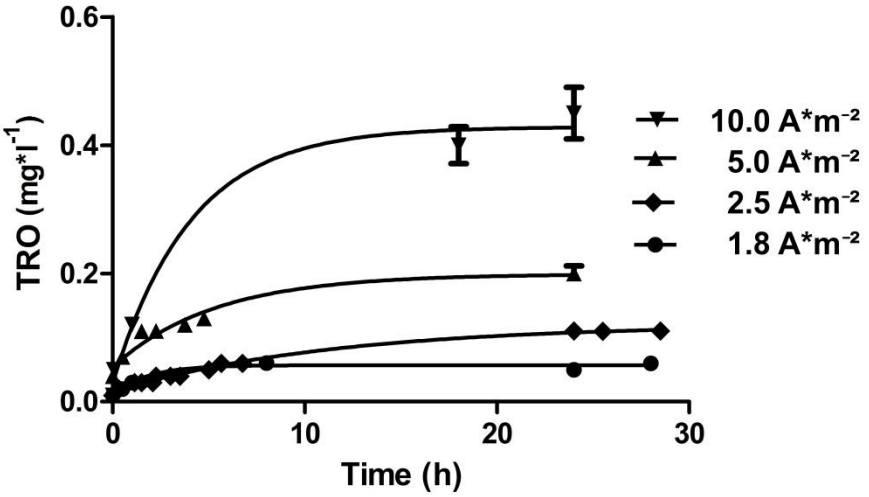

Figure 4. Accumulation of TRO (mg/l Cl_2) over a period of 30 hours at different current densities. Perpendicularly cross-section of BDD-electrodes: 202 cm², flow rate 500 l/h, total volume 300 l. All data show average ± SD, n=15-42.

3.3 Shrimp culture experiment

3.3.1 Shrimp performance

Shrimp feeding behavior was affected in treatment IV (intermittent BDD treatment) and thus was terminated after two weeks because shrimp were stressed and stopped feeding and thus biased water quality parameters. Therefore data are included for disinfection performance, but not for other parameters. For other treatments shrimp performance in terms of mortality, growth and feed utilization did not significantly vary between treatments (table 2).

Table 2. Shrimp biomass gain, specific growth rate (SGR), relative growth rate (RGR) and feed conversion ratio (FCR). No significant differences were observed (ANOVA, n=3, $p>0.05$).

Parameter	(I) control	(II) $OH^{\bullet}$	(III) O_3
Initial biomass (g)	252 ± 21	258 ± 39	254 ± 23
Final biomass (g)	587 ± 52	564 ± 51	593 ± 60
Biomass gain (g)	335 ± 32	306 ± 13	339 ± 37
SGR (% bdw/d)	3.05 ± 0.10	2.77 ± 0.45	3.05 ± 0.34
FCR (g/g)	1.62 ± 0.15	1.77 ± 0.08	1.61 ± 0.19
Mortality (%/d^1)	0.13 ± 0.12	0.12 ± 0.19	0.17 ± 0.11

3.3.2 Addition of oxidants and total residual oxidants in the water

Average TRO values in all tanks were managed to keep at ≤ 0.06 mg/l Cl_2, no significant differences between tanks or treatments (table 3) were observed. To maintain desired TRO values in the BDD treatment applied current on the electrodes varied between 0.01 and 0.04 (A) (0.5 – 2.0 A/m²). Although TRO values were measured and regulated intensively, high variation within days and between days was observed in both ozone and BDD treatment. Peak values did regularly exceed the maximum of 0.06 mg/l Cl_2 and varied between 0.00-0.16 mg/l Cl_2 for treatment II ($OH^{\bullet}$) and between 0.00-0.11 mg/l Cl_2 for treatment III (O_3). The occurrence of TRO values above the safety level of 0.06 mg/l Cl_2 was significantly higher for treatment (II) $OH^{\bullet}$ compared with treatment (III) O_3. Although not significant, the trend in all tanks was that oxidant addition per day with respect to TRO safety levels, increased with feed load per day, suggesting that TRO breakdown in the tank is dependent on feed gift. Ozone addition was measured but due to analytical limitations values are given as maximum values to assure that no overestimations are made. Total energy consumption was significantly higher for treatment (II) compared to treatment (III).

Table 3. Total residual oxidants produced, oxidants added and energy input and energy consumption during the 30 day experimental period.

Parameter	N	(II) $OH^{\bullet}$	(III) O_3
TRO (mg/l Cl_2)	126	0.06 ± 0.02	0.04 ± 0.02
TRO > 0.06 (mg/l Cl_2) %	126	33.3 ± 7.4[a]	15.0 ± 4.0[b]
Oxidant added (mol/m³/d)	84	0.07 ± 0.02	< 0.03
Oxidant added (mol/kg feed)	84	1.16 ± 0.45	< 0.36
Applied charge (A/ m³/d)	84	0.08 ± 0.02	n.d.
Applied current density (A/m²)	84	1.2 ± 0.3	n.d.

3.3.3 Water quality parameters

Temperature and salinity were regulated and thus did not vary between treatments. pH and total alkalinity decreased over the experimental period as a result of the nitrification process but did not vary between treatments. Total ammonia nitrogen (TAN) and nitrite fluctuated within and between days, no accumulation took place and thus average concentrations over the experimental period were compared. No significant differences between treatments were observed (table 4).

Table 4. Water quality parameters recorded within the shrimp culture experiment (temperature, salinity, pH, total alkalinity, total ammonia nitrogen (TAN), nitrite, nitrate, ortho-phosphate, total iron, total organic carbon and colored dissolved organic matter). Different superscripts in the same row indicate significant differences (ANOVA, n=3-15).

Parameter	N		(I) Control	(II) $OH^{\bullet}$	(III) O_3
Temp (°C)	12	Average	28.0 ± 0.2	28.2 ± 0.3	27.9 ± 0.3
Salinity (psu)	12	Average	24 ± 3	23 ± 3	23 ± 3
pH	3	Initial	8.173 ± 0.095	8.073 ± 0.211	8.167 ± 0.106
	3	Final	7.293 ± 0.102	7.143 ± 0.081	7.063 ± 0.222
Total alk. (mM/l)	3	Initial	1.70 ± 0.01	1.67 ± 0.03	1.69 ± 0.02
	3	Final	1.04 ± 0.14[a]	0.64 ± 0.15[b]	0.64 ± 0.17[b]
TAN (mg/l)	15	Average	0.29 ± 0.14	0.25 ± 0.17	0.32 ± 0.12
NO_2-N (mg/l)	15	Average	0.11 ± 0.07	0.12 ± 0.10	0.08 ± 0.04
NO_3-N (mg/l)	3	Initial	98 ± 2	93 ± 6	93 ± 6
	3	Final	179 ± 7	181 ± 10	187 ± 0
Ortho-P (mg/l)	3	Initial	6.74 ± 0.10	6.11 ± 0.40	6.09 ± 0.40
	3	Final	11.75 ± 0.79[a]	9.81 ± 0.80[b]	13.60 ± 1.42[c]
Fe (mg/l)	3	Initial	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	3	Final	0.00 ± 0.00[a]	0.01 ± 0.01[ab]	0.02 ± 0.01[b]
TOC (mg/l)	3	Initial	8.40 ± 0.32	8.08 ± 0.70	8.18 ± 0.71
	3	Final	23.98 ± 0.81[a]	20.87 ± 2.08[b]	18.15 ± 1.66[c]
Abs. 436 nm	3	Initial	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.001
	3	Final	0.027 ± 0.001[a]	0.012 ± 0.002[b]	0.005 ± 0.003[c]

3.3.4 Effect of treatment on accumulating substances

NO_3-N, ortho-P, TOC and absorbance (436 nm) did significantly ($p>0.000$) accumulate in the control groups and showed high correlation with the CFB (figure 5) and therefore they were considered inert and thus effect of treatments could be found comparing slopes using ordinary least squares (OLS).

Nitrate showed significant accumulation in all treatments and showed high correlation with CFB but no significant effect of treatment on nitrate accumulation was found (table 5).

Ortho-P showed significant accumulation in all treatments, and significant differences between treatments were observed (figure 5). Accumulation in the hydroxyl radicals treatment (II) was significantly lower ($p = 0.038$) than control, accumulation of ortho-P in ozone treatment was significantly higher than in control ($p = 0.000$). TOC showed significant accumulation in all

treatments, and significant differences between treatments were observed (figure 5). Accumulation in the hydroxyl radicals (II) and ozone (III) treatments were significantly lower ($p = 0.038$ and $p = 0.000$) than control, and accumulation in ozone treatments was significantly lower than in hydroxyl treatment ($p = 0.037$).

CDOM did accumulate in the control groups, but not in both treatments. Therefore accumulation was significantly higher in control compared to hydroxyl radicals and ozone treatments ($p = 0.000$ and $p = 0.000$) but did not significantly vary between treatments ($p = 0.097$).

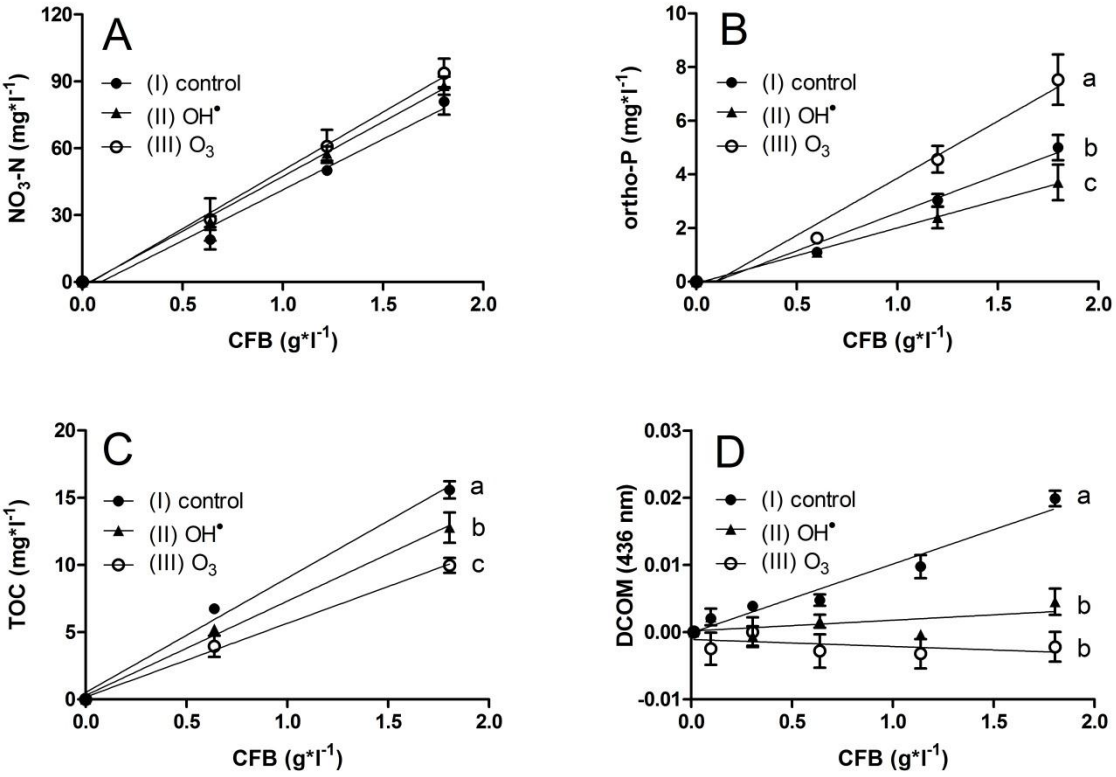

Figure 5. Increase in accumulating substances; A) nitrate-N, B) ortho-P, C) total organic carbon (TOC) D) colored dissolved organic matter (CDOM) over the experimental period against cumulative feed burden (CFB).

3.3.5 Disinfection performance

All disinfection treatments, (II) (III) and (IV) showed significant lower *vibrio* counts at the outflow of the respective disinfection unit compared to the inflow, no significant differences between treatments were observed. For total bacteria counts no significant disinfection was found for continuous hydroxyl (II) treatment, whereas ozone and intermittent treatment showed significant lower bacterial counts at the outflow (table 5).

Table 5. Reduction efficiency for total bacteria CFU and *Vibrio sp.* CFU for treatments (II), (III), (IV) taken at the inflow and outflow of the disinfection unit. P-value indicates outcome of one-sampled T-test, compared against no effect, different letters in different rows indicate significant differences between treatments (ANOVA, n = 9).

Parameter	Treatment	C TRO (mg/l)	T HRT (s)	C*T	Reduction efficiency %	p
Total-CFU	(II) $OH^{\bullet}$	0.06 ± 0.02	0.3	0.018	5.49^{ab} ± 12.63	0.168
	(III) O_3	0.04 ± 0.02	60	2.350	37.12^{ab} ± 36.02	0.042
	(IV) $OH^{\bullet}$	0.09 ± 0.03	0.3	0.027	76.66^{c} ± 15.79	0.000
Vibrio-CFU	(II) $OH^{\bullet}$	0.06 ± 0.02	0.3	0.018	59.12 ± 44.57	0.007
	(III) O_3	0.04 ± 0.02	60	2.350	81.99 ± 12.53	0.000
	(IV) $OH^{\bullet}$	0.09 ± 0.03	0.3	0.027	88.29 ± 21.62	0.000

3.3 Effect of treatment on bacterial pressure

Although very high deviations within treatments were observed for control and BDD treatment figure 3 shows clearly that total bacterial pressure in the culture tank was lower for the ozone treatments compared to control and hydroxyl treatment. Although significant reduction for *Vibrio* sp. was observed after addition of oxidants (table 5), no effect of treatment on *Vibrio* sp. pressure in the culture tanks was observed (figure 6).

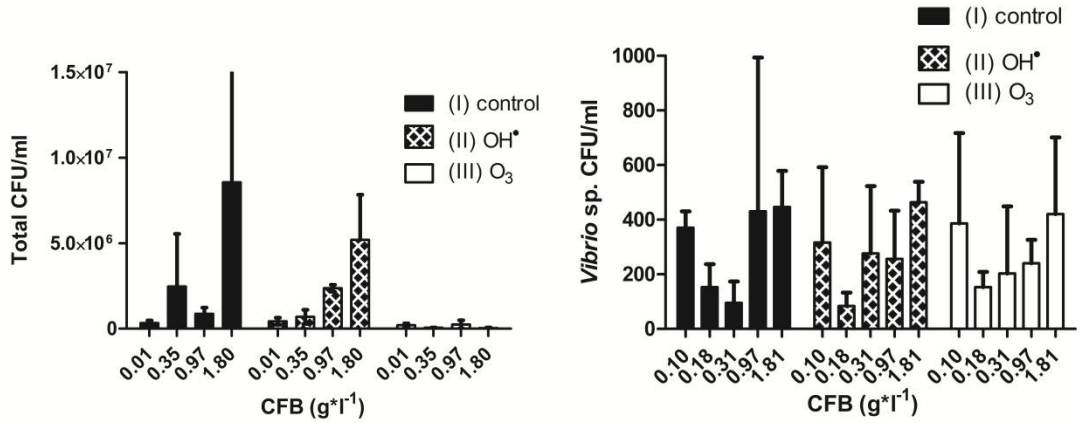

Figure 6a, 6b. Average a) total colony forming units (CFU) bacteria and b) *Vibrio* colony forming units against cumulative feed burden (CFB) during the 30 day experimental period.

3.7 Electrodes performance, maintenance and status

The relation between current (A) and potential (V) did not change during the 30 days of the experiment. This indicates that performance of the BDD-electrodes did not decrease during the experiment. Although the performance did not measurable decrease, electrodes built up a film of limestone between electrode-pairs.

4. Discussion

4.1 Total residual oxidant formation on BDD-electrodes and implications for RAS

Oxidation of saline water, containing halogen ions like Cl^- and Br^-, with hydroxyl radicals leads to formation of mono- di and tri- forms of chlorine and bromine species (Bergmann and Rollin 2007, Echardt and Kornmueller 2009). The reactive forms can be measured with the DPD method and the sum of the reactive oxidants is called total residual oxidants (TRO). TRO are known to be very toxic to shrimp and fish. Concentrations as low as 0.1 mg/l Cl_2 significantly decrease survival of shrimp (*L. vannamei*) at chronic exposure and 0.06 mg/l Cl_2 was given as safety value (Schroeder et al. 2010) although more sensitive parameters such as feed intake and growth performance were not considered. When saline water also contains ammonia and organics, such as RAS system water, chlor- and brom-amines, chlor- and bromo-acetic acids, bromate, chlorate, perchlorate and chloroform and bromoform are other main formation products (Echardt and Kornmueller 2009, Diaz et al 2011). Due to their lower reactivity bromate, chlorate and bromoform accumulate at much higher levels than other species (Echardt and Kornmueller 2009) but these oxidants are not detected and measured by the DPD-method used in this study. LC-50 values of bromoform to marine fish (*Brevoortia tyrannus)* and shrimp *(Penaeus aztecus)* were found at 10 mg/l and 26 mg/l respectively. However sublethal effect revealing stress, such as unselective rapid swimming and hitting the walls, were already observed for concentration of 0.4 mg/l bromoform, indicating that stress already occurs at low concentrations (Gibson et al. 1979).

Considering the extreme toxicity of reactive oxidants and the stress reaction of shrimp to stable accumulating oxidants the prevention or minimization of TRO formation should be the key point in BDD-treatment of aquaculture water. In the present research formation of TRO was dependent on current density with lower TRO values than one can expect when linearity is assumed, at current densities below 27 A/m² (figure 1). Minimum TRO formation was found at 17 A/m² whereas maximum change in ORP was found at 22 A/m². These both indicates that at a current density of 17-22 A/m² larger molecules or particles are oxidized to smaller fractions, reducing TRO formation and lowering ORP. Lowest current efficiencies for TRO formation, which are desired due to toxicity of TRO, were observed at lowest current densities applied with current efficiencies close to 0 (figure 2).

Even using such low current densities, TRO did accumulate (figure 3) limiting the applied doses. During the present research TRO levels for ozone treatments were 0.04 ± 0.02 mg/l Cl_2 and 0.06 ± 0.02 mg/l Cl_2 for BDD-treatment. No significant differences in growth or feed efficiency were observed, however one has to consider that the present experimental design is not optimal for testing growth and production performance. Trends in growth and feed efficiency showed lower values for BDD-treatments compared to control and ozone treatments, although not significant. However one can assume that, when experimental period would be longer effects would be more pronounced and an average TRO value of 0.06 might be too high for an optimal shrimp culture performance. Further research is needed to quantify safe TRO concentrations for commercial aquaculture with respect to feed intake and feed utilization efficiency. When BDD-electrocell treatment is up-scaled to commercial size marine RAS, current densities should be kept as low as possible to minimize TRO formation. Furthermore optimization of hydrodynamics in the electrocell, a turbulent flow instead of a laminar flow, might optimize performance of the electrocell.

4.2 Inorganic nitrogen accumulation and conversion on BDD-electrodes in RAS

Continues increased levels of TAN, NO_2-N or NO_3-N all decrease fish and shrimp production performance (Colt, 1996). The conversion of TAN to N_2 and NO_3 to N_2 are processes that remove nitrogen from the culture water, and therefore might improve production in RAS. Several authors have studied the behavior of inorganic forms of nitrogen in waste water after electrolysation with BDD-electrodes (Kraft 2003, Georgaud et al. 2011, Levy-Clement 2005, Bergmann and Rollin 2007, Michels et al. 2010). A simplified summary of processes observed by these authors is:

$N_2 \leftarrow$ TAN $\leftrightarrow NO_2 \leftrightarrow NO_3 \rightarrow N_2$

Both oxidation of TAN and reduction of NO_3 have the advantage that N_2 is formed and nitrogen is removed from the culture water. Disadvantages of the oxidation of TAN to N_2 is the requirement of high, fish and shrimp toxic levels of TRO, formation of several chlorine, bromine and THM species (Diaz et al. 2010), the formation of chloramines (Bergmann and Rollin 2007) and formation of the byproducts NO_2 and NO_3 (Kraft, 2003). Furthermore TAN oxidation is inhibited by nitrite and yellow substances, decreasing its efficiency and suitability for aquaculture waste water (Diaz et al., 2010, Schroeder et al. 2011) and thus is considered risky and economic unviable for marine RAS purposes (Schroeder et al., 2011). Disadvantages of the cathodic reduction of NO_3 to N_2 is the formation of byproducts TAN and NO_2 and chloramines and that the reduction process is slow (Levy-Clement 2005). Due to this slow cathodic reduction process, at the anodic side TRO are formed, facing the same disadvantages as by TAN oxidation. Therefore authors suggest cathodic reduction of nitrate only an option by highly concentrated or toxic waste waters until biological treatment is possible (Levy-

clement 2005, Georgaud et al. 2011). In the present study no effect on nitrogen accumulation was found, only oxidation of nitrite-N was found but the effect was low compared to biofiltration. Opportunities for anodic conversion of nitrate to nitrogen gas in marine RAS water needs further research.

4.3 Organic carbon accumulation and conversion on BDD electrodes in RAS

Total organic carbon in RAS originates from feed but carbon species present in the water are diverse. Generally TOC is divided in a particulate and a dissolved organic fraction. The particulate fraction can be removed by mechanical filtration but removal efficiency is dependent on densities and size. Due to pump sharing solids are decreased in size and fine solids accumulate in RAS and the accumulation of fine solids can affects health of cultured animals. Furthermore TOC is the limiting nutrient for the growth of heterotrophic bacteria in RAS, and thus by reducing TOC the total amount of heterotrophic bacteria can be reduced. If chemical removal of TOC leads to a reduced pathogenic bacterial load is questionable, because a reduced system HRT or increased disinfection performance leads to less stable and more pathogenic bacterial communities (Schneider et al., 2007, Attramadal et al., 2012). The dissolved carbon fraction consists of humic acids which accumulate in RAS. Effects of humic acids on fish growth are not known, however humic acids might promote health and reduce stress in fish raised in RAS (Meinelt et al. 2008). The chemical removal of TOC in RAS is thus still questionable. During the present research significant removal of TOC and CDOM by hydroxyl radicals was observed. Current efficiency for TOC removal was estimated to be 0.11 (table 6). Other data on removal of TOC in aquaculture waste water are not available, however reduction of chemical oxygen demand (COD) in aquaculture waste water as a measurement of organic load, was also observed by Diaz et al. (2010) using BDD-electrodes at current densities between 5 and 50 A/m², and by Feng et al (2003) using Ti/RuO2 electrodes in pond water. During the present research ozone removed significantly more TOC compared to hydroxyl radicals. Molar conversion efficiency was estimated to be 0.19 and this was solely due to chemical oxidization of TOC. Ozone is known for its ability to improve flocculation of organic matter and thereby improving mechanical removal in RAS (Summerfelt and Hochheimer, 1997). Removal efficiencies of ozone in combination with foam fractionating would likely be higher than values obtained during the present study. Further research is needed to see if BDD-treatment also can improve mechanical removal due to flocculation of organic matter and if a combination of BDD-treatment and foam fractionating would be useful.

4.4 Ortho-P accumulation and conversion on BDD-electrodes in RAS

The effect of accumulated ortho-phosphate levels on fish or shrimp performance and health are unknown. However, phosphate is the main factor in eutrophication of coastal areas and thus phosphate

removal from RAS water, in system or at the end-of-the-pipe, is relevant. Anodic removal of total phosphorus with hydroxyl radicals, produced with Ti/RuO2 anodes, is reported, but reaction mechanisms are not described (Feng et al., 2003). In the present study significant removal of orthophosphate was observed in the BDD treatment, although current efficiencies for ortho-phosphate removal were very low (table 6). Hydroxyl radicals are known to carry out electron transfer with HPO_4^- and PO_4^- to form hydroxide ions and a phosphoric acid ion with a changed valence state (Thomas, 1965). A possible pathway of phosphate removal during our study is precipitation of the orthophosphate anion with a cation. Several authors reported the formation of limestone on BDD-electrodes (Diamadopoulos et al., 2009). In our study growth of biofilm and limestone ($CaCO_3$) at the BDD-electrodes was observed, and limestone was mechanically or chemically removed by cleaning of electrodes, or removed by re-polarization of the electrodes and then filtered out of the water by the filter mats. Because orthophosphates are absorbed by $CaCO_3$ in seawater (Millero et al., 2001) it might be that the removal of orthophosphates is due to absorption of orthophosphates on calcium carbonate, and then removed by mechanical filtration. Further research is needed to find out reaction mechanisms and to optimize phosphate removal by BDD-electrolysation.

Table 6. Conversion efficiency of accumulating substances by ozone and hydroxyl radicals, expressed in mole per mole during the present research. Values for NO_2-N and TRO are calculated from short term experiment (equation 17&19) and values for NO_3-N, ortho-P and TOC from long term experiments (figure 5). n.s.= no significant effect measured.

Conversion efficiency	**1 mole O_3**	**1 mole $OH^\bullet$**
TAN (mole)	n.s.	n.s.
NO_2-N (mole)	0.124*	0.064
NO_3-N (mole)	n.s.	n.s.
Ortho-P (mole)	-0.03± 0.02	0.022 ± 0.018
TRO (mole)	0.0 - 0.4*	0.0 - 0.056
TOC (mole)	0.19±0.03	0.11 ± 0.04

*Values for ozone are based on study of Schroeder et al. (2010) figure 1 and 3, using similar tanks, same ozone injection system and same species.

4.5 BDD-treatment disinfection in RAS

During the present research a disinfection efficiency of roughly 80% was achieved for total bacteria and 90% for *Vibrio* sp., during intermittent disinfection with current densities of 14.8 ± 3.0 A/m² at the BDD-electrodes. When hydroxyl radicals were applied at continues safe concentrations at a current density of 1.2 ± 0.3 A/m² disinfection efficiency decreased to 0% for total bacteria count and 60 % for

Vibrio sp. counts. Continues applied ozone resulted in higher disinfection efficiencies, 40% for total bacteria counts and 80 % for *Vibrio sp.* counts (table 7). Differences between disinfection efficiencies between total bacteria and *Vibrio* species might be explained by the fact that total bacteria are mostly particle-aggregated which protects them against oxidants, and Vibrio are free living and thus more susceptible (Hess-Erga et al., 2008). Literature values for BDD-disinfection in saline water are limited to the data set of Echardt and Kornmueller (2009) who achieved a disinfection rate of >97 % for E. coli bacteria, unfortunately current densities are not given but TRO levels of 1.4 mg/l Cl_2 were observed. Therefore achieving significant disinfection in RAS culture water using BDD-electrodes seems impossible due to TRO formation. Differences between disinfection efficiency of hydroxyl radicals and ozone might be shorter half-life time of hydroxyl radicals in water (Westerhoff et al., 1999). In RAS water disinfection efficiency is expressed by the factor C*T were C is the concentration of oxidants in mg/l and T is the exposure time in minutes. For ozone, applied to ozone demand free water, C*T values of 0.01 obtain a 99.9 % disinfection, and in aquaculture effluent or system water C*T values of 0.2 – 0.5 are mostly sufficient for a 99.9 % disinfection with exposure time around 1 minute (Timmons and Ebeling, 2010).

The bacterial pressure in the culture tanks was not affected by continues treatment with hydroxyl radical. In both control and hydroxyl radicals total bacterial counts increased with increasing CFB. Treatment with ozone resulted in significant lower total bacteria counts in the culture tanks, but not in lower *Vibrio sp.* counts. Hydroxyl radicals are active disinfectants itself, additionally in marine water the secondary formed bromine and chlorine compounds are also active disinfectants (Echardt and Kornmueller, 2009). Increased concentrations of residual oxidants do increase disinfection performance by equal current densities suggesting that secondary formed oxidants are more effective than hydroxyl radicals itself (Furuta et al. 2005). However during the present research TRO levels were on average 50 % higher in the hydroxyl treatments (0.06±0.02) than in the ozone treatments (0.04±0.02). If equal forms of TRO are produced by ozone and hydroxyl radicals, the differences in disinfection efficiency can be explained by the difference in half-life time between ozone and hydroxyl radicals making ozone a more favorable disinfectant.

5. Conclusions

Electrolyzation of marine RAS water with BDD-electrodes leads to formation of TRO which can accumulate in RAS to shrimp toxic levels, limiting the applied doses of hydroxyl radicals. By applying low current densities (1.2 ± 0.3 A/m²) significant removal of TOC, CDOM and ortho-P was achieved without significantly affecting shrimp growth, feed efficiency and survival. No significant removal of nitrate was observed. Disinfection performance at these low current densities is very low, therefore no effects on bacterial pressure in culture tanks could be observed. Although current was re-poled twice a day rapid formation of limestone on BDD-electrodes was observed changing the actual applied current

density. When formation of limestone can be prevented, BDD-treatment can become a safe way of water treatment in marine RAS systems to decrease accumulation of TOC and CDOM, but especially to decrease ortho-P accumulation which is not possible using conventional ozonation. Further research on BDD-electrochemical denitrification in marine RAS is suggested.

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Chapter 5:

Improving water quality and reducing nutrient emission in commercial scale marine recirculating aquaculture systems by using Boron Doped Diamond (BDD) electrodes – Effect on N, P, C and metal removal, residual oxidant formation and disinfection performance.

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Abstract

The use of Boron Doped Diamond (BDD) electrodes is a relative new water treatment technique, but until now application in RAS is limited to laboratory or aquarium scaled research. Therefore, we studied the effects of a BDD electro cell, the EctoSys operated at 10 m^3/h, in a 45 m^3 cold water marine RAS on disinfection and water quality. RAS water was treated for 10 days using only mechanical and biological treatment as control, 10 days with additional ozonation at 5.2 $g/m^3/d$ without foam fractionating, and 10 days with additional BDD produced hydroxyl radicals at current densities of 14 A/m^2 instead of ozonation.

The accumulation of nitrates and total phosphorus was not influenced by treatment. The accumulation of ortho-phosphate and total organic carbon was significantly reduced by BDD treatment, but not by ozonation. The accumulation of colored dissolved organic matter was significantly reduced by BDD treatment but even more by ozonation. Metal concentrations did not significantly vary between treatments. TRO production by BDD electrodes was lower compared to ozone, but formed oxidants were more persistent resulting in higher TRO concentrations in the culture tanks. Although in both treatments some disinfection was observed no effects of treatment on bacterial densities in the culture tanks were observed.

Results, with respect to organic carbon and orthophosphate removal, are promising to improve culture conditions in RAS system water and to reduce nutrient emissions. Further research on cathodic nitrate removal and coupling BDD treatment to mechanical removal methods such as flocculation or foam fractionating, to increase removal efficiencies of particulate matter, is advised.

1. Introduction

Aquaculture production in recirculating aquaculture systems (RAS) offers several benefits over against common production systems as pond or cage culture. Benefits include improved control of water quality, improved disease management, no risk of escapees, less dependence on surface waters, better waste stream management and reduced water consumption (Martins et al. 2010). By recirculating and re-use of water, substances originating from feed or make up water do accumulate in such systems which can negatively affect survival, production or fish quality (Davidson et al., 2009, Martins et al. 2009, Martins et al. 2011, Schrader et al. 2010, van Bussel et al, 2012). These substances include ammonia, nitrite, nitrate, phosphate, metals and dissolved and particulate organic matter. Although waste streams are treated separately inside the RAS, water exchange is still inevitable. Therefore the re-use of water is limited by the system's ability to maintain water quality at acceptable levels. Thus by improving water treatment in RAS, water consumption per amount of feed applied, or fish produced, can be reduced. Consequently energy requirements for water treatment do increase, making RAS production, less favorable compared to common production systems in terms of global warming, acidification and land competition (Fitwi et al., 2013). Therefore reducing energy consumption, next to water consumption, is a future challenge for RAS development.

Advanced oxidation process using Boron Doped Diamond (BDD) electrodes has proven its ability for removing ammonia, nitrite, nitrate and COD in saline waste water (Kraft et al., 2003). Furthermore treatment with BDD electrodes can be an effective way of disinfection in saline water (Echardt and Kornmueller, 2009). Energy consumption of electrolysis is lower compared to ozonation due to low energy use by electrodes and no need for additional use of chemicals such as oxygen (Oturan and Brillas, 2007). Both disinfection and nutrient oxidation are an effect of the direct oxidation by hydroxyl radicals or oxidation by formed by-products such as free bromine and chlorine species. In fresh water hydroxyl radicals are produced at the anodic side of BDD electrodes according to

$$H_2O \rightarrow OH^{\bullet} + H^{+} + e^{-}$$

Hydroxyl radicals react very fast, are unselective and extremely short-living. However when marine water is electrolyzed with BDD-electrodes simultaneously to hydroxyl radicals, free chlorine is formed according to:

$$2\ Cl^{-} \rightarrow Cl_2 + e^{-}$$

In marine water chlorine gas is immediately converted into hypochloric acid which further dissociates depending on the pH:

$Cl_2 + H_2O \rightarrow HCl + HOCl$
$HOCl + H_2O \leftrightarrow OCl^- + H_3O^+$

In marine water not only chloride but also bromide is present. The chloride ion in hypochloric acid will be substituted with a bromide ion forming the stable hypobromous acid

$HOCl + Br^- \rightarrow HOBr + Cl^-$

(Echardt and Kornmueller, 2009). Next to the simultaneous formation of free chlorine small amounts of free bromine are produced:

$2\ Br^- \rightarrow Br_2 + e^-$

Like chlorine gas, bromine gas is immediately converted into hypobromous acid which further dissociates depending on the pH:

$Br_2 + H_2O \rightarrow HBr + HOBr$
$HOBr + H_2O \leftrightarrow OBr^- + H_3O^+$

Thus in marine water with pH around 8, hypobromous acid is the dominant residual oxidant formed (Kornmueller 2007). Furthermore chlorate, perchlorate and bromate are formed (Bergmann and Rollin 2007, this thesis chapter 6). When saline water additionally contains organics and ammonia, which is inevitable in RAS, organic chloramines, organic bromamines, chloracetic acid, bromoacetic acids, chloroform and bromoform are other main formation products (Bergmann and Rollin 2007, Echardt and Kornmueller 2009, Diaz et al 2011).
With the exception of perchlorate, bromate and chlorate which are relatively non-toxic, all other by-products are harmful to fish and shrimp causing stress, morphological changes and mortality at concentrations less than 0.5 ppm (Gibson et al., 1979, Hutchinson et al., 1997 Reiser et al. 2010, Schroeder et al. 2010, van Wijk and Hutchinson, 1995). Thus formation of by-products should be minimized and accumulation in system water must be avoided.
Data on the use of BDD electrodes in marine aquaculture water are limited to only two studies. Diaz et al. (2010) treated marine aquaculture water with BDD electrodes in lab scaled systems and promoted BDD-treatment as alternative to biological filtration for the removal of ammonia, nitrite and COD. However experiments were carried out in a statical optimal lab environment, not in a dynamic biological environment, and flow rates used were 100-200 times higher than optimal flow rates suggested for RAS (Timmons and Ebeling, 2010). Furthermore concentrations of toxic TRO exceeded safety levels for fish and shrimp culture 100 times, thus even the suggested removal of TRO with

active carbon is risky, because by possible failure the complete stock will die within minutes. Data obtained by Diaz et al. (2010) are therefore useful to obtain basic knowledge about the processes taking place in marine RAS water, but are hard to implement in commercial scaled systems.

A recent study of van Bussel et al. (2013a) showed that water treatment with BDD electrodes in small scaled RAS, is possible using low current densities and optimal flow rates, without exceeding TRO safety levels and without significant negative effects on shrimp (*Litopeneaus vannamei*) health. In addition significant reduced accumulation of TOC, CDOM and ortho-phosphate (ortho-P) could be observed.

Goal of the present study was to see if results obtained in the small scaled RAS (300 l, van Bussel et al., 2013a), could also be obtained in a commercial scale RAS. Therefore, in several short term experiments the formation of TRO using BDD electrodes and the behavior of the oxidation reduction potential (ORP) in marine RAS culture water was studied. Thus the intensity of water re-use was artificially increased over a period of 10 days while water treatment consisted of mechanical and biological filtration only, and the accumulation of substances was monitored. Afterwards this procedure was repeated but additionally ozone was added to the culture water, then this procedure was repeated a third time but instead of ozone addition water was treated with BDD-electrodes. Next to the accumulation of substances in the culture water, bacterial pressure and disinfection performance was monitored. To guarantee fish welfare BDD and ozone treatment intensity were kept below 0.06 mg/l Cl_2 TRO levels in accordance to Schroeder et al., 2010)

2. Material & Methods

2.1 System setup

2.1.1 Electrodes and electro cell set up

The electro cell used in this experiment was a prototype ECTOSYS® (RWO GmbH, Bremen, Germany) equipped with 3 pair of electrodes consisting of a Niobium carrier material and a 20 µm thick Boron doped diamond layer, type DIACHEM® 100 mm diameter (CONDIAS GmbH, Itzehoe, Germany). Flow rate through the electrocell was kept constant at 10 m³/h using an ultrasonic clamp-on flow meter (Optisonic 6400, KROHNE Messtechnik GmbH, Duisburg, Germany).

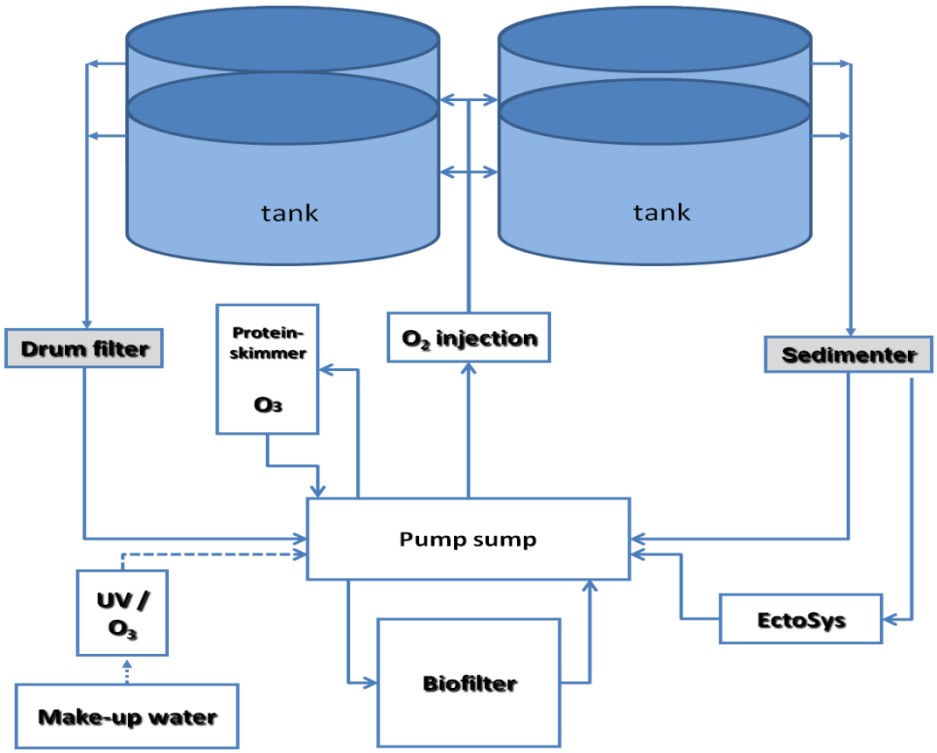


Figure 1. Schematic drawing of the RAS used in the present study.

2.1.2 RAS

Experiments were conducted in a commercial scale RAS which consisted of 10 cylindrical tanks (radius of 1.8 m, 2.3 m³ each) constituting a total volume of 41 m³. RAS were stocked with 630 adult turbot (*Psetta maxima*) in the range of 550-1800 gram with an initial total biomass of 619 kg. The effluent water of the fish tanks is drained via central bottom effluent and a second effluent-close to the surface (Dual-Drain-Design). The bottom effluent is regulated by stand pipes. The effluent water can be split into the brush sedimenter (Prototype, Spranger Kunststoff, Germany) with flow rates of 10 m³/h and the drum filter, with flow rates of 80 m³/h and 60µm pore size (type KTS8/12, Dryden aqua, Scotland).The flow through the drum filter was led directly into the pump sump, the flow through the brush sedimenter was pumped through the ECTOSYS electrocell into the pump sump. Water from the pump sump is directed by three pumps (max. 50 m³/h each) into one of the following circuits: fluidized bed filter and back into the pump sump; protein skimmers (10 m³/h, type Helgoland 500, Sander skimmers, Germany) or a plate heat exchanger and back into the pump sump or via an oxygen saturator cone back into the fish tanks. Temperature and oxygen were measured and regulated automatically. pH, conductivity and water exchange were measured and recorded automatically. Optionally ozone was produced by an ozone-generator (Ozone Generator C-Lasky, AirTree Ozone Technology Co., Taiwan) using liquid oxygen. Foam produced in the protein skimmer was not discarded but led back into the pump sump to compare solely the chemical effect of ozone, and not

additional the mechanical removal through foam fractionating. Optional addition of hydroxyl radicals was realized with the ECTOSYS system. The electric current required for electrolyzation was generated by a laboratory power supply (model EA-PS 3032-05 B, HEIDE power GmbH, Pürgen, Germany). The applied potential (V) and current (A) on the BDD-electrodes were recorded by hand.

2.2 Experimental set up and procedure

2.2.1 Pre- experiments

Pre-experiments were carried out to determine TRO formation and ORP behavior, energy consumption and nitrite and ammonia removal by different current densities and different water matrices. Differences in water matrices were obtained by changing the applied feed load and make-up water flow.

2.2.3 *Longterm experiment*

The long term experiment consisted of three experimental periods of 14 days each, 4 adaptation days and 10 experimental days. The first 4 days, the adaptation period, fish were fed 1 kg feed per day and water exchange rates were set at 10% total volume per day (4.1m^3/kg feed applied/d). Additionally foam fractionating was enhanced by ozone injection and in contrast to experimental periods foam was discarded to maximize nutrient removal from the system water (see table 1).
During the next 10 day experimental period fish were fed until apparent satiation twice a day. After 5 days (120 hours) experimental period the makeup water flow was closed to artificially increase CFB. Because water was discarded through the backwashing of the drum filter, water level in the tanks was lowered to maintain constant water levels in the pump-sump. Lowering of water in tanks was equal for all 3 experimental periods. Water treatment during the 3 experimental periods of 10 days consisted of:

- Control (I): Drum filter, moving bed biological nitrification, sedimentation
- BDD (II): equal to control + continues applied current on BDD-electrodes.
- Ozone (III): equal to control + continues ozone addition

In order to ensure comparability of treatment (II) and (III), dosages of hydroxyl radicals and ozone were standardized at TRO concentration $\leq$ 0.06 mg/l in the culture tanks. Therefore, the TRO concentration was measured at the inflow and outflow of the disinfection unit (electrocell or ozonation) and in the culture tanks, once a day in the control treatment and 5 to 6 times a day during ozone or BDD treatment.

Table 1. Experimental phases and treatments with the main parameters measured.

Experimental phase	Time	Treatment (water exchange rates)	Focused parameters	Samples
Pre-experiment	3 d	Electrocell 0-5 A	TRO & ORP	3
		(4 m^3 / kg)	NO_2 & NH_4	3
			energy	1
Pre-experiment	3 d	Electrocell 0-5 A	TRO & ORP	3
		(1 m^3 / kg)	NO_2 & NH_4	3
			energy	1
Pre-experiment	3 d	Electrocell 0-5 A	TRO & ORP	3
		(0.2 m^3 / kg)	NO_2 & NH_4	3
			energy	1
Cleaning	*4 d*	*Protein skimming with O_3 (10 m^3 / kg)*		
Control	10 d	Manually increase CFB	N,P,C, CDOM	10
			metals	3
			bacterial counts	6
Cleaning	*4 d*	*Protein skimming with O_3 (10 m^3 / kg)*		
Ozone	10 d	Manually increase CFB additional ozonation	N,P,C, CDOM	10
			metals	3
			bacterial counts	6
			disinfection	6
Cleaning	*4 d*	*Protein skimming with O_3 (10 m^3 / kg)*		
BDD	10 d	Manually increase CFB additional applied current (1A) on electrocell	N,P,C, CDOM	10
			metals	3
			bacterial counts	6
			disinfection	6

2.3 Water quality analysis

Water samples for the pre-experiments were taken at the inflow and outflow of the electrocell or ozonation, respectively. During the long-term experiment water quality samples were taken at the inflow, however for TRO samples were directly measured at the in- and out-flow of the protein skimmer, respectively electrocell. TP and O-P were measured spectrophotometrically with the ascorbic acid method using a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland) according to the protocols of Grasshoff et al. (1999) and adapted to microplate format. TAN, NO_2-N, NO_3-N and TRO were determined spectrophotometrically using a HACH-DR 2800 spectrophotometer

(Hach-Lange GmbH, Berlin, Germany) and powder pillow detection kits (Hach-Lange GmbH, Berlin, Germany) based on the ammonia salicylate method, diazotization method, the cadmium reduction method, 1,10 Phenantrolin method and the N,N-diethyl-p-phenylenediamine (DPD) method, respectively. Total residual oxidants (TRO) is the name used for all substances that oxidize N,N-diethyl-p-phenylenediamine (DPD). Oxidants known to measure with the Hach-Lange DPD method include all free and total chlorine and bromine. Interference with trihalomethanes (THM) chloramines, bromamines or organic chloramines is minimal due to quick reaction of free chlorine and colormetric determination within seconds after measurement (Harp 2002) . TRO is measured in mmol/l but notated during the present study as clorine (Cl_2) equivalents in mg/l. TOC was analyzed as non purgable organic carbon using a total organic carbon analyzer TOC-V CPH/CPN (Shimadzu corp., Kyoto, Japan). Dissolved metals were analyzed in duplicate using Inductively coupled plasma mass spectrometry (ICP-MS) method (TeLa, Technische Lebensmittel- und Umweltanalytik GmbH, Bremerhafen, Germany) in 100 ml samples. Temperature and pH were measured with a WTW 340i portable multiparameter instrument (WTW GmbH, Weilheim, Germany), salinity with a digital refractometer HI 96822 (HANNA Instruments, Woonsocket, USA) and dissolved oxygen with a Handy Polaris oxygen probe (Oxygard International A/S, Birkenrød, Denmark) ORP with a redox probe (Hach Lange GmbH, 1200-S sc). Dissolved colored organic matter (CDOM) was determined by direct absorption at 436 nm of pre-filtered (0.22 µm) culture water and compared against a blank of artificial seawater (ASW) at 20 g l^{-1} Cl^-. Absorption was measured with a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland). A BMT ozone analyser 963 (BMT Messtechnik GmbH, Berlin, Germany) was used to determine ozone addition by measuring flow rate and ozone concentration in the gas stream.

2.4 Microbial analysis

Water samples for the pre-experiments were taken at the inflow and outflow of the electrocell or ozonation, respectively. Samples were taken one by one. Directly after each sample was taken, the respective sample was diluted and analyzed by the spread plate method using an unspecific marine agar (Difco Marine Agar, BD, Sparks, USA) for the detection of total viable cell counts, as well as a *Vibrio sp.* specific agar (Difco TCBS Agar, BD, Sparks, USA) for the detection of Vibrios. Colony forming units per plate were recorded after 24 h and 96 h of incubation at 28 °C.

2.5 Assumptions and formulas

Current density (CD) was calculated as follows:

CD = Applied current (A) / working surface electrode (1)

Working surface electrode (WSE) was calculated as follows

WSE = surface electrode * active area electrode * surface factor (2)

In this case the surface of an electrode = radius² * Π, the radius was 50 mm. The active area of an electrode is the area functioning actively as anode, which is dependent on the three dimensional arrangement of the electrodes pair which depends if an electrode is active on one or both sides. Because we used 3 pairs, 2 anodes were active at both sides and 1 electrode was active at only 1 site. Thus the total active area in our case was 5. The surface factor is a correction for the roughness of the diamond surface and a correction for the fact that a mesh is used, not a plate, the surface factor used here is 1.8.
Thus value used for WSE is (0.05*0.05*Π)*5*1.8= 0.0707 m².

To compare ozonation and BDD treatment the amount of charge applied was converted into mole according to:

1 Faraday = charge of one mole e^- (3)
1 Faraday = 96485.3399 C (4)
1 C = 1 A*s (5)

When oxidized or produced substances are plotted against Faraday, the oxidant equivalent of 1 mole, the current efficiency can be estimated.

Current efficiency = substance X (mole) / Faraday (6)

For ozone, molar efficiencies can be calculated in the same way

Molar efficiency = substance X (mole) / O_3 (mole) (7)

To compare the accumulation of substances in the culture water the function cumulative feed burden (CFB) is used. This function is describes the amount of feed added per volume of water, corrected for water exchange and is calculated as follow:

CFB_{x+1} = (CFB_x + Feed added (g) / V_{total} (l)) ^ (-V/V_{total}) (8)

where “feed added” is the amount of feed added per tank in g, “V_{total}“ is the total culture volume and “–V” is the amount of water replaced.

As CFB was set to 0 at the start of the experiment although the initial process water matrix was not free of accumulating substances, accumulating water parameters (NO3-N, OP, TP, TOC, metals and dissolved matter) are presented as increase of the parameter:

$$F(x) = mx + b \quad (9)$$

where x is the cumulative feed burden, F(x) is the accumulation of the water parameter and b is the consumption or production of the parameter at a CFB = 0.

Reduction efficiency for disinfection was calculated as the difference in CFU between inflow and outflow

$$RE\ (\%) = (CFU_{inflow} – CFU_{outflow} / CFU_{inflow})*100 \quad (10)$$

Reduction of CFU was calculated for *Vibrio sp* (Vibrio %), total bacterial counts after 24 hours (24 h %) and for total bacteria after 96 hours (96 h %).

2.6 Data analysis

Data are presented as mean ± standard deviation (SD) of n samples. Statistical analysis of variances was performed using SPSS 20.0 (IBM inc, Armonk, USA). Data were tested for normality and for equal variances using Levene’s test and the Shapiro-Wilk test, respectively. Consequently the Tukeys-HSD post-hoc test, Dunett-T3 test or the Kruskal-Wallis test followed by the Mann-Whitney U test for pairwise comparisons was used. For the comparison of two means with a normal distribution Students T-test was used. For comparison of two means with a non-normal distribution the Mann-Withney U test was used. Single comparisons were carried out by paired or unpaired t-tests. Linear regression was conducted using Graphpad (Graphpad software inc., La Jolla, USA). Differences were considered as significant at $p<0.05$.

3. Results

3.1 Short term experiments

The relation between the applied current and the potential needed to apply a charge on the electrocell could be described with a 2nd order polynomial regression (figure 1). The minimum potential needed to apply a charge was 2.7 V. From 2 A onwards, the relation between applied current and the potential needed could be described by a linear function, meaning that direct energy costs (V*A) to apply a charge (C), is favorable below 2 A (28 A/m²).

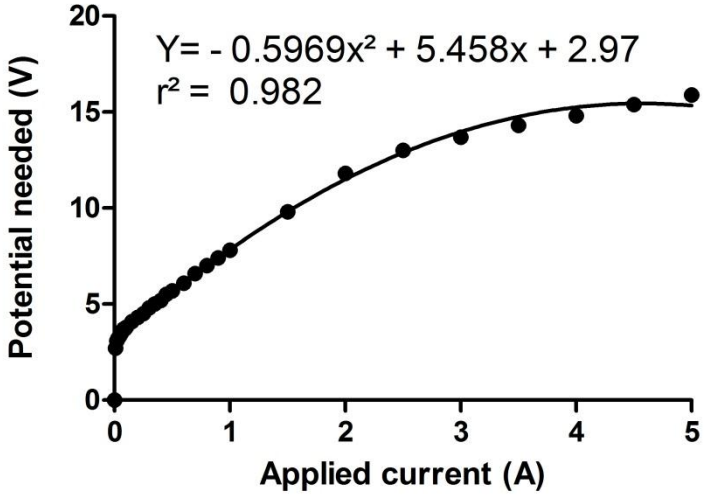
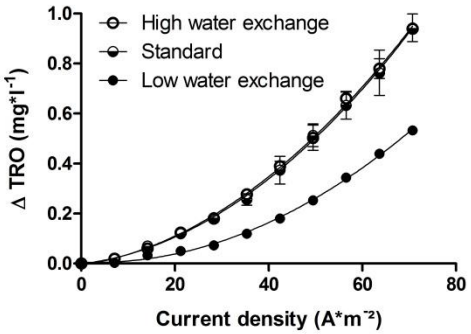
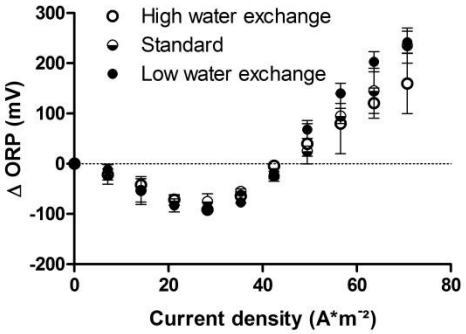


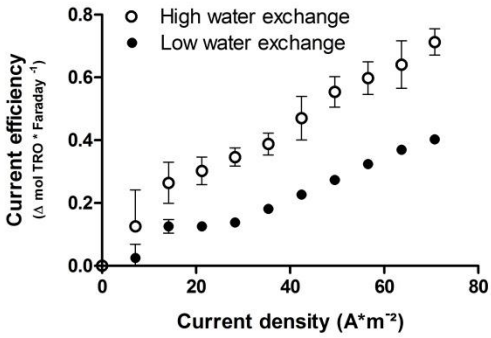
Figure 2. Relation between applied current on the electrocell and potential needed to apply charge.

Although different water exchange rates were applied, differences in water quality were only distinctive in color absorption and pH, but not in other water quality parameters measured (figure 2). The relations between TRO formation and applied current density at different water exchange rates could be accurately (r^2 = 0.986-0.997) described by a 2nd order polynomials. Accordingly current densities followed an almost linear relation with current efficiency for the high and standard water exchange rates, for the low water exchange rates current efficiency was constant between 7 and 28 A/m² also followed by a linear increase (figure 2). The slope of the increase in current efficiency was significantly lower for low or standard water exchange rates compared to high exchange rates (F-test, n=7, $p<0.001$) but not between low and standard exchange rates.

Oxidation reduction potential showed similar behavior for all three water exchange rates tested. A clear decrease in ORP between 0 and 28 A/m² followed by an increase in ORP.







	High water exchange	Standard	Low water exchange
TAN (mg l^{-1})	0.1	0.1	0.2
NO_2-N (mg l^{-1})	0.2	0.2	0.2
NO_3-N (mg l^{-1})	59	64	79
PO_4-P (mg l^{-1})	4.7	5.6	6.2
TOC (mg l^{-1})	7.4	8.7	9.7
Abs. 436 nm.	0.0007	0.0012	0.0063
Salinity (ppt)	24	25	25
pH	7.6	7.6	7.2

Figure 3. Differences in TRO and ORP between in- and out-flow of the electrocell at different current densities (n=3) and calculated current efficiencies in different water matrices. Main water quality parameters of the different matrices are described in the table.

Concentrations of TAN, and TOC were compared between inflow and outflow at a current densities between 0 and 71 A/m², however no significant differences were observed (paired t-test, n=6, $p>0.05$), for nitrite and yellow substances significant differences for the highest 2 current densities, 57 A/m² and 71 A/m² were observed (paired t-test, n=6, $p<0.05$), data are not shown.

3.2 Long term experiment

No significant differences in daily feed intake between treatments were observed (Tukey-HSD, n=10, $p<0.05$) however total feed intake for control group (36.6 kg) was much lower compared to feed intake during BDD and ozone treatments, 45.4 and 45.5 kg respectively (table 1). Consequently the maximum CFB achieved was 0.82 kg/m³ for the control group and 0.97 kg/m³ and 0.98 kg/m³ for the BDD and ozone treatments.

No significant differences in temperature, pH and nitrite-N concentrations between treatments were observed (Tukey-HSD, n=10, $p>0.05$). Salinity was significantly higher for BDD treatment compared to control (Tukey-HSD, n=10, $p<0.05$) but not between ozone and control or BDD treatment (Tukey-HSD, n=10, $p>0.05$). Conductivity was found significant between all treatments (Tukey-HSD, n=10,

p<0.05). Total ammonia nitrogen was significantly higher for ozone treatment compared to the other two treatments (Tukey-HSD, n=10, p<0.05) (table 1).

Table 2. Average feed intake, temperature, salinity, conductivity, pH, total ammonia nitrogen (TAN) and nitrite nitrogen concentrations over the 10 day experimental periods for different treatments.

Parameter	N	Control	BDD	Ozone
Feed intake (kg/d)	10	3.66 ± 0.60	4.54 ± 2.00	4.55 ± 1.05
Temp (C°)	10	17.8 ± 0.4	17.2 ± 0.2	17.4 ± 0.2
Salinity (ppt)	10	27 ± 1^{a}	30 ± 0^{b}	30 ± 2^{ab}
Conductivity (ms/cm)	10	34.8 ± 0.5^{a}	35.4 ± 0.3^{b}	36.5 ± 0.3^{c}
pH	10	7.37 ± 0.07	7.44 ± 0.08	7.33 ± 0.10
TAN (mg/l)	10	0.31 ± 0.08^{a}	0.27 ± 0.07^{a}	0.59 ± 0.24^{b}
NO_2-N (mg/l)	10	0.12 ± 0.04	0.16 ± 0.03	0.15 ± 0.06

The amount of oxidants added was different between BDD and control treatment (table 2). From day 4 on, applied doses of ozone was limited by the maximum output of the ozone generator at 240 g/d. Consequently average TRO concentrations in the culture tanks were found significantly different between treatments (Kruskall-Wallis, n=10, 64, 55, p<0.001) and differences were considered significantly different between all treatments (Mann-Whitney, p<0.01). The amount of TRO produced, calculated as the difference between out-flow and in-flow of the electrocell or protein skimmer combined with ozonation, was significantly higher for the protein skimmer (Mann-Whitney, n=64, 55, p<0.01) (table 2).

For both treatments significant reduction of *Vibrio* counts were observed (One-sample T test, n=6, p<0.05). For total bacteria counts after 24 hours significant lower counts were observed for the BDD treatment (One-sample T test, n=6, p<0.05) but not for ozone. For bacterial counts after 96 hours no significant effect for both treatments was observed. However no significant differences between treatments, for any of the bacterial counts, were found (independent T test, n=6, p>0.05).

Although some disinfection effects were found, no significant differences in bacterial load in the culture tank between different treatments were found (Tukey-HSD, n=6, p>0.05) (table 2).

Table 3. Amount of oxidants added, residual oxidant formation, disinfection performance and bacterial pressure during the 10 days experimental periods.

Parameter	N	Control	BDD	Ozone
Oxidant addition				
Oxidant (mmol/m^3/d)	10	0	22 ± 2	107 ± 30
A/m^3/d	10	0	0.024 ± 0.02	Nd
g/m^3/d	10	0	Nd	5.2 ± 1.2
Residual oxidant formation				
TRO tank (mg/l)	10, 64, 55	0.03 ± 0.01[a]	0.06 ± 0.01[b]	0.04 ± 0.02[c]
TRO in (mg/l)	nd, 64, 55		0.05 ± 0.01	0.05 ± 0.02
TRO out (mg/l)	nd, 64, 55		0.11 ± 0.02	0.13 ± 0.08
Δ TRO out-in (mg/l)	nd, 64, 55		0.05 ± 0.02[a]	0.09 ± 0.07[b]
Disinfection performance				
Δ *Vibrio* (%)	6		30 ± 22	62 ± 38
Δ 24h (%)	6		55 ± 33	49 ± 47
Δ 96 h (%)	6		9 ± 19	30 ± 35
Bacterial pressure tank				
Vibrio (log CFU/ml)	6	3.0 ± 0.9	3.2 ± 0.3	2.9 ± 0.5
24h (log CFU/ml)	6	4.5 ± 0.3	5.0 ± 0.8	4.3 ± 0.7
96 h (log CFU/ml)	6	5.2 ± 0.9	6.0 ± 0.8	5.7 ± 0.7

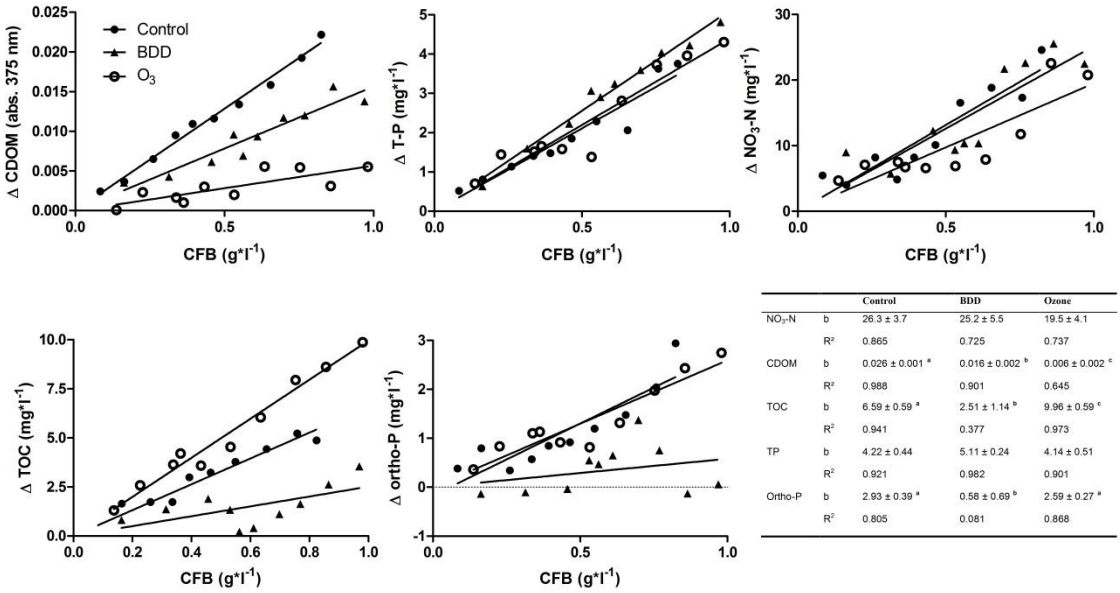
A significant accumulation of CDOM was found in the control treatment and the correlation coefficient was high. Accumulation of CDOM in the BDD treatment was significantly lower compared to control, but significantly higher compared to ozone treatment (Tukey-HSD, n=10, $p<0.01$).

In the control group the accumulation of TOC was also found significant and highly dependent on the CFB. In contrast to CDOM, the accumulation of TOC in the ozone treatment was not significantly different compared to control (Tukey HSD, n=10, $p>0.05$). In the BDD treatment no significant accumulation of TOC took place, and was therefore significantly lower compared to control or ozone treatment (Tukey HSD, n=10, $p<0.001$).

Nitrate showed significant accumulation in all groups and no significant differences between treatments were observed (Tukey HSD, n=10, $p>0.05$).

Total phosphorus showed significant accumulation in all groups and no significant differences between treatments were observed (Tukey HSD, n=10, $p>0.05$). Ortho-P showed significant accumulation in the control and ozone group and no significant differences were observed, in the BDD treatment however no significant ortho-P accumulation was observed, and the accumulation was

therefore significantly lower compared to control or ozone treatment (Tukey HSD, n=10, $p<0.05$) (figure 3).



		Control	BDD	Ozone
NO_3-N	b	26.3 ± 3.7	25.2 ± 5.5	19.5 ± 4.1
	R^2	0.865	0.725	0.737
CDOM	b	0.026 ± 0.001 ^{a}	0.016 ± 0.002 ^{b}	0.006 ± 0.002 ^{c}
	R^2	0.988	0.901	0.645
TOC	b	6.59 ± 0.59 ^{a}	2.51 ± 1.14 ^{b}	9.96 ± 0.59 ^{c}
	R^2	0.941	0.377	0.973
TP	b	4.22 ± 0.44	5.11 ± 0.24	4.14 ± 0.51
	R^2	0.921	0.982	0.901
Ortho-P	b	2.93 ± 0.39 ^{a}	0.58 ± 0.69 ^{b}	2.59 ± 0.27 ^{a}
	R^2	0.805	0.081	0.868

Figure 4. Change in nutrient concentration in the water by changing cumulative feed burden for the three treatments during the 10 day experimental periods.

Although increase in Iron and Zinc was observed with increasing CFB, accumulation was not found significant (one sampled t-test, n=3, $p>0.05$) and no significant differences in average concentrations between treatments were found (Tukey HSD, n=3, $p>0.05$). Arsenic concentrations were comparable between treatment and no differences were observed. For Cobalt and Manganese higher, values were observed in the control group compared to BDD or ozone groups, but because some data were below detection limits no statistics was done. For Cadmium and Copper no concentrations above the detection limits were found (table 3).

Table 4. Concentrations of metals in the culture water for the three different treatments, n=3. No significant correlation between metal concentration and CFB was observed. No significant differences in average concentration between treatments were observed.

Metals (µg/l)	Control	BDD	Ozone
Arsenic (As)	4 ± 1	4 ± 0	4 ± 1
Cadmium (Cd)	nd	nd	nd
Cobalt (Co)	≤ 7	nd	nd
Copper (Cu)	nd	nd	nd
Iron (Fe)	14 ± 5	14 ± 7	14 ± 4
Manganese (Mn)	≤ 7	nd	≤ 5
Zinc (Zn)	100 ± 31	76 ± 19	157 ± 34

nd = not detected, detection level; Cd = 5 µg/l, Co = 4 µg/l, Cu 10 µg/l, Mn 4 µg/l

4. Discussion

In general in RAS ozonation is combined with foam fractionating or flocculation which improves the removal efficiency of both particulate and dissolved metabolites. Such a system is not yet developed for BDD treatment in RAS. Therefore during the present study only the chemical oxidation capacity of both ozone and BDD treatment were compared, to exclude biased effects on mechanical removal efficiency. Both methods were standardized on a TRO safety level of 0.06 mg/l in the culture tanks (Reiser et al., 2010). Irrespective of ozone doses applied, average TRO maxima in the culture tanks were not reached. As a result ozone doses was limited by maximal ozone generator output at 240 g/d, providing an ozone doses of 53 g/kg feed which is roughly double of the maximum doses advised under normal usage (Timmons and Ebeling, 2010). Therefore by combining oxidation with mechanical removal, the removal efficiency of nutrients is likely to increase, likewise an increased formation of disinfection by-products can then be expected.

4.1 TRO formation and ORP behavior

Treatment of natural waters containing bromide and chloride with BDD electrodes results in the formation of disinfection by-products. Observed by products are free bromine and chlorine species, bromamines and chloramines which are called total residual oxidants (TRO), trihalomethanes (THMs) with bromoform being the major THM species, and bromate, chlorate and perchlorate (Bergmann and Rollin, 2007, Diaz et al., 2011, Echardt and Kornmueller, 2009, van Bussel 2013b).

TROs are extremely toxic to marine fish and shrimp species, adverse effects on morphology and survival already observed at concentrations of 0.06 respectively 0.10 mg/l Cl_2 (Reiser et al., 2010, Schroeder et al., 2010) although more sensitive parameters such as feed intake or feed conversion were

not assessed. Bromoform is less toxic to marine fish and shrimp but abnormal behavior such as rapid swimming in random directions thereby hitting the tank walls, are observed at concentrations as low as 0.4 mg/l (Gibson et al., 1979) but again feed intake or feed conversion were not assessed. Bromate and chlorate are least toxic to marine fish with reported long term LC-50 values for bromate in the range of 200-500 mg/l (Hutchinson et al., 1997). For chlorate no toxicity data on marine fish are available but fresh water fish species 96h LC-50 vary between 800 and 10,000 mg/l (van Wijk and Hutchinson 1995).

Observed TRO concentrations in marine waters after BDD treatment can exceed safety levels for aquaculture in magnitudes. Echardt and Kornmueller (2009) reported TRO concentrations of 1.4 mg/l in BDD treated marine ballast water, and Diaz et al. (2011) reported concentrations up to 8 mg/l in BDD treated marine aquaculture water. However the present study and other recent studies (van Bussel 2013a, van Bussel 2013b) showed that in both fresh and marine aquaculture water effective water treatment by TRO concentration ≤ 0.06 mg/l is possible.

The present short term experiments showed that the TRO formation increased with the applied current. This is a logical consequence of an increased amount of charge, and thus mole electrons available to oxidize ions such as bromine and chlorine. This increased TRO formation at increased applied current on DBB electrodes is also observed in other studies (Bergmann and Rollin, 2007, Diaz et al., 2010, van Bussel 2013a). During the present study the formation of TRO was depending on water quality, whereas TRO formation in `dirty` water, with low exchange rate, was significantly lower as in standard or `cleaner` water. As a result of TRO formation the amount of charge needed to form a defined amount of residual oxidants, expressed as the current efficiency, was also dependent on water matrix. An increase in current efficiency with increased current densities was observed for all treatments, except for the low water exchange treatment where constant current efficiency was observed between 7 and 28 A/m². The ORP behavior was similar in all three water matrixes, where a decrease in ORP was observed below 28 A/m², followed by a linear increase. Simultaneously to TRO formation, oxidation of yellow substances and nitrite was observed during the pre-experiments as it could be observed in small-scaled systems in a previous study (van Bussel 2013a). The presence of both nitrite and yellow substances is known to scavenge oxidants, and therefore reduce the formation of TRO during ozonation (Schroeder et al., 2011). Because nitrite concentration was comparable between water matrices in the present pre-experiments, the difference in TRO formation is likely a result of different concentrations CDOM, which was a factor 5-9 lower in the high and standard water exchange compared to low exchange rates. Other differences in the water matrix, such as in salinity, pH and temperature were minimal and are therefore not likely to explain differences in TRO formation (Hsu, 2005).

Next to the scavenging of ozone or hydroxyl radicals by humic acids, breaking the large molecules into smaller bio-available fractions, bromoform is formed (Cooper et al. 1985). During the present study THMs were not measured. Reported THM concentrations after BDD treatment do also exceed

safety values. Reported concentrations of THMs ranged between 0.3 – 1.6 mg/l, with bromoform being the major THM accounting for 85-96% on weight basis (Echardt and Kornmueller, 2009, Diaz et al., 2010). Diaz et al. (2010) observed that the amount of total THM formed by equal applied charges (Ah) was much higher at lower current densities (10-30 A/m²) than at 50 A/m². Because THM are not detected by the DPD method, oxidant scavenging by humic acids and successively THM formation can be an explanation for the relative low current efficiencies of TRO formation observed below 28 A/m². The decrease in redox potential below 28 A/m², is a second indication for oxidant scavenging by humic acids and/or formation of persistent residual oxidants such as THMs, instead of the formation of reactive chlorine/bromine species.

During all three periods of the long-term experiment average TRO concentrations were within acceptable levels for turbot culture (Reiser et al., 2010). The amount of TRO produced by ozonation was higher than amounts of TRO produced by BDD treatment. The TRO degradation in the system was higher during the ozonation treatment than for BDD-treatment, resulting in higher TRO levels in the culture tank during BDD treatment. This indicates that the TROs produced with the BDD treatment were more persistent than ozone produced oxidants.

Thus TRO and THM formation and accumulation is limiting the application of BDD electrodes in RAS. Minimizing of residual oxidant formation is therefore a key-point in RAS water treatment with BDD electrodes.

4.2 Disinfection performance and bacterial pressure

Disinfection performance of both ozone and BDD treatment were low for *Vibrio* sp. and low or absent for total bacteria counts after 24 and 96 hours of incubation. Upon ozonation, particles present in the water protected bacteria, increasing the c*t values needed to achieve 90% reduction roughly a 3-fold up to 0.19 mg/l minute (Hess-Erega et al., 2008). The c*t values during the present study in the protein skimmer, 1.5 minutes HRT * TRO 0.13 mg/l = 0.195, were in the same range. However the produced TRO in the protein skimmer, thus the effective c*t values, were only 0.135 mg/l minute resulting in average reduction rates of 30-62 %, similar disinfection efficiencies as observed by Hess-Erega et al. (2008).

For BDD treatments data on disinfection efficiency in saline water are limited to two studies. Echhardt and Kormueller (2009) observed disinfection efficiencies of >95 % for saline water (15 ppt) with high organic loads during ballast water treatment. However in this study water was treated twice with BDD-electrodes producing TRO values up to 1.4 mg/l and time between treatments was 5 days, resulting in extremely high c*t values which are not applicable for RAS.. In the other study (van Bussel et al. 2013a) disinfection efficiencies of 5-60 % were observed and applied doses of charge was limited by TRO formation and accumulation, similar to the present study.

No differences in bacterial counts between different treatments were observed. The number of bacterial counts in RAS water is dependent on the CFB, e.g. the water exchange rates per amount of feed given can also affected by disinfection (Leonard et al. 2000, van Bussel et al., 2013). The absence of effects on bacterial counts during the present study is the result of bacterial doubling time and time between water passes the disinfection unit. Leonard et al. (2000) estimated the doubling time of bacteria which were not bound to a biofilm in marine RAS by 25 °C at 2 h 50 minutes. In studies with significant reduction of bacterial numbers due to disinfection, the time between water passing the disinfection unit twice was ≤1 hour and thus less than the bacteria doubling time resulting in an effective reduction potential (Park et al., 2013, Leonard et al. 2000, Schroeder et al., 2010, 2011, van Bussel et al. 2013a).

Thus to achieve effective reduction of bacterial counts in marine RAS, reduction efficiency and thus disinfectant exposure must be relatively high due to particle protection. Furthermore the HRT of the system before water enters the disinfection unit again must be short enough to encounter re-growth. Therefore this can only be obtained by treatment of large parts of the water flow, not in by-passes such as in regular protein skimmers. Beside the limitations of TRO formation and disinfection of large water flows, it can be questioned if in-system disinfection is desirable. After strong disinfection there is a destabilization of the microbial community in RAS (Attramadal 2012) and due to high availability of nutrients and a lack of competition the growth of opportunistic bacteria could be supported (Hess-Erega et al., 2010). Opportunistic bacteria in RAS are mainly species which are potentially pathogenic and favor a short HRT (Schneider et al., 2007). Therefore managing constant water exchange rates to maintain stable bacterial populations (Leonard et al., 2000) and reducing organic load to decreased overall bacterial growth (Maeda et al., 1997) can be a better strategy to control healthy microbial populations. The use of oxidation processes must therefore be optimized for nutrient removal, in order to maintain low bacterial pressure, and not for direct disinfection.

4.3 Accumulation of (in)-organics and treatment effects

During the present study nitrate was not influenced by treatment with ozone or BDD electrodes. Ozone is known to affect nitrate concentrations during ozonation (Tanga and Gagnon 2000) however this is due to better organic removal and therefore less protein being degraded to nitrate in the biofiltration unit. BDD treatment is known to prevent nitrate formation by an anodic process known as breakpoint bromination or by nitrate removal via cathodic processes (Kraft, 2003, Georgaud et al., 2011, Levy-Clement 2005, Bergmann and Rollin 2007, Michels et al. 2010, Cabeza et al., 2007). However both methods have serious disadvantages when applied in RAS water. Breakpoint bromination, in marine water often falsely described as breakpoint chlorination, is a bromine ion catalyzed oxidation of ammonia to N_2 via the intermediated dibromamine ($NHBr_2$), and tribromamine (NBr_3). But TRO concentrations of <0.6 mg/l are needed before this reaction takes place (Schroeder et

al., 2011). Furthermore the presence of nitrite and yellow substances is preventing the accumulation of free bromine and thus preventing breakpoint bromination (Diaz et al. 2011, Schroeder et al., 2011). Beside the highly toxic TRO concentrations needed before breakpoint bromination is realized unwanted by-products such as THMs, chloramines, NO_2 and NO_3 are formed (Diaz et al. 2010, Bergmann and Rollin 2007, Kraft 2003). The cathodic reaction mechanism of nitrate to N2 on DBB electrodes is not fully understood, but reaction mechanisms are slow and by-products such as TAN, NO_2, TRO and chloramines are formed (Kraft 2003, Levy-clement 2005, Georgaud 2011). Therefore anodic breakpoint bromination is considered risky and unpractical for RAS purposes. Cathodic nitrate removal on electrodes combined with anodic oxidation on BDD electrodes at low current densities to prevent high TRO formation is a future technique that requires further research.

During the present study the accumulation of DCOM was reduced by both ozone and BDD treatment but could not be prevented. The removal of DCOM is not a complete oxidation of organic carbon to CO_2 but a breakdown of double bindings in long non-biodegradable molecules into smaller biodegradable molecules (Tanga and Gagnon, 200). Accumulation of DCOM was lowest in the ozone treatment, similar observations have been made in small-scaled experiments (van Bussel et al., 2013) and can be explained by a preferred reaction of ozone with humic acids over other accumulating metabolites (Schroeder et al., 2011). Next to a lower addition of oxidant compared to ozone, BDD produced hydroxyl radicals are more unspecific in their reaction partners resulting in a lower removal of DCOM. The overall accumulation of TOC was lower in the BDD treatment compared to ozonation and control treatment. Both ozone and BDD treatment are known to reduce the accumulation of TOC in RAS (van Bussel et al., 2013). Other data on TOC removal by BDD electrodes in marine water are lacking but the formation of THMs, as discussed above, is an indication for the oxidation of organics by both free bromines and free chlorines (Diaz et al., 2010, Echardt and Kornmueller, 2009). The absence of effects on TOC during ozonation might be the preferred reaction with humic acids over other metabolites (Schroeder et al., 2011). In contrast to CDOM removal, TOC must be fully oxidized to CO_2 by oxidation or by heterotrophic bacteria conversion before it is removed from the system. Furthermore due to a lack of particle removal by protein skimming or improved flocculation, the rate of chemical oxidation of TOC to CO_2 is expected to be low (Tagno and Gagnon, 2003). Further research is needed to see if BDD-treatment also can improve mechanical removal due to flocculation of suspended particles and if a combination of BDD-treatment and foam fractionating would be useful to improve TOC removal by BDD treatment even more.

The accumulation of total phosphorus was equal in treatments suggesting that no phosphorus was removed from the water due to treatment. Ortho-P accumulation was not influenced by ozone treatment. Ozone does improve particle removal due to flocculation or improved protein skimming, reducing the leaching effect of particulate phosphorus to ortho-P. Due to the absence of protein

skimming no reduced leaching can be expected. In the present study ortho-P accumulation during the BDD treatment was significantly reduced. Because total phosphorus accumulation was not effected by treatment, ortho-P must be converted to another chemical form. Hydroxyl radicals are known to carry out electron transfer with HPO_4^- and PO_4^- to form hydroxide ions and a phosphoric acid ion with a changed valence state (Black and Hayon, 1970, Thomas 1965). Because ortho-P could be absorbed by $CaCO_3$ in seawater (Millero et al., 2001), it might be that ortho-phosphate was converted to calcium phosphate. Other studies in aquaculture water and agricultural waste water have also reported ortho-P or total phosphorus removal after hydroxyl radical addition by electrolysation methods (Feng et al., 2003, van Bussel et al., 2013a, van Bussel et al., 2013b). The lack of total phosphorus removal but significant ortho-phosphate removal during the present study is probably a result of the conversion of ortho-P to particulate phosphorus, but a lack of suspend solids removal. Combining electrochemical treatment of aquaculture water, combined with particle removal by flocculation, protein skimming or sedimentation might therefore be a promising technique to reduce phosphate emissions of fish production in RAS.

4.4 Energy consumption and maintenance

The relation between applied current and potential needed showed a logarithmic function up to 28 A/m² followed by a linear increase. Consequently the energy consumption is more efficient at current densities below 28 A/m² compared with higher current densities. Similar results were observed by Diaz et al. (2011) who observed lowest energy consumption to completely oxidize ammonia in marine RAS water at a current density of 10 A/m², higher energy consumption at a current density of 20 A/m² and equal but higher energy consumption at 30 and 50 A/m².

During the 10 days experiment no differences in potential needed to apply the same current were observed, meaning that the electrical resistance of the electrodes did not change during the experiment. Energy consumption was therefore constant over the 10 days and energy consumption, without considering the energy costs of the electric power converter, was as low as 96 Wh or 2.3 Wh m^{-3}. Although no increase in electrical resistance was observed, build-up of limestone on the electrodes was observed at the end of the 10 day period although current was changed twice daily (figure 4). The buildup of limestone is a phenomenon often observed when calcium rich water is treated (Fryda et al., 2003, Diamadopoulos et al., 2009, van Bussel et al., 2013a, 2013b) and is considered problematic because it change the surface available for hydroxyl radical production and thus changing the applied current density. For future application in RAS the formation of limestone has to be prevented in order to maintain optimal current densities and prevent increased TRO formation due to a reduction in available electrode surface and thus higher current densities.

Figure 5. Limestone/biofilm formation at BDD electrode after 10 days of water treatment at 14 A/m^2, function 12 hours per day as anode.

5. Conclusions

Results from the present study confirm findings of previous experiments in small-scaled RAS that safe and effective water treatment with BDD electrodes directly in the culture water of a marine RAS is possible. By applied current densities of 14 A/m^2 and 0.02 Ah m^{-3} d^{-1} the concentrations of TRO in the culture tanks could be kept at $\leq$ 0.06 mg/l which are considered safe for marine fish and shrimp culture. As a result of oxidant addition by BDD electrodes significant lower accumulations of DCOM, TOC and ortho-P were observed, although TP levels were not changed. The formation and accumulation of TRO in the culture water is a serious limitation for the applied doses of oxidant addition. Furthermore the formation of a limestone/biofilm, which changes the actual current density should be prevented for optimal application. Additional filtration such as flocculation or protein skimming is advised to reduce TOC levels further and to effectively remove TP from the culture water.

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Chapter 6:

The effect of different oxidantion processes on nutrient accumulation and by-product formation in fresh water RAS.

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Abstract

Hydroxyl radicals, which can be produced on Boron Doped Diamonds (BDD) electrodes could be an alternative water treatment in fresh water recirculating aquaculture systems (RAS) due to their high oxidation potential. Therefore small scale RAS were equipped in triplicate with biological and mechanical filtration (control), with additional ozonation (ozone) or additional BDD electrodes with an electrolyte-membrane between electrodes (OS) or without (SS). Tanks were stocked with 40 European eels (*Anguilla Anguilla*) (250 ± 37 g) each and cultured for 30 days. Applied doses of oxidants were limited by a residual oxidant concentration of 0.06 mg/l Cl_2 in the culture tanks.

In control and ozone treatments chloride and bromide accumulated, in OS and SS treatments practically all chloride and bromide were converted into chlorate, bromate and other residual oxidants. Average concentrations of nitrite were significantly elevated in both BDD treatments, average concentrations of total ammonia, Na, Ca, Mg, Fl did not significantly vary between treatments.

All oxidation processes significantly reduced the accumulation of TOC, but no significant differences between the different processes were observed. Nitrate and ortho-phosphate accumulation were lower in both BDD treatments, although differences were only considered significant between ozone and OS or SS respectively between control or ozone and SS. Significant disinfection was only found for ozone, however bacterial densities in culture tanks were not affected by any treatment and no clear effects on prevalence of bacterial infections was observed. Infection with gill parasites (*Dactylogyrus* sp.) was significantly increased in BDD treatments. Like in marine water, limestone formation was observed on the electrodes requiring intensive maintenance and affecting applied current densities.

We conclude that the accumulation of toxic residual oxidant does limit application in RAS, even in fresh water. Compared to ozonation the formation and accumulation of stabile residual oxidant is a clear disadvantage. Therefore minimization of residual oxidant formation is needed. Further research on end-of-the-pipe treatment instead of in system treatment of aquaculture waste water with BDD electrodes is advised.

1. Introduction

Clean fresh water is scarce on global scale, and aquaculture production is one source of eutrophication. Water consumption per amount fish produced is dependent on the production system used. Extensive systems, most widely used worldwide, consume roughly 500 m^3 per kg fish produced, intensive raceway systems 50-5 m^3 whereas recirculating aquaculture systems (RAS) consume only 0.1 m^3 per kg fish produced making RAS production favorable with respect to nutrient release (Fitwi et al., 2012, Martins et al., 2010). However due to this low water consumption and thus intensive production waste products due accumulate in the culture water, potentially affecting health or growth performance (Martins et al., 2009, Davidson et al., 2009). Technical improvements in water treatment loops of RAS are therefore needed to optimize production, however increased operating costs can negatively affect profitability of such systems (Blancheton et al., 2007, Martins et al. 2010). Therefore there is a need for low cost water treatment units in fresh water RAS.

Electrolyzation is price effective way of (drink/industry) water treatment and boron doped diamond (BDD) electrodes are the most suitable and economical electrode type of electrode to remove organic pollutants from waste water, due to low electrochemical formation of oxygen and high oxidant formation, and thus effective pollutant oxidation (Kapalka et a. 2008). In marine RAS BDD electrodes have successfully been used to continuously treat water resulting in lower accumulation of organic matter and ortho-phosphate however the formation of fish toxic total residual oxidants (TRO) limits the applied doses of oxidants by BDD electrodes (van Bussel 2013a, 2013b) which are mainly free bromine and free chlorine species (Echardt and Kornmueller 2009). However the accumulation of more stable residual oxidant such as bromate, chlorate and chlorinated or brominated organics were not assessed and might also accumulate and limit application in RAS. Data available on the use of BDD electrodes are limited to marine RAS (Diaz et al., 2010, van Bussel et al. 2013a, 2013b). All these studies indicate that effective oxidation/conversion of organics, phosphates and nitrogen species are possible, but also indicate that application is limited by residual oxidant formation.

In fresh water RAS concentrations of chloride and bromide ions are roughly a factor 500-1000 lower than in marine RAS. Due to this difference in chloride/bromide concentration the formation of free chlorines and bromines (TRO) on BDD electrodes might be a less limiting factor in fresh water RAS.

Thus the aim of the present study was to test suitability of BDD electrodes for water treatment in fresh water RAS. Effects of waste product accumulation, bacterial counts, disinfection performance and infection of eel (*Anguilla anguilla*) with bacteria (*Aeromonas* sp.) and parasites (*Dactylogyrus* sp*)* were assessed.

2. Material & Methods

2.1 System setup

2.1.1 Electrodes and electro cell set up

The electro cell used in this experiment was the CONDIACELL® CP450 (CONDIAS GmbH, Itzehoe, Germany) equipped with 4 pair of electrodes consisting of a Niobium carrier material and a 20 µm thick Boron doped diamond layer, type DIACHEM® 24*50 mm (CONDIAS GmbH, Itzehoe, Germany), called salt water stacks (SS). Three other electrocells were equipped with a solid polymer electrolyte membrane to increase ozone production (Kraft 2007) so called ozone stacks (OS). Electrodes were once weekly cleaned with water and acid (1 molar HCL) to remove the formed limestone and biofilm.

2.1.2 RAS set up

The experiments were conducted in 12 small scaled experimental RAS, each with a system set-up as exactly as described in van Bussel et al. (2013a).
Optionally ozone was produced in an ozone-generator (Aquamedic 200 mg/h, Germany) using compressed air and was injected at the bottom of the aeration tower by a wooden air-stone diffuser in counter current. Optional addition of hydroxyl radicals took place in the reaction chamber by electrolyzing the passing water with BDD-electrodes. The electric current required for electrolyzation was generated by a laboratory power supply (model EA-PS 3032-05 B, HEIDE power GmbH, Pürgen, Germany). In order to measure and compare the energy consumption (kWh) of electrochemical treatment and ozonation, an electricity meter was mounted prior to the laboratory power supply and the ozone generator, respectively. The applied potential (V) and current (A) on the BDD-electrodes were recorded manually.

2.2 Experimental set up and procedure

The 12 small-scaled RAS (figure 1) were divided in 4 system designs with 3 replicates each, providing 3 different treatments by chemical oxidation and a control without oxidation process:

- Control: filter mats, MBBR, aeration tower.
- Ozone: equal to control + continuous applied ozone addition.
- Ozone stack (OS): equal to control + continuous applied current on ozone stacks
- Saltwater stack (SS): equal to control+ continuous applied current on saltwater stacks

In order to ensure comparability of treatment Ozone, OS and SS the dosages of hydroxyl radicals and ozone were standardized with regard to residual oxidant concentration in the tanks. Therefore, the TRO concentration was measured at the tank inflow in regular time intervals and adjusted to a value of

$\leq$ 0.06 mg Cl_2 l^{-1} by regulating the current applied for the generation of OH-radicals and ozone, respectively.

RAS were filled with a tap water and eel were acclimatized in systems for 7 days. Then a total of 480 eels with a mean average weight (250 ± 37 g) were randomly divided in groups of 40 individuals and distributed to the 12 RAS. Average total wet weight per system was 10,002 ± 45 g resulting in an initial density of 33,339 ± 140 g/m^3. Temperature was automatically adjusted to 25°C with a temperature-controlled 500 W heater (Aqua Medic, Bissendorf, Germany). A photoperiod of 14 h light (06:00-20:00) 10 h dark was provided. Eel were fed by hand with a commercial eel diet (Aller Ivory ex 54/20 3 mm., Aller Aqua, Christianfeld, Denmark). Feed load was restricted, resulting in equal amounts of feed, and thus nutrient input, for every tank. Feeding regime once a day, 7 days per week. Feed load was 15 g per day. Tab water was added to maintain total water volume and thus compensate for evaporation and cleaning losses.

The experimental period lasted 30 days. The performance of ozonation as well as the two different BDD electrode stacks were evaluated by determining the accumulation of inorganic (TAN, NO_2-N, NO_3-N, ortho-P) and organic (TOC) substance, disinfection performance and the bacterial pressure in the culture tanks. Eel health was assessed as mortality, clinical infection with bacterial diseases and infection by the parasites *Ichthyophthirius multifiliis* and *Dactylogyrus spp.*

2.3 Water quality analysis

Nitrate, sulfate, chlorate, bromate, chloride, bromide, sodium, calcium, magnesium and fluoride were analyzed using high pressure liquid chromatography (LC2010-HT, Shimadzu corp., Kyoto, Japan). TP and O-P were measured spectrophotometrically with the ascorbic acid method using a microplate reader Infinite 200 (Tecan, Männedorf, Switzerland) according to the protocols of Grasshoff et al. (1999) and adapted to microplate format. TAN, NO_2-N and TRO were determined spectrophotometrically using a HACH-DR 2800 spectrophotometer (Hach-Lange GmbH, Berlin, Germany) and powder pillow detection kits (Hach-Lange GmbH, Berlin, Germany) based on the ammonia salicylate method, diazotization method and the N,N-diethyl-p-phenylenediamine (DPD) method, respectively. TRO is measured in mmol/l but notated during the present study as clorine (Cl_2) equivalents in mg/l. TOC was analyzed as non purgable organic carbon using a total organic carbon analyzer (TOC-V CPH/CPN, Shimadzu corp., Kyoto, Japan).

Temperature and pH were measured with a WTW 340i portable multiparameter instrument (WTW GmbH, Weilheim, Germany), salinity with a digital refractometer HI 96822 (HANNA Instruments, Woonsocket, USA) and dissolved oxygen with a Handy Polaris oxygen probe (Oxygard International A/S, Birkenrød, Denmark). A BMT ozone analyser 963 (BMT Messtechnik GmbH, Berlin, Germany) was used to determine ozone addition by measuring flow rate and ozone concentration in the gas stream.

2.4 Microbial analysis

Water samples were taken from the tanks of all treatments for the estimation of bacterial density. Furthermore, water samples at the inflow and the outflow of both tested chemical water treatment processes, ozonation and BDD electrolysation, were taken to assess bactericidal efficiencies. Samples were taken one by one. Directly after each sample was taken, the respective sample was diluted and analyzed by the spread plate method using an unspecific agar (TSB Agar, BD, Sparks, USA) for the detection of total viable cell counts. Colony forming units per plate were recorded after 24 and 96 h of incubation at 28° C on.

2.5 Fish health analysis

Fish were daily inspected for mortality. At the start and end of the experiment all eels were clinically assessed for health appearance. Bacterial infection with e.g. *Aeromonas sp.* was assessed according to typical clinical signs of infection according to Schaeperclaus, 1979. Eels were scored as positive when stripes and bands on side as well as and red spots on belly were visible. Infection was scored as severe when the mucus was damaged mucus and white/blue circles were visible, open wounds mainly at the tail were visible or when fish were lying at the surface with bent body shape. At the end of the experiment three eels were killed and gills and swimbladder were dissected. Gills were sampled using a binocular and number of *Dactylogyrus spp*, were counted.

2.5 Assumptions and formulas

Current density (CD) was calculated as follows:

CD = Applied current (A) / working surface electrode (1)

Working surface electrode (WSE) was calculated as follows

WSE = (length (m) * width electrode (m)) * active area electrode * surface factor (2)

Length of an electrode was 75 mm, width was 32 mm. Active areas during the present study using 4 electrode pairs was 7. The surface factor used here is 1.2. Thus total active area is 20160 mm^2 or 0.020160 m^2.

To compare the accumulation of substances in the culture water the function cumulative feed burden (CFB) is used. This function is combining the amount of feed added per volume of water, corrected for water exchange and is calculated as follow:

1 mole e^- = 1 mole oxidant (3)

1 Faraday = charge of one mole e^- (4)

1 Faraday = 96485.3399 C (5)

1 C = 1 A*s (6)

When oxidized or produced substances are plotted against Faraday, the mole $OH^\bullet$ equivalent, the current efficiency can be estimated.

Current efficiency = substance X (mole) / $OH^\bullet$ (mole) or Faraday (7)

For ozone, molar efficiencies can be calculated in the same way

Molar efficiency = substance X (mole) / O_3 (mole) (8)

To compare the accumulation of substances in the culture water the function cumulative feed burden (CFB) is used. This function is combining the amount of feed added per volume of water, corrected for water exchange and is calculated as follow:

CFB = Feed added (g) / V_{total} (l) ^ ($-V/V_{total}$) (9)

where "feed added" is the amount of feed added per tank in g, "V_{total}" is the total culture volume and "–V" is the amount of water replaced.

As CFB was set to 0 at the start of the experiment although the initial process water matrix was not free of accumulating substances, accumulating water parameters (NO3-N, OP, TP, TOC and dissolved matter) are both presented as absolute value and as increase of the parameter:

F(x) = mx + b (10)

where x is the cumulative feed burden, F(x) is the accumulation of the water parameter and b is the consumption or production of the parameter at a CFB = 0.

Reduction efficiency for disinfection was calculated as the difference in CFU between inflow and outflow

$$RE = (CFU_{inflow} - CFU_{outflow} / CFU_{inflow})*100 \quad (11)$$

2.6 Data analysis

Data are presented as mean ± standard deviation (SD) of n samples. Statistical analysis of variances was performed using SPSS 17.0 (IBM inc, Armonk, USA). Linear regression was conducted using Graphpad (Graphpad software inc., La Jolla, USA). Data were tested for normality and for equal variances ($p<0.05$) using Levene's test and the Kolgomorov-Smirnov test, respectively. Multiple comparisons were carried out by the parametric Tukey's HSD or the non-parametric Dunnet's T3 test. Single comparisons were carried out by paired or unpaired t-tests. Differences were considered as significant at $p<0.05$.

3. Results

Do to the low feed load no significant changes in eel biomass were observed (data not shown).Water temperature, oxygen concentrations did not significantly vary between treatments (n=27, Tukey, $p>0.05$). Total ammonia nitrogen (TAN) levels did not accumulate and no significant differences between treatments were observed (n=15, Tukey, $p>0.05$). Nitrite-nitrogen concentrations did not accumulate and average concentrations were comparable between control and ozone but were significantly higher (n=15, Tukey, $p<0.05$) for both OS and SS treatments. The microelements Na, K, Mg, Ca and Fl did not accumulate and did not significantly vary between treatments (n=9, Tukey, $p>0.05$) (table 1). Although not significant there was a trend that average Ca concentrations were lower in SS treatment compared to control or ozone (n=9, Tukey, $p<0.1$).

Table 1. Temperature, pH and average concentrations of non-accumulating substances during the 30 day experimental period for the four different treatments. Different subscripts in same lines indicate significant differences between treatments.

	Control	Ozone	OS	SS
Temp (C°)	24.8 ± 0.5	24.7 ± 0.5	24.9 ± 0.2	24.8 ± 0.3
O_2 (mg l^{-1})	6.9 ± 0.7	7.2 ± 0.4	6.7 ± 0.3	6.6 ± 0.2
pH	6.5 ± 1.1	6.5 ± 1.2	6.6 ± 1.1	6.6 ± 1.1
TAN (mg l^{-1})	0.24 ± 0.02	0.25 ± 0.03	0.27 ± 0.07	0.24 ± 0.03
NO_2-N (mg l^{-1})	0.16 ± 0.10^{a}	0.16 ± 0.11^{a}	0.88 ± 0.56^{b}	0.85 ± 0.47^{b}
Na (mg l^{-1})	41.4 ± 3.2	41.8 ± 3.8	42.0 ± 3.3	39.8 ± 4.4
K (mg l^{-1})	19.4 ± 2.7	18.8 ± 2.7	18.9 ± 2.8	17.6 ± 2.8
Mg (mg l^{-1})	8.2 ± 2.0	8.4 ± 1.5	6.9 ± 1.0	6.5 ± 1.4
Ca (mg l^{-1})	116 ± 10	118 ± 11	112 ± 6	104 ± 7
Fl (mg l^{-1})	0.24 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.24 ± 0.02

Nitrate, total organic carbon (TOC), ortho-phosphate and sulfate did accumulate in all treatments (figure 1).

Nitrate accumulation was significant in all treatments but no significant effect of treatment compared to control was found, however accumulation of nitrate in the ozone treatment was significantly higher compared to OS and SS treatments ($n=3$, Tukey, $p<0.05$).

TOC accumulation was significant in all treatments and significant lower accumulation ($n=3$, Tukey, $p<0.05$) was found for all three treatments compared to control, but no significant differences between different oxidation processes were found.

Accumulation of orthophosphate was comparable between control and ozone, and was lower for both BDD treatments (OS and SS), although differences were only found significant between SS and control or ozone ($n=3$, Tukey, $p<0.05$).

Sulfate accumulated in all treatments but no significant differences between treatments were observed ($n=3$, Tukey, $p>0.05$).

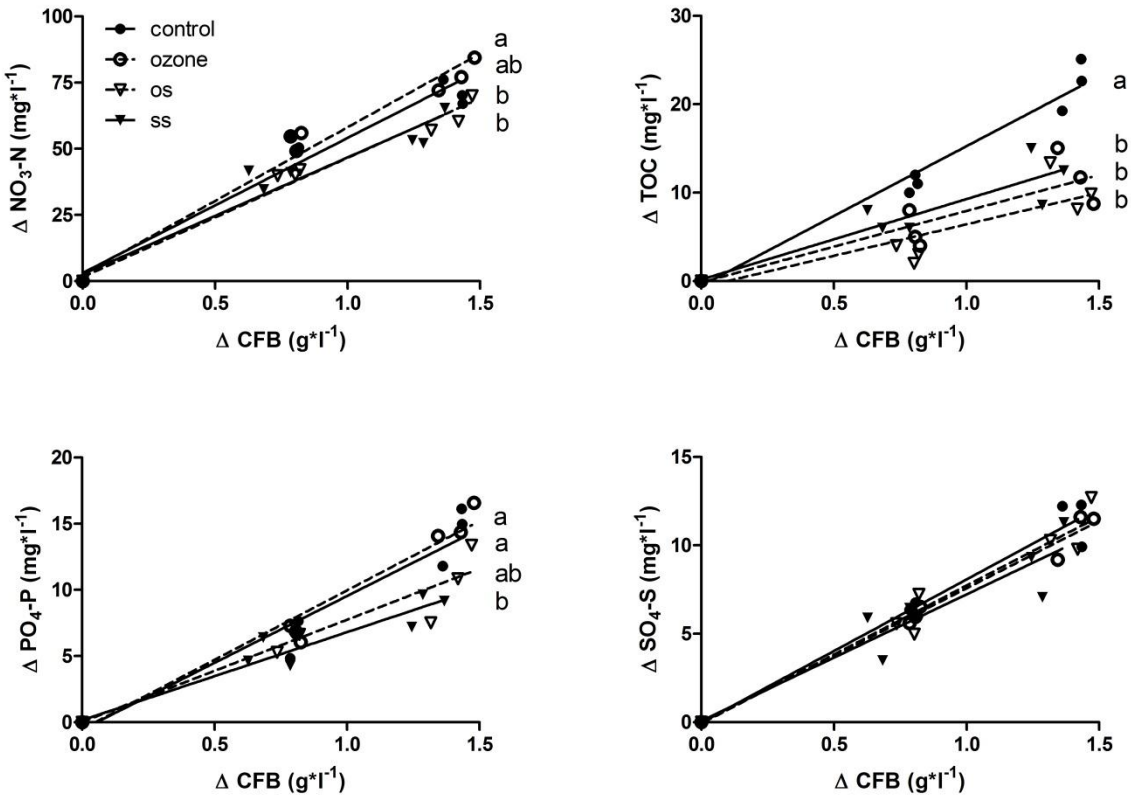

Figure 1. Observed changes in nitrate, total organic carbon (TOC), ortho-phosphate and sulfate concentrations plotted against the cumulative feed burden (CFB). Different letters indicate significant differences in slopes between treatments (n=3, Tukey, $p<0.05$).

Average concentrations of TRO in the culture tank were within the safety level of ≥ 0.06 mg/l in all tanks. In control groups no TRO was measured, in all other groups significant amounts of TRO were measured and concentrations in the ozone groups were significantly lower compared to both BDD treatments (table 1). In ozone treatments TRO formation was not limited by applied doses, therefore we assume that maximal ozone saturation of the water was reached. After initial regulation on day 1-3 of applied current on BDD electrodes, a constant applied doses of 2.00 A on the OS and a constant applied current of 0.75 A on the SS electrodes resulted in relative constant TRO values. TRO levels at the outflow of the ozonation or BDD electro cells was equal or higher compared to tank levels, but no significant differences between tank and outflow within treatments (n=30, paired t-test, $p<0.05$) or between treatments (n=90, Tukey, $p<0.05$) were observed. Although average TRO concentrations were lower in the ozone groups, a significant disinfection effect was only found in the ozone treatments not in OS or SS treatments, both for opportunistic and total bacterial counts. As a result average bacterial counts for both types were also lower in ozone treatments compared to all other treatments although differences were not found significant (n=6, Tukey, $p>0.05$).

Table 2. Average concentrations of total residual oxidant (TRO) in the culture tank (tank) and out flow of disinfection units (out), bacterial counts of 1 day (24 h) or 4 days (96 h) of incubation and reduction efficiency (RE) of bacteria.

	Control	ozone	OS	SS
TRO tank (mg l^{-1})	0.00 ± 0.00a	0.04 ± 0.02b	0.06 ± 0.03bc	0.06 ± 0.04c
TRO out (mg l^{-1})	0.00 ± 0.00a	0.06 ± 0.01b	0.06 ± 0.04b	0.10 ± 0.05b
24h (CFU ml^{-1})	4.5 ± 4.7	4.1 ± 4.2	4.3 ± 4.1	4.4 ± 4.4
RE 24h (%)	Nd	56 ± 23*	22 ± 31	12 ± 31
96 h (CFU ml^{-1})	6.6 ± 6.6	5.2 ± 5.4	5.9 ± 6.0	6.5 ± 6.8
RE 96h (%)	Nd	50 ± 28*	19 ± 28	14 ± 45

*disinfection performance significant different from 0 (n=6, paired t-test, $p<0.05$).

Chloride and bromide did significantly accumulate with increasing CFB in both the control and ozone treatments, in contrast no significant effect (n=3, OLS, $p>0.05$) on chloride was found for both OS and SS treatments and for bromide in SS treatment whereas bromide in OS treatment showed significant negative (n=3, OLS, $p<0.05$) accumulation (figure 3).

No significant accumulation of chlorate was observed in both control and ozone treatments (n=3, OLS, $p>0.05$) whereas significant accumulation in both OS and SS was found (n=3, OLS, $p<0.05$) and accumulation was therefore found significantly higher (n=3, Tukey, $p<0.05$) in both BDD treated groups (OS and SS) compared to control or ozone. Final concentration of chlorate were 1.1 ± 1.0 mg/l and 0.1 ± 0.0 mg/l for control respectively ozone and 24.8 ± 5.0 mg/l and 32.5 ± 6.4 mg/l for OS respectively SS.

No bromate was observed in any of the sampled tanks at any time point in both control and ozone treatments, in contrast bromate was observed in measurements in OS and SS tanks, except for initial sample. The accumulation of bromate was found significant in both BDD treatment (n=3, OLS, $p<0.05$) and accumulation rate did not vary between OS and SS but was significantly higher compared to control or ozone (n=3, Tukey, $p<0.05$) (figure 3). Final concentrations of bromate were 0.04 ± 0.03 mg/l and 0.07 ± 0.02 mg/l for OS respectively SS.

Final concentrations of total-chloride (Cl^- + ClO_3-Cl) and total bromide (Br^- + BrO_3-Br) were standardized to a CFB of 1. Final standardized concentrations of total-chloride were 47.0 ± 1.7 mg/g/l and 47.1 ± 2.6 for control respectively ozone treatment and 34.5 ± 1.6 mg/g/l and 37.6 ± 6.6 for OS respectively SS. Final standardized concentrations of total-bromide were 0.11 ± 0.01 mg/g/l and 0.11 ± 0.01 mg/g/l for control respectively ozone treatment and 0.05 ± 0.02 mg/g/l and 0.08 ± 0.03 mg/g/l for OS respectively SS. Differences were only found significant between control or ozone and OS for both total chloride and total bromide (n=3, Tukey, $p<0.05$).

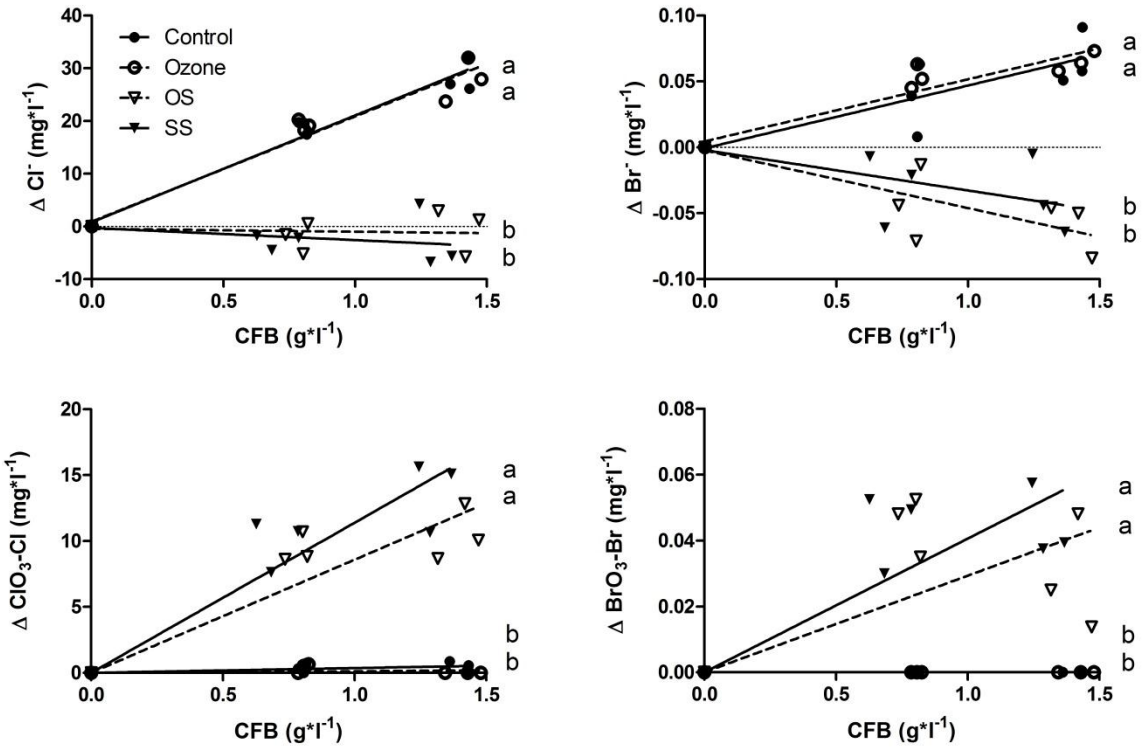

Figure 2. Observed changes in chloride, bromide, chlorate and bromate plotted against the cumulative feed burden (CFB). Different letters indicate significant differences between treatments (n=3, Tukey, $p<0.05$).

Effects of treatment on survival and health of eel are presented in table 2. Eel mortality did not significantly vary between treatments (n=120, Tukey, $p>0.05$). The majority of eels did show clinical signs of infection with *Aeromonas* sp. although significant differences between treatments were not observed (n=113-119, Tukey, $p>0.05$).

Infection of eels with *Dactylogyrus* sp. was higher in both BDD treatments, although significant differences were only observed between control or ozone and OS treatment (n=9, Tukey, $p>0.05$). Number of *Dactylogyrus* sp. per eel varied widely and most severe cases 3500 and 1400 individuals per eel were observed for OS respectively SS, due to high variations no significant differences between treatments were observed (n=9, Dunett-T3, $p<0.05$).

Table 3. Total observations of dead eels and disease occurrence after the 30 day experimental period.

Clinical signs of:	Control	Ozone	OS	SS
Mortality	0.06 ± 0.04	0.01 ± 0.02	0.03 ± 0.04	0.01 ± 0.01
Prevalence bacterial infections	0.55 ± 0.16	0.70 ± 0.09	0.59 ±0.06	0.44 ± 0.14
Prevalence *Dactylogyrus* sp.	0.44 ± 0.51^{a}	0.33 ± 0.00^{a}	0.89 ± 0.20^{b}	0.78 ± 0.39^{ab}
Dactylogyrus sp. ($fish^{-1}$)	80 ± 122	21 ± 39	437 ± 1149	241 ± 449

4. Discussion

4.1 Oxidants and by-product formation on BDD electrodes in fresh water RAS.

When fresh water is treated with BDD electrodes primarily hydroxyl radicals ($OH^{\bullet}$) are formed. Secondary formation products are hydrogenperoxide (H_2O_2), ozone (O_3) and peroxidisulphate ($S_2O_8^{2-}$) (Bergmann and Rollin, 2007). When fresh water which contains small amounts of Br^- and Cl^- is treated with BDD electrodes, hydroxyl radicals ($OH^{\bullet}$), chlorine (Cl_2) and bromine (Br_2) are simultaneously formed. Hydroxyl radicals have a very short half-life time in the range of milliseconds, and both chlorine and bromine are transferred to hypochlorous acid (HOBr) and hypobromous acid (HOCl). When sufficient bromine is present hypochlorous acid is substituted by bromine making hypobromous acid the main disinfection by-product formed (Echardt and Kornmueller 2009).

During the present study in both BDD treatments (OS and SS) concentrations of TRO were observed at the outflow of the elctrocells and equal or lower concentrations in the tank. Applied currents were 2.00 respectively 0.75 A on the OS and SS stacks, resulting in current densities of 37 A/m² respectively 99 A/m². Observed TRO values in the BDD treatments reached values as high as 0.20 mg l^{-1} and free bromide available to be oxidized varied between 0.02 and 0.09 mg l^{-1}. Consequently the measured TRO values in the BDD treatments must be a combination of at least oxidized bromide and one or more other by-products.

Next to these active by-products, measured as TRO, chlorate and bromate were formed (figure 2) which is a second indication that the measured TRO consisted both of free chlorine and bromine species. Furthermore, next the formation of TRO, chlorate and bromate it is likely that other disinfection by-products were formed during the present study in the BDD treated groups. In the OS and SS treatment roughly 12 mg l^{-1} Cl and 0.06 mg l^{-1} Br respectively 9 mg l^{-1} Cl and 0.03 mg l^{-1} Br were not recovered. Most likely these are chlorinated and brominated organics called trihalomethanes (THMs) and haloacetic acids (HAAs) as were also found after BDD treatment of fresh water by other authors (Echardt and Kornmueller 2009).

4.2 Conversion of waste products by BDD electrodes

The accumulation of nitrate was lower in both BDD treatments compared to ozone but not to control. Both oxidation processes, ozone and BDD, do oxidate protein bound nitrogen to nitrate, and differences between treatments are therefore not expected. However next to anodic oxidation, some electrodes including BDD electrodes can reduce nitrate to nitrogen gas at the cathodic site. However conversion efficiencies are mostly low and the by-products ammonia and nitrite might occur (Kraft

2003, Georgaud 2011, Bergmann and Rollin 2007). Because significant differences between BDD treatment and control were not observed, cathodic nitrate reduction was low or ineffective.

Next to lower nitrate concentration, nitrite concentrations were significantly elevated compared to control groups. This is unexpected because oxidation processes, including BDD treatment, oxidize nitrite to nitrate (Kraft 2003, van Bussel et al., 2013a, 2013b). However the nitrite can also be a by-product of cathodic nitrate reduction as described above. A second explanation can also be an effect of negative effect of BDD treatment on biofilter performance. Although TRO levels in the range of 0.05-0.15 mg l^{-1} can improve nitrification rates under circumstances (Klatt, 2012), it might well be that elevated levels of chlorate, bromate and possible THMs and HAAs negatively influence nitrification rates of the biofilter.

Ortho-phosphate accumulation in the BDD treatments was lower as in ozone and control treatments. The reduction of orthophosphate during BDD treatment is observed in several studies, both in marine and fresh water levels (Feng et al., 2003, van Bussel et al., 2013a, 2013b). Reaction mechanisms are unclear however binding of phosphate to calcium complexes followed by precipitation is suggested (van Bussel et al., 2013a). Although not considered significant, there was a trend that average concentrations of calcium were lower due to BDD treatment. Although recently it was found that elevated levels of orthophosphate in RAS might improve fish growth in RAS (Eding et al., 2012, van Bussel et al., 2013c) and is thus not beneficial to remove from the culture water, ortho-phosphate is a pollutant in RAS effluent water. Therefore further research to describe and optimize phosphate removal is needed.

The accumulation of TOC was significantly lower by all three oxidation processes applied. This is due to a processes that breakdowns larger organic molecules into smaller fractions and eventually into carbon dioxide (Martínez-Huitle & Ferro 2006). However in RAS it is still unknown to which extend TOC reduction by ozone is due to improved particle removal part and thus reduced leaching, or by conversion to carbon dioxide. Further research is needed both for BDD and ozone treatment to quantify effects of chemical oxidation and improved particle removal to optimize treatment of dissolved and particulate organic carbon in RAS.

4.3 Disinfection and effects on fish health.

Although significant amounts of TRO were produced within the electro-cells no significant bacterial reduction or effect on bacterial counts in the culture tanks were observed in the BDD treatments. In contrast disinfection with ozone was observed with lower TRO concentrations, however no effects on bacteria densities in the culture water were found. Clinical infections with bacteria, most probably of the *Aeromonas* sp. (Schaeperclaus, 1979) were neither affected by treatment. Although the general consensus is that reducing bacterial loads decrease bacterial diseases and therefore disinfection in RAS is useful. However it is known that by reducing hydraulic retention time, which can be obtained by

effective disinfection, fast growing pathogenic bacteria can outcompete other bacteria (Schneider et al., 2007). Therefore relating (the lack of) disinfecting performance and fish health should be done with care. Reducing substrate and nutrient availability in RAS in the form of TOC might therefore be a better future strategy to control bacterial diseases.

The infection rate of eel with *Dactylogyrus* sp. parasites and the number of parasites found per eel were higher in both BDD treatments. Concentrations of TRO as low as 0.06 mg/l Cl_2 are known to negatively affect gill morphology (Reiser et al., 2010). Although interactions between parasites and residual oxidants are unknown, it might be that gills damaged by TRO are more susceptible to parasites.

5. Conclusion

Based on the present study effects on TOC removal are comparable between ozonation and BDD treatment. In contrast to ozonation BDD treatment reduces ortho-phosphate accumulation, however this is of minor importance to fish health or growth. The formation of chlorate, bromate and other residual oxidants is a serious risk for both biofilter and fish performance, and is limiting the application of BDD electrodes in fresh water RAS. Furthermore the formation of limestone on electrodes, thereby changing current densities and effectiveness of BDD treatment is a serious problem limiting the practicality of BDD treatment in RAS. With respect to systems tested during the present study ozonation has clearly benefits over BDD treatment. However end-of-the-pipe treatment, where residual oxidant formation is not a limiting factor, might be an cheap and effective way to reduce nutrient emissions from fresh water RAS.

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General discussion

Effects of inorganic waste accumulation in RAS on fish health and performance.

The accumulation of metabolites in RAS

Fish production in land based recirculating aquaculture systems (RAS) is characterised by a trend towards intensification of water re-use (Blancheton et al., 2007). Due to excretion of metabolics by fish and leachning of nutrients from spilled feed and faeces 'inert' substances such as nitrate, ortho-phosphate and metals originating from make-up water and feed accumulate in the culture water. These substances are suggested as possible cause for negative effects on fish production performance (Davidson et al., 2009, Davidson et al., 2011, Deviller et al., 2005, Martins et al., 2009, Martins et al 2011). However the the causal effects of many of these substances are still unknown.

Nitrate toxicity is generally considered as low to fresh water fish (Camargo et al., 2005), and especially to marine fish (Brownell, 1980) however findings in chapter 1 suggest the opposite.

The effect of elevated nitrate levels in the culture water on the performance and health of a marine fish, the turbot (*Psetta maxima*) was studied. A negative linear relation between any concentration of nitrate applied (125, 250 or 500 mg / l NO_3-N) and growth was observed. Although effects on health were only considered significant for the two highest treatments (250 and 500 mg/l), the most sensitive parameters such as the relative spleen size and survival were also slightly affected at the 125 mg/l NO_3-N treatment. These effects on growth and health indicate that there is not a safety limit or threshold, the optimum for fish culture is 0 mg/l NO_3-N. The same effects can also be observed by two other studies done on marine cultured species that focused on growth (Frankes and Hoff, 1982, Kuhn et al., 2010). Nitrate does convert hemoglobin into methemoglobin, and similtanously the reductase systems converts methemoglobin back to hemoglobin. No differences in methemoglobin levels were observed suggesting that turbot are capable of maintaining hoemostasis. The reduced growth performance and negative effects on health at any nitrate concentration can thus be explained by energetic costs to maintain homeostasis by converting and excreting the up-taken nitrate. This increased energy costs are not compensated by extra feed intake as voluntary feed intake declined. Furthermore the decreased feed conversion efficiency and reduced health are other indicators for an increased energy demand for detoxification to maintain homeostasis.

Ortho-phosphate is not considered toxic to fish, however in natural water concentrations found in natural waters are a fraction of concentrations found in intensive RAS. Thus in chapter 2 the effect of elevated phosphate levels (25, 50 and 75 mg/l OP_4-P) in the culture water on performance and health of turbot was studied. No indications of ortho-phosphate toxicity on the health status of turbot were observed. Production performance was neither negatively affected by ortho-phosphate, however a

slightly improved growth was observed at moderate elevated ortho-phosphate levels. When turbot were fed a low phosphorus diet, growth rates were significantly improved by the elevated water ortho-phosphate concentrations. The improved growth was a result of increased feed intake, but not of increased feed conversion efficiency. However a trend was found for increased fat content in the control group and increased protein retention efficiency in the elevated ortho-phosphate treatments. Other studies on effects of phosphate on fish performance in marine water are lacking. A recent study with a fresh water fish species showed that elevated phosphate levels do not increase voluntary feed intake but do reduce metabolic costs resulting in higher feed efficiencies (Eding et al., 2012). This suggests that turbot can uptake and utilize ortho-phosphate from the culture water and thereby increases voluntary feed intake.

Like nitrate and phosphate, trace metals are considered as relatively low toxic in existing concentrations but effects on fish are only studied in fresh water species. Thus in chapter 3 the effect of the elevated concentrations of the five main dietary trace metals (Fe, Zn, Cu, Co and Mn) on the performance and health of turbot was studied. Fe levels showed a quick decrease in the culture water, due to oxidation and successive precipitation. Manganese showed a slower breakdown and precipitation. Other trace metals were relatively stable over time, although observed copper concentrations were much lower as nominal concentrations; the remaining trace metals concentrations were comparable with nominal concentrations. Fish growth or feed efficiency was not affected by any elevated level of metals, neither was health affected. However significant relations between exposure and body concentrations of Zn, Mn and Co were observed. Furthermore it was observed that with increased metal accumulation the dry matter content of the body decreased. This suggest that turbot do uptake and incorporate these trace metals from water into their body, and consequently compensate by incorporating extra water in the muscle tissue. The relation between water exchange rates and heavy metal concentrations in the body is also observed by other authors in both fresh and marine species (Deviller et al., 2005, Martins et al., 2011) but proximate body composition was not determined. However the increased incorporation of water, resulting in a decreased dry matter and caloric content, is also a known effect of exposure of fish to aquatic metals (Belanger et al., 1986). Toxic concentrations of Zn, Mn and Co to fish are relatively equal (Taylor et al., 1985, Partridge and Lymbery, 2009, Diamond et al., 1992). However requirements of fish for Zinc are a factor 5-50 higher compared to manganese respectively cobalt requirments, and observed concentrations in the diets was even a factor 30-500 higher. Consequently Zinc is potentially the metal that can become first toxic in marine RAS and should be monitored carefully when marine RAS production is very intensive.

Limitations on the intensification of RAS

To reduce the potential for the accumulation of nitrate, phosphate and trace minerals feed formulation should be optimized for RAS. A high digestability of the feed combined with a high retention efficiency of nutrients reduces the potential or waste production. This is desirable for any aquaeed, but is of specific interest in RAS production. Nitrogen, phosphorus and the trace metals are the main inorganics that are suspected to affect fish performance.

For turbot RAS operating at water exchange rates at around 400 liter make up water per kg feed applied, theoretical nitrate-nitrogen levels will be around 125 mg/l. At this level negative effects on turbot growth were observed. With respect to ortho-phosphate, turbot RAS operating at water exchange levels as low as 50 liter make up water per kg feed applied, theoretical ortho-phosphate levels will be around 75 mg/l. This was the highest level tested and no negative effects on growth were observed. With respect to trace minerals, at water exchange levels as low as 10 liter make up water per kg feed applied no negative effects on growth were observed. Thus from these inorganics, nitrate is most liming fish performance in RAS production.

Nitrate removal is thus, after the nitrification process and solids removal, first priority in intensive marine RAS production. However the linear relationship between nitrate and growth suggest that nitrate concentration and should be kept as low as possible. Costs for denitrification and costs for water exchange should be balanced for optimal RAS management. Feed formulation should also be balanced between extra costs for optimal protein retention and less costs due to nitrate removal/water consumption decrease. In contrast ortho-phosphate is not limiting growth at any levels which would be relevant in RAS. Ortho-P does have positive effects on fish performance and should therefore preferably not be removed in the system water, but in the discharge water so called end-of-the-pipe treatment. Trace minerals do not effect growth or health and are therefore less relevant. However the reduced dry matter content in the fish and the high potential for zinc to reach toxic levels are reasons to optimize retention of metals in fish, to minimize accumulation and to monitor metal concentrations in very intensive marine RAS.

The effect of BDD electrodes on water quality in RAS

In chapters 4, 5 and 6 the effects of BDD electrodes on organic accumulation, in-organic accumulation and disinfection were studied. Several forms of oxidized forms of chloride and bromide were determined, commonly called disinfection by-products. This disinfection by products form serious limitations on the use of BDD electrodes, and are a serious disadvantage compared to ozone.

The formation of disinfection by-products on BDD electrodes

In the present studies in both in fresh and marine water significant amounts of TRO were produced on the BDD electrodes. These TRO are formed simultaneously with hydroxyl radicals, because chloride in the water is oxidized to chlorine gas and bromide to bromine gas. Due to different equilibriums and follow up reaction, as discussed in chapter 6, hypobromous acid is the main form in seawater and hypochlorous acid in fresh waters (Echardt and Kornmueller, 2009). The formation of TRO after BDD electrolysation is inevitable and is dependent on current density and salinity (Diaz et al., 2011, Echardt and Kornmueller 2009, Bergmann and Rollin, 2007). Such TRO are very harmful to fish and shrimp (Reiser et al., 2010, Schroeder et al., 2010). Furthermore it was observed that these TRO are relatively stable and do accumulate. In contrast ozone produced TRO have a tendency for a quicker breakdown, and thus the rate of TRO accumulation is less. Differences in TRO stability are caused by the fact that BDD electrodes are less selective and can oxidize any ion present (e.g. bromide, chloride, ammonia), while ozone prefers the oxidation of bromide (Schroeder et al., 2011). The accumulation of these TRO produced by BDD electrodes was observed in small scaled RAS in both fresh water (chapter 6) and marine water (chapter 4) but also by other authors (Diaz et al., 2011). Even in a commercial scaled RAS with a hydraulic retention time of 4.5 hours (chapter 5) TRO accumulated. This means that, in contrast to drinking water or ballastwater treatment, applied doses is limited by the amount of TRO directly produced and the amount of remaining TRO in the culture water. Minimation of TRO formation is thus the keypoint for safe and effective use of BDD electrodes. Low current efficiencies for TRO formation were found in the range between 0 and 30 A/m^2. Further research should focus on such low current densities to oxidize waste products in RAS without the accumulation of TRO and other disinfection by-products.

Besides the formation of hypobromous and hypochlorous acid (measured as TRO), production of the persistant by-products bromate and chlorate was observed in the fresh water experiment (chapter 6). In contrast ozonation did not result in any bromate or chlorate formation. In marine water (chapter 4 and 5) bromate and chlorate were not measured due to analytical limitations; however other studies suggest that formation of perchlorate, chlorate and bromate increase with salinity (Echardt and Kornmueller, 2009) Although chlorate and bromate are not acute toxic to fish, they have been proven to be toxic to algae and bacteria (van Wijk and Hutchinson, 1995) which cause adverse effects on biofiltration as was observed in chapter 6. However minimizing TRO formation by only applying low current densities might also be a strategy to reduce the accumulation of perchlorate, chlorate and bromate.

Although not directly measured during the present studies, ion balances (chapter 6) suggest the production of significant amounts of chlorinated and brominated organics, when aquaculture water is treated with BDD electrodes. These products are formed when organics are present, and bromoform is the major form present (Echardt and Kornmueller, 2009, Diaz et al., 2011). In contrast ozonation did not suggest any formation of bromoform or chloroform. Although not as acute toxic as hypobromous or hypochlorous acid, bromoform and chloroform affect fish and bacteria at relevant low concentrations (Gibson et al., 1979). Therefore negative interactions on fish and biofilter performance can be expected. Further research is needed to investigate if bromoform formation can also be minimized by applying only low current densities

Waste conversion by BDD electrodes

Although the dose of oxidants was limited by TRO formation, BDD treatment did also decrease the accumulation of some waste products in RAS. Organics, in the present study measured as dissolved colored organic matter (DCOM) and total organic carbon (TOC), was significantly reduced by BDD treatment. Similar findings, COD reduction, both in fresh and marine aquaculture water have been reported in literature (Feng et al., 2003, Diaz et al., 2011). For RAS production this means that based on organics, water quality can be improved and water consumption can be reduced. However it might be that BDD treatment, like ozonation improves the removal of small particulate matter due to improve coagulation. To reveal the full potential for BDD electrodes in RAS, the combination of BDD treatment and mechanical treatment of the water needs further investigations.

Besided the removal of organics, BDD treatment reduced concentrations of ortho-phosphate in the culture water. This process is also observed in another study (Feng et al., 2003) but mechanism of removal remains unclear. Binding/coagulation with other molecules, followed by precipitation or mechanical filtration might be a possible pathway. However, based on finding in chapter 2, orthophosphate accumulation is not a factor limiting water exchange rates in RAS systems.

Effects of BDD treatment on nitrogen compounds were observed in the several pre-studies. BDD treatment oxides nitrite to nitrate, but both ammonia and nitrate levels are unaffected. This is in

contrast to other studies which described nitrogen removal due to nitrogen gas formation (Kraft et al., 2003, Georgeaud et al., 2011, Diaz et al., 2011). However in these studies applied currents were not limited by TRO formation and thus processes such as breakpoint chlorination and anodic nitrate reduction can effectively take place. As was seenrevealed during the present long term experiments these processes could not be observed during the safe application in RAS. Further studies are needed to see if electrochemical nitrogen removal is a safe and cost effective alternative to biological denitrification is needed.

Disinfection with BDD electrodes

In our pre-experiments as well as in previous studies (Echardt and Kornmueller, 2009) significant disinfection effects were found using BDD electrodes. Due to their high reactivity, hydroxyl radicals react very fast with any substance present in the water, resulting in a low, contact time between pathogen and hydroxyl radicals. Therefore it is rateher the more persistant secondary oxidants than the hydroxyl radicals that enable effective disinfection (Furuta et al. 2005). As discussed above these by-products are the main drawback of BDD electrodes in RAS and should be minimized. Consequently in both fresh and marine water, no effective disinfection performance was observed for bacterial counts under practical RAS circumstances. Furthermore in all three studies disinfection took place in a by-pass, therefore not the whole water flow was disinfected. For effective disinfection the whole water flow should be treated to avoid mixing of disinfected and non-treated water. Furthermore biofilm formation on the electrodes was observed, and the formed biofilm served as substrate for bacterial growth and bacterial densities of $4.4*10^7$ CFU / cm² were observed, making the BDD electrodes a source of bacteria. This might also partly explain the high standard deviations observed in bacterial disinfection experiments.

Usability and energy costs of BDD electrodes

The energetic costs to treat water with BDD electrodes is low, and is mainly dependent on the energy use by the transformer. The electrical power consumed by the Ectosys system to treat a 45 m³ RAS was only 8 Watt (1 A * 8 V) but was limited by TRO formation. Electrochemical treatment of RAS water is therefore considered to be very low in energy consumption. However in all three studies, both in fresh and marine water, formation of limestone was observed within hours. Because limestone formation changes the actual electrode surface it will change applied current densities and thus TRO formation. As a result electrodes should be cleaned intensively to maintain optimal current densities. When done manual this is labour intensive. Furthermore after continues use, in all types of electrodes used and in both fresh and marine water damage of the diamond layer on the electrodes could be observed. Like limestone formation, a reduction of the diamond surface will change the actual current density applied, and will quickly decrease the maximal doses of oxidants applied to keep TRO levels

within limits set. For continues effective working diamonds, a constant renewal of the diamond layer is needed.

Is BDD treatment an alternative for ozonation in RAS?

BDD treatment in RAS can be a way to decrease organic load and ortho-phosphate concentrations in RAS. However the formation of stabile TRO, perchlorate, chlorate, bromate and chlorinated and brominated organics is a major drawback compared to much lower formation of such byproducts during ozonation. Keeping applied current densities low might be a way to minimize the formation of these unwanted by-products. Consequently this means that a large area of diamond is needed which increase investment costs. The formation of limestone, requiring constant maintenance is a second drawback compared to ozonation. The low power consumption is a benefit compared to ozonation, however the deterioration of the diamond layer means a constant additional operating costs.

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Summary

To reduce water and energy consumption in recirculating aquaculture systems (RAS) there is a trend over the last years to reduce water exchange levels. Due to this reduction in water exchange several substances accumulate in the system water. The most important inorganic water compounds originally are applied by the feed and are nitrate, orthophosphate and the trace elements iron (Fe), zinc (Zn), copper (Cu), manganese (Mn) and cobalt (Co). The effects of these inorganics in the culture water on marine fish species are unknown. Therefore, in the first part of this thesis, the effects of these inorganics on growth, feed utilization and several health parameters of a marine fish species, the turbot (*Psetta maxima*) were studied.

In 12 independent RAS nitrate levels were artificially increased. In a dose-response study the effects of four different nitrate levels on the performance of juvenile turbot were evaluated. It was observed that the voluntary feed intake was negatively affected by elevated nitrate-nitrogen concentrations in the range of 125-500 mg/l NO_3-N compared to a control treatment at 4 mg/l NO_3-N. Effects on fish health were only observed above 125 mg/l NO_3-N and included increased mortality and increased spleen size. Blood parameters were not affected, suggesting that turbot are capable of keeping homeostasis up to 500 mg/l NO_3-N, although this increased energetic demands.

Next to nitrogen compounds other accumulating substances possibly affect the growth performance of turbot. In a second dose-response study the effect of ortho-phosphate on production performance parameter and health parameter of juvenile turbot was studied. It was found that elevated ortho-phosphate concentrations did not negatively affect health and growth of turbot in RAS. Neither voluntary feed intake nor health is negatively affected by elevated ortho-phosphate concentrations in the range of 3-75 mg/l PO_4-P. However fish cultured under ortho-p concentrations of 25 mg/l showed a tendency for higher feed intake and growth compared to the control groups. When fed a low-P diet growth at elevated ortho-p concentrations (25 mg/l), growth was significantly higher compare to a control, suggesting that turbot are capable of taking up and utilize waterborne phosphorus.

This resulted in the hypothesis that turbot are capable to absorb trace elements from the culture water, which was tested in another study. In detail the effects of the accumulation of the elements Fe, Zn, Cu, Co, Mn and Co in the culture water on turbot were evaluated. In 5 individual RAS the accumulation of these metals at 5 different water exchange rates (between 1000 and 10 l/kg feed) were simulated, and effects on turbot performance and health were studied. It was observed that the accumulation of metals (Fe, Zn, Cu, Co, Mn) did not negatively affect turbot growth up to water exchange rates as low as 10 l/kg feed applied. However Zn, Co and Mn bioaccumulate in turbot resulting in decreased dry matter content. Based on the concentration observed in a commercial RAS and in commercial turbot diets, Zn is the most important parameter which should be monitored when turbot is raised in intensive RAS.

In the second part of this thesis water treatment to reduce accumulating substances and disinfection at low water exchange rates was investigated. So called Boron Doped Diamonds (BDD) electrodes are artificial diamond electrodes that are applied in wastewater-, drinking water- and ballast water-treatment. These BDD electrodes are used to remove organic and inorganics, such as nitrates, phosphates and trace metals. Furthermore BDD electrodes are used to disinfect drinking water and wastewater. Until now it was unknown if BDD electrodes also could be applied in RAS.

In a fourth experiment it was observed that in small scaled RAS, concentrations of total organic carbon (TOC), colored dissolved organic matter (DCOM) and orthophosphate could be reduced by BDD treatment. The concentration of total residual oxidants (TRO) could be kept below critical concentrations for turbot. However due to the production and accumulation of these TRO the applied current on the electrodes was limited, reducing the disinfection potential. An effective disinfection was possible but will directly lead to fish critical TRO concentrations. In comparison to BDD treatment, conventional ozonation resulted in a higher reduction of TOC and CDOM, but not to orthophosphate removal. Additionally an effective disinfection was not realized by ozonation, due to TRO formation.

In a fifth experiment BDD electrodes were tested in a commercial scaled RAS. Also here a reduction of TOC and orthophosphate could be observed, compared to a control treatment. However, accordingly to the small scaled RAS, TRO formation was limiting the applied current and consequently an effective disinfection.

The last experiment in fresh water small-scaled RAS showed that, also in fresh water, BDD treatment led to a reduced accumulation of TOC and ortho-phosphate compared to a control treatment. In accordance to marine water, also in fresh water the formation of TRO was limiting the applied dose and thus the disinfection performance. The removal of TOC was on a comparable level between ozonation and BDD treatment, but no orthophosphate removal during ozonation was observed.

In all three experiments it was observed that the accumulation of organics and inorganics can be reduced by BDD treatment without exceeding fish critical TRO concentrations. However the formation of these TRO, which are stable and accumulate in the culture water, is limiting the application of BDD electrodes in RAS. Furthermore the accumulation of perchlorate, chlorate, bromate, and chlorinated and brominated organics is a serious drawback for the use of BDD electrodes in RAS. By applying low current densities formation of these by-products can be minimized. Due to the formation of stabile TRO, low current densities must be applied. Consequently effective disinfection is not possible by safe application. Effective disinfection is possible, however this will result immediately in fish toxic TRO concentrations.

Electrical energy consumption for the oxidation of waste products on BDD electrodes is very low and most energy is consumed by the transformer. In this way BDD treatment can be a cheap and efficient way of removing waste products from RAS. However because current densities must be kept low, large diamond surfaces are needed resulting in high investment costs. By applying continues current on the BDD electrodes a rapid buildup (within hours) of limestone and biofilm was observed. This

biofilm was found to be a substrate for bacterial growth, but more important the limestone reduced the available surface for oxidation of waste products. As a result current density will increase leading to increased formation of TRO. Furthermore after continuous use for two to four weeks clear deterioration of the diamond surface was observed. Besides optimization of BDD electrocell adjustment further studies should focus on reduction of TRO formation to obtain effective removal without exceeding fish toxic TRO concentrations. Furthermore possibilities for the electrochemical reduction of nitrate with BDD electrodes could be a cost effective way to enhance RAS productivity.

Zusammenfassung

Um den Einsatz von Energie und Wasser in Kreislaufanlagen (KLA) in der kommerziellen Fischzucht zu optimieren, gibt es den Trend die Wasseraustauschraten in KLA weiter zu minimieren. Durch diese Reduktion akkumulieren verschiedene Substanzen im Produktionswasser. Die wichtigsten anorganischen Substanzen, die sich in KLA akkumulieren, werden über das Futter eingetragen und sind Nitrat, Orthophosphat und die Spurelemente Eisen (Fe), Zink (Zn), Kupfer (Cu), Mangan (Mn) und Cobalt (Co). Wie sich diese Substanzen auf marine Fischarten auswirken ist weitestgehend unbekannt. Die Erfassung und Beurteilung dieser Substanzen im Systemwasser stellen den Ausgangspunkt der Untersuchungen dar. In dem ersten Teil der vorliegenden Arbeit wurden die Effekte dieser anorganischen Substanzen auf Wachstum, Futterverwertung und verschiedene gesundheitsrelevante Parameter einer marinen Fischart, demSteinbutt (*Psetta maxima*) untersucht.

So wurde in einer ersten Dosis-Wirkungsstudie der Einfluss der Nitratkonzentration auf das Wachstum juveniler Steinbutt in 12 separaten KLA untersucht. Es konnte festgestellt werden, dass die freiwillige Futteraufnahme durch erhöhte Nitrat-Konzentrationen bereits ab einer Konzentration von 125 mg/l NO_3-N im Vergleich zu einer Kontrollbehandlung mit nur 4 mg/l NO_3-N negativ beeinflusst wird. Der Gesundheitsstatus der Fische, gemessen an den Parametern Sterblichkeit sowie relative Größe der Milz, war bereits bei einer Konzentration von 125 mg/l NO_3-N negativ beeinflusst. Da gemessene Blutparameter bis zu einer Konzentration von 500 mg/l NO_3-N unbeeinflusst waren, ist davon auszugehen, dass der Steinbutt in der Lage ist, die Homöostase trotz hoher energetischer Kosten aufrecht zu erhalten.

Neben Abbauprodukten des Stickstoffs, haben noch weitere Nährstoffe und chemische Elemente Einfluss auf das Wachstum und die Gesundheit von insbesondere in Kreislaufanlagen kultivierten Fischen. In einer weiteren Dosis-Wirkungsstudie wurde der Einfluss des Abbauprodukts Orthophosphat in 4 Stufen mit jeweils 3 Replikaten auf Leistungs- und Gesundheitsparameter am Steinbutt untersucht. Bei einer Erhöhung der Orthophosphat-Konzentrationen von 3 auf 75 mg/l PO_4-P wurden keine negativen Auswirkungen auf gemessene Gesundheitsparameter festgestellt. Allerdings zeigten Fische, die bei einer Konzentrationen von 25 mg/l PO_4-P kultiviert wurden, eine Tendenz zur gesteigerten Futteraufnahme einhergehend mit höherem Wachstum im Vergleich zu den Kontrollgruppen. Bei der Verabreichung einer Phosphor-limitierten Diät, in Kombination mit einer erhöhten Orthophosphat-Konzentration (25 mg/ l), war das Wachstum signifikant höher als in der Kontrollgruppe. Diese Ergebnisse belegen, dass der Steinbutt Orthophosphat aus dem Wasser absorbieren und metabolisieren kann.

Die daraus resultierenden Hypothesen zur Absorption von anorganischen Mikronährstoffen aus dem Systemwasser wurden in einer weiteren Studie überprüft. Hierbei wurde der Einfluss von akkumulierenden Metallen (Fe, Zn, Cu, Co, Mn) auf Produktionsparameter beim Steinbutt untersucht.

In fünf KLA wurde die Akkumulation dieser Metalle für fünf verschiedenen Wasseraustauschraten (zwischen 1000 und 10 l/kg Futter) simuliert, und der Effekt auf Leistungs- und Gesundheitsparameter am Steinbutt untersucht. Es konnte festgestellt werden, dass keine Bioakkumulation von Cu und Fe im Steinbutt erfolgt. Allerdings wurden Zn, Co und Mn aus dem Wasser aufgenommen und im Körper akkumuliert, und führten außerdem zu einer geringeren Trockenmasse des Steinbutts. Basierend auf der Konzentration dieser Spurenelemente im Wasser und in kommerziellen Futtermitteln, sowie der Funktionalität dieser Elemente in biochemischen Prozessen im Metabolismus des Fisches, kann Zink die größte Bedeutung zugesprochen werden und sollte in intensiv geführten marinen KLA überwacht werden.

Die sogenannte Bor-dotierte Diamant (BDD) Elektrode ist eine künstlich produzierte Elektrode, die in Abwasser-, Ballastwasser- und Trinkwasserbehandlung eingesetzt wird. Sie dient der Entfernung verschiedener organischer Substanzen, sowie der Entfernung unterschiedlicher anorganischer Substanzen, wie u.a. Nitrat, Phosphat und Metallen. Außerdem werden BDD Elektroden zur Desinfektion von Trinkwasser und Ballastwasser angewendet. Potential und Grenzen dieser BDD Elektroden für den Einsatz in der kreislaufgeführten Aquakultur sind bislang nahezu unerforscht und stellen den Fokus des zweiten Teils dieser Arbeit dar.

In einem vierten Versuch in marinen kleinskaligen KLA konnte gezeigt werden, dass die Konzentration von Gesamt- organischem Kohlenstoff (TOC) und gelösten organischen Substanzen (CDOM) sowie Orthophosphat durch den Einsatz der BDD-Elektroden im Vergleich zu einer unbehandelten Kontrolle deutlich verringert werden kann.. Die Bildung von Restoxidantien (Total residual oxidants - TRO) konnte hierbei unterhalb der für Steinbutt kritischen Höhe gehalten werden. Das Desinfektionspotential der BDD-Elektroden scheint jedoch durch die Dosis-limitierende Bildung toxischer Restoxidantien stark begrenzt zu sein. Eine effektivere Desinfektion ist möglich, diese führt jedoch direkt zu einer für Fische kritischen TRO Konzentration. Im Vergleich zur BDD-Behandlung resultierte die konventionelle Ozonisierung zu einem erhöhten Abbau von TOC und CDOM, nicht aber von Orthophosphat. Eine effektive Desinfektion war auch hier limitiert durch TRO-Akkumulation im Prozesswasser.

Unter Verwendung einer marinen KLA im Technikumsmaßstab konnten in einem fünften Versuch die bereits aufgezeigten Effekte auch für den kommerziellen Maßstab verifiziert werden. Die BDD-bedingte Reduktion von TOC und Orthophosphat fiel sogar höher als bei konventioneller Ozonisierung aus. Zudem bestätigte sich die stetige Anreicherung von TRO, welche wiederum die Effizienz der Desinfektion einschränkte.

Ein sechster Versuch in kleinskaligen Süßwasser-KLA zeigte, dass die Konzentrationen von TOC und Orthophosphat auch in Süßwasser deutlich verringert werden konnten, wenn das Systemwasser mit der BDD-Elektrode behandelt wurde. Aber auch im Süßwasser limitierte die Bildung von TRO den Einsatz der Elektrode und die Desinfektionsleistung. Der Abbau von TOC mittels Ozonisierung war

gleichwertig zur BDD-Behandlung, obwohl der Abbau von Orthophsophat mittels BDD effektiver war.

In allen 3 Versuchen wurde gezeigt, dass sowohl organische als auch anorganische Substanzen deutlich reduziert werden können, ohne kritische TRO-Werte zu überschreiten. Da die Bildung dieser TRO kontinuierlich verläuft und stets neue TRO gebildet werden, limitiert dies die Anwendung der BDD-Elektroden in KLA. Zudem ist die Entstehung von Perchlorat, Chlorat, Bromat, sowie chlorierte und bromierte organische Verbindungen ein stark limitierender Faktor für die Verwendung von BDD-Elektroden in KLA. Nur der Einsatz geringer Stromdichten kann die Bildung dieser Nebenprodukte begrenzen. Der sichere Einsatz bei ausreichender Desinfektion ist somit nur begrenzt möglich. Eine weitere Erhöhung der Desinfektionsleistung führt umgehend zu einem Anstieg der TRO-Werte und damit zu Fisch-kritischen Konzentration. Die elektrische Energie, die für die Oxidation der Abfallprodukte in der BDD Elektrode benötigt wird, ist sehr gering, und wird überwiegend vom Transformator verbraucht. Somit stellt die Desinfektion von Systemwasser mit der BDD Elektrode eine Betriebskosten-günstige Methode dar. Dementgegen stehen hohe Investitionskosten einer Diamantbeschichtung großer Elektrodenflächen, um auch bei geringen Stromdichten effektive Leistungen erreichen zu können. Beim Stromfluss durch die BDD Elektrode kommt es binnen weniger Stunden zur Bildung eines Biofilms und Kalksteins auf dem Diamantträger. Die Bildung des Kalksteins verringert die für die Oxidation von Abfallprodukten nötige Oberfläche entscheidend. Diese geringere Diamantfläche führt zu einer Erhöhung der Stromdichte, welche wiederum die Bildung der TRO steigert. Die Diamantfläche in der BDD Elektrode war nach einem Einsatz von 2 bis 4 Wochen deutlich abgenutzt.

Der Einsatz von BDD Elektroden sollte dennoch weiterhin mit dem Ziel der Verringerung der sich bildenden TRO Werte untersucht werden. Außerdem wird empfohlen die elektrochemische Reduktion von Nitrat mittels BDD Elektroden weiter zu optimieren, da die Akkumulation des Nitrats größte Auswirkungen auf die Produktivität mariner KLA besitzt.

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