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Effect of oxidation–reduction potential on performance of European sea bass (*Dicentrarchus labrax*) in recirculating aquaculture systems

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Abstract:

The direct impact of oxidation–reduction potential (ORP) on fish welfare and water quality in marine recirculating aquaculture systems (RAS) is poorly documented. In this study, the effects of the fish size (S_1, S_2, S_3) and ORP level (normal, four successive levels) on the performance of European sea bass (*Dicentrarchus labrax*) were investigated. Three size fish were distributed into two RAS (RAS and RAS O_3). Ozone was injected into RAS O_3 to increase the ORP level. The ORP was stabilized to four successive levels: 260–300, 300–320, 320–350, and 300–320 mV in fish tanks during four periods ($P_{1–4}$). At the last day of each period, the hematological parameters, plasma protein and mortality of sea bass were analyzed. Two-way ANOVA revealed that several hematological parameters, including pH, hematocrit, concentrations of oxygen, carbon dioxide, glucose (Glu), ionized calcium, kalium, and hemoglobin, were significantly influenced by the increased ORP levels over the experimental period. The alteration in blood Glu and plasma protein concentration showed that ORP around 300–320 mV started to stress sea bass. Once the ORP exceeded 320 mV in the tanks during the P_3 period, mortality occurred even when total residual oxidants/ozone-produced oxidants was only 0.03–0.05 mg L^{-1} in the fish tanks. At the same time, plasma protein decreased notably due to appetite depression. After the decrease in ORP during the P_4 period, mortality continued. In conclusion, the results strongly suggest that for European sea bass in RAS, the ORP should not exceed 320 mV in the tanks. Once ozonation damaged fish, the effect seemed to be irreversible. However, how ORP affected related hematological parameters still need the further investigations.

Keywords: ORP ; Performance ; European sea bass ; RAS ; Ozone

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1. Introduction

With limited land and clean seawater resources, and increasing demand for seafood, the expanding aquaculture industry is facing many challenges (Zhang et al. 2011). In response, recirculating aquaculture systems (RAS) or semi-RAS are frequently used instead of flow-though systems (FTS). Compared with traditional FTS, RAS offer advantages such as a stable environment in terms of physico-chemical parameters and microbial flora, which provides an effective control or pre-control of diseases and improves the welfare of culture animals and less environmental impact (Blancheton 2000; Tal et al. 2009).

Many studies have focused on optimizing the RAS environment, especially physicochemical indexes, in order to optimize the living conditions of cultured animals. The impacts of water quality parameters such as pH, temperature, salinity, and dissolved gas in RAS on the biological performance of fish have been studied (Kristensen et al. 2009; Soria et al. 2007). Seawater is a complex medium with strong interactions among chemical parameters, as pH interacts with the gas balance (mainly CO_2), and pH and temperature affect the balance between un-ionized and ionized ammonia (Sanni and Forsberg 1996; Whitfield 1974).

The oxidation-reduction potential (ORP) results from all reactions involving both oxidations and reductions and varies as a function of the standard potential, relative ion concentration, temperature, and the number of electrons transferred (Banhidi 1995). It is dependent on all oxidants and reducers present in the system. In water, the ORP is strongly related to temperature, pH, salinity, and concentrations of dissolved oxygen and dissolved oxidants such as ozone (Liu et al. 2009; Summerfelt et al. 2009; Tango and Gagnon 2003). Nowadays, ozone is wildly used in aquaculture for disinfection, water treatment, and bacteria control (Buchan et al. 2006; Forneris et al. 2003).

During ozonation, ozone reacts with several compounds resulting in the formation of ~~-o~~zone-produced oxidants" (OPO) also mentioned as total residual oxidants (TRO) including free bromine (HOBr/OBr−) and bromamines (NH2Br, NHBr2), which are more stable than ozone. The toxicity of TRO and particularly of related bromate compounds to aquatic animals at low dosage is focused (Reiser et al. 2010). However it is also proved that bromate will not be formed when ammonia is present in the seawater (Tango et al. 2003, Schroeder et a. 2011).

To date, few studies mentioned a direct connection between ORP and biology, although most existing reports describe the ORP as an easy tool for monitoring ozonation in aquaculture systems (Buchan et al. 2005). Summerfelt et al (2009) reported that an ORP of 375–525 mV was required to reach the mean daily ozone concentration necessary to obtain full-flow disinfection in freshwater RAS. For southern rock lobster larvae, survival was higher and bacterial contamination was

lower when the ORP was between 330 and 500 mV(Ritar et al. 2006). With moderate ozonation corresponding to an ORP value of 250 mV in a low exchange freshwater RAS, rainbow trout showed improved performance compared to a system without ozonation (Good et al. 2011). However, the influence of ORP modification using ozonation on marine fish physiology in RAS has rarely been studied (Silva et al. 2011). In particular little is known about European sea bass which is widely cultured in the Mediterranean Sea (Blancheton 2000).

In this study, the impacts of (1) four successive ORP levels induced by ozonation and (2) fish sizes on physiological and hematological parameters of European sea bass (*Dicentrarchus labrax*) were studied and compared to data from a RAS without ozonation. The goals of this study were to identify the safe range of ORP for sea bass and to evaluate how European sea bass respond to ORP alterations.

2. Materials and method

2.1. RAS structure

Two RAS located at the French Research Institute for Exploration of the Sea (Ifremer, Palavas les Flots, Montpellier, France) have been running for several years. Each consists of three 1 m^3 fish tanks, a mechanical filter, UV disinfection, a protein skimmer, a warm-cold exchanger, moving bed biofilters, a degassing unit, and storage and pumping tanks organized in four loops (Fig. 1). In the first loop, water flowing out of the fish tank is filtered in a mechanical filter and pumped from the storage tank back to the fish tanks. Three parallel loops connected to the pumping tank were used for the other water treatments (1) biofiltration, (2) ozonation and protein skimming and (3) UV disinfection, a heat exchange, and degassing. The first of the two RAS was equipped with an O_3 generator (BMT 802N, BMT Messtechnik, Berlin, Germany), which converted pure oxygen to ozone. This system was named RAS O_3. The O_3 generator was controlled by an ORP sensor placed after the biofilters and delivered a maximum of 4 g O_3 per hour. In the second system, named RAS, air was used as feed gas in the skimmer. Two systems were set up in the same way before ozone injection into RAS O_3.

pH was controlled by injecting sodium hydroxide into the pumping tank in order to keep the value around 7.2–7.8 in the fish tanks. Temperature was maintained at 21.5 °C. Salinity was between 20-30 ppt. Oxygen concentrations in all fish tanks of both systems were almost similar at a super saturation level between 120-140% except it in fish tanks of RAS O_3 was 10% higher than RAS in the first two days at the P_3 period. The water flow rate to each fish tank was 1 m^3 h^{-1}. The daily water renewal rate was between 1 and 2 m^3 of new water per kg feed.

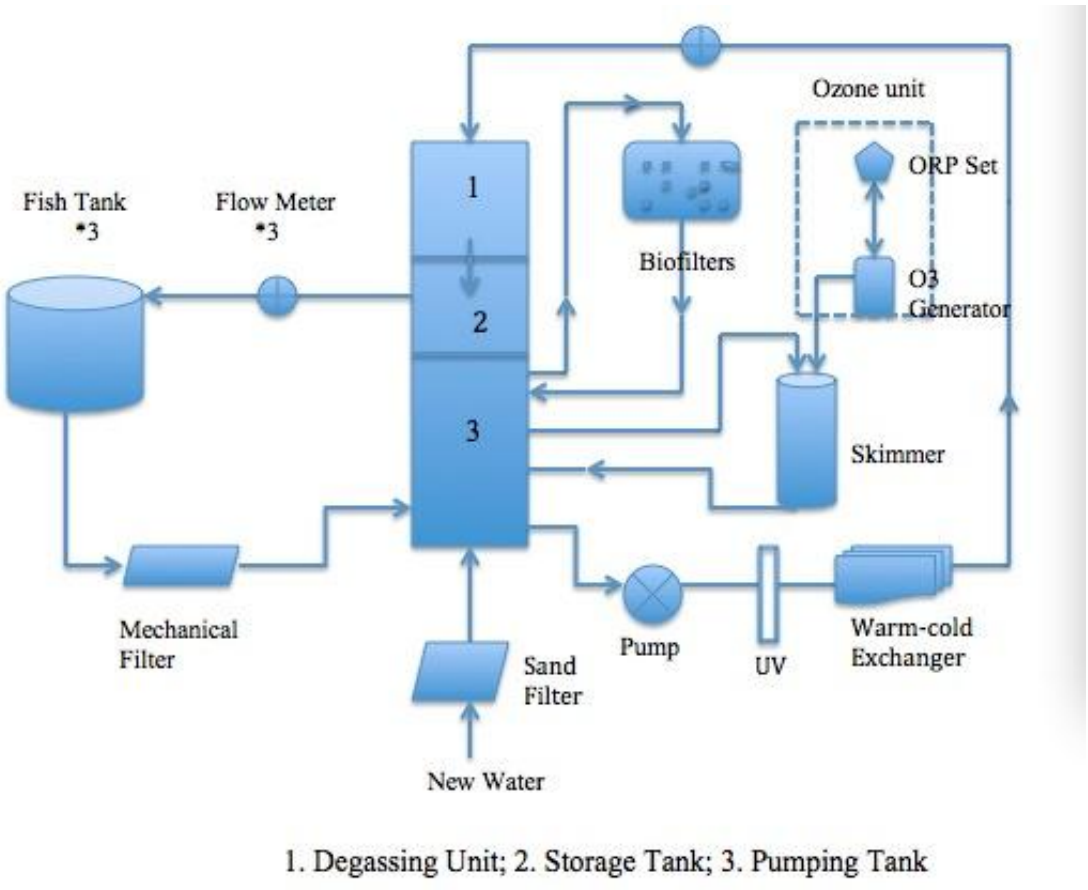

Fig 1. The structure of RAS O_3

2.2. ORP levels control

The ORP was stabilized at four successive levels: 260–300 mV, 300–320 mV, 320–350 mV and 300–320 mV in fish tanks during four periods ($P_{1\text{-}4}$) as shown on Table 1. During the P_1 period, the ORP level was increased gradually over 4 days to reach ~290 mV in the fish tanks, and this value was maintained for 8 days. During the P_2 period, the ORP in the fish tanks was between 300 and 320 mV. During the P_3 period, the ORP in the fish tanks was 320–350 mV. Three days after the ORP reached 330 mV, mortality occurred. Two days later, ORP was decreased to the same level as that of the P_2 period (320 mV), and it was maintained for 15 days during the P_4 period.

Table 1 : ORP levels and oxygen concentrations in fish tanks during four periods in both RAS O_3 and RAS

Period	RAS O_3				RAS
	P_1	P_2	P_3	P_4	P_1-P_4
Duration	12	29	13	15	69
ORP (mv)	260-300	300-320	320-350	300-320	250-270
TRO （mg L^{-1} cl）	--------	--------	0.03-0.05	--------	--------

----------: below detectable limits (0.02 mg L^{-1})

2.3. Acclimation and feeding of fish

European sea bass were reared at Ifremer. They were first stored in one big tank for several months. Two months before the trial, they were transferred to the RAS. During this time they were fed at 1.0–1.5% of the biomass per day.

2.4. Stocking density and distribution of fish

Before distribution into the tanks, fish were starved for 24 h and anaesthetized using eugenol (40 mg L^{-1}). Three fish sizes were selected as follows: S1: 100 g; S2: 150 g; and S3: 200 g. Each size class was distributed into one of the three tanks in each of the two RAS at a stocking density of 20 kg m^{-3} (Table 2). During the experiment, fish were fed Ep 3-5 dry pellets (Le Gouessant, Brittany, France) at 1.0–1.5% of biomass per day using auto feeders. The ingested feed quantity was calculated from the feed trap.

Table 2 : Distribution of three sizes classes in RAS and RAS O_3

	RAS			RAS O3		
	S1	S2	S3	S1	S2	S3
Mean weight (g)	107.24±14.28	150.36±13.74	201.6±26.70	106.74±14.04	152.67±14.28	201.6±26.62
Density (kg m^{-3})	20.05	20.15	20.2	20.07	20.00	20.2

2.5. Measured parameters of water quality

The temperature, salinity, pH, and oxygen concentration in the fish tanks were monitored and recorded daily using an ODEON device (Ponsel, France). Water samples from the fish tanks were collected at 10:00 am twice a week and stored at –20

°C after being filtration on GF/C porous membrane. TAN (total ammonia nitrogen), NO_2-N (nitrite-nitrogen) and PO_4-P (orthophosphates) were measured using an Alliance Futura instrument (Sociele AMS France, Frepillon, France) mentioned as Sammouth et al (2009). Total suspended solid (TSS) concentration in the fish tanks was determined twice a week after filtration on GF/C porous membrane; data were expressed in the unit of mg per 1 L water. Total resident ozone (TRO equal to OPO) in the fish tanks was measured three times a week at the P_1, P_2, P_4 periods while three times a day at the P_3 period using the colorimetric N,N-diethyl-p-phenylenediamine (DPD) method (Aqualytic, Langen, German). Its concentration was expressed as mg L^{-1} of chlorine.

All fish were starved for 24 h before sampling. At the last day of each period, fish were removed from the tanks and blood was taken from the caudal vein with syringes within 30 s. Immediately, one or two drops of the blood were used for hematological parameter measurements using an I-STAT portable analyzer (Abbott Laboratories, Abbott Park, IL, USA) equipped with CG8+ cartridges (Good et al. 2011). The pH, pCO_2, pO_2, HCO_3, total CO_2 (TCO_2), O_2 saturation (sO_2%), sodium (Na), potassium (K), ionized calcium (iCa), glucose (Glu), hematocrit (Hct), and hemoglobin (Hb) of the whole blood were measured. The remaining blood was placed in dry heparinized Eppendorf tubes and centrifuged for 15 min at 5000 rpm at 4 °C. The upper plasma was collected in the Eppendorf tube and stored at –80 °C until analysis. At the end of P_1 and P_2 period, four fish more from S_1 group and one fish more from S_2 group were removed to avoid density interference.

The concentration of protein in the plasma was determined by spectrophotometer using a kit (BCA Protein Assay Kit, Thermo Scientific, Hudson, New Hampshire, USA). Based on the reaction of protein with alkaline Cu^{2+}, the plasma was first diluted 2000 times in a four-step operation to obtain the best test range. Tests were performed, and run in duplicate (Smith et al. 1985).

Since the P_3 period, some fish in bad status were lying on the bottom. The fish sampled in RAS O_3 at the P_3 and P_4 periods were only swimming.

2.6. Data analysis

All results were expressed as mean ± S.D. All statistical analyses were performed using SPSS 16.0 for Windows. The data were first tested for homogeneity using Levene's *F*-test. The percent data were analyzed after arcsine transformation. The differences in water quality parameters between RAS O_3 and RAS at the P_1 and P_2 periods were evaluated using paired *t*-tests. The differences in hematological parameters and plasma protein were compared using a two-way ANOVA (fish size, ORP and fish size*ORP). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Water quality

There was no significant difference on all water parameter concentrations between the RAS and RAS O_3 groups during the P_1 and P_2 periods ($p > 0.05$) (Table 3). During these periods, the TSS concentration in the RAS O_3 fish tanks was slightly higher than that in the RAS tanks, but the difference was not significant ($p > 0.05$). The TSS and TAN concentrations of the RAS O_3 group peaked during the P_2 period and then decreased during the P_3 and P_4 periods.

Table 3. Water parameters (mg L^{-1}) in fish tanks of RAS and RAS O_3 during four periods

	RAS				RAS O3			
	P1	P2	P3	P4	P1	P2	P3	P4
TSS	6.33±7.64	6.91±7.50	6.91±7.43	6.91±1.80	9.21±6.0	17.10±9.6	9.10±8.6	6.64±1.47
TAN	0.54±0.12	0.73±0.11	0.71±0.24	0.78±0.12	0.51±0.13	0.71±0.12	0.68±0.21	0.54±0.24
NO_2-N	0.04±0.02	0.05±0.01	0.06±0.02	0.07±0.02	0.04±0.01	0.05±0.01	0.05±0.02	0.04±0.03
PO_4-P	0.70±0.12	0.95±0.11	1.01±0.13	0.89±0.24	0.66±0.34	0.89±0.21	0.89±0.22	0.67±0.33

Two-paired t-test

3.2. Mortality

There were no dead fish in RAS group during P_{1-4} periods.Although the ORP was set back to 300–320 mv during the P_4 period, mortality during P_4 was two times higher than that during P_3 (Fig. 2). The mortality of medium size fish (S_2) was slightly higher than that of the two other sizes, whereas mortality of the largest-sized fish (S_3) was the lowest, especially during the P_4 period.

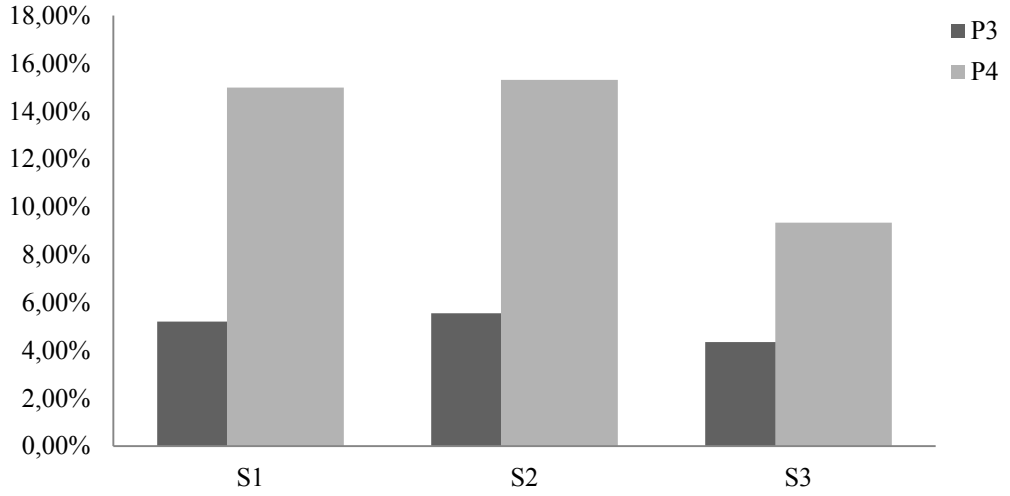

Fig. 2 : Mortalities of three sizes fish in RAS O_3 during the P_3 and P_4 periods

3.3. Hematological parameters

3.3.1. P_1 period

At the end of the P_1 period, blood O_2 concentration, measured as PO_2 and sO_2% , was first significantly influenced by the increased ORP ($p < 0.05$) (Table 4). At the same time, Hct and Hb were lower in both the S_1 and S_2 groups of RAS O_3 and significantly affected by ORP ($p < 0.05$). Besides ORP had a significant effect on iCa concentration.

No effects of fish size and ORP/fish size interaction on related parameters wererecorded ($p > 0.05$) (Table 5).

Table 4 : Hematological parameters of three size classes in RAS and RAS O_3 during the P_1 period

Parameters	***S1***		***S2***		***S3***	
	RAS	***RAS O3***	***RAS***	***RAS O3***	***RAS***	***RAS O3***
pH	7.33±0.05	7.30±0.05	7.34±0.18	7.33±0.06	7.32±0.02	7.34±0.03
pCO_2(mmol $L^{-1)}$	19.27±0.84	22.02±1.00	18.30±1.67	20.48±4.21	20.10±2.08	17.95±1.17
pO_2(mmHg)	44.75±2.28	54.75±2.86	43.50±2.38	56.00±5.48	47.75±12.42	46.00±4.08
HCO_3(mmolL^{-1})	10.43±1.20	10.10±0.19	09.98±0.71	10.40±0.43	10.43±1.06	9.75±0.21
tCO_2(mmol L^{-1})	11.00±1.22	10.75±0.43	10.5±0.58	11.00±2.00	10.75±0.56	10.25±0.50
sO_2 (%)	78.25±4.26	85.25±0.83	78.25±2.37	87.00±2.94	78.75±11.93	80.75±3.86
Na (mmol L^{-1})	160.50±2.60	157.75±3.27	159.50±1.73	155.50±2.89	159.00±3.92	160.25±2.87
K (mmol L^{-1})	3.90±0.19	4.15±0.32	3.95±0.19	3.70±0.72	4.03±0.10	3.63±0.17
iCa (mmol L^{-1})	1.34±0.07	1.49±0.03	1.37±0.08	1.45±0.03	1.41±0.09	1.48±0.08
Glu (mg dL^{-1})	70.50±5.02	68.75±1.78	67.50±7.59	63.50±7.72	74.50±13.60	71.75±10.87
Hct (%PCV)	23.75±2.59	18.85±2.17	20.75±3.50	15.50±1.50	22.00±5.48	19.00±5.48
Hb (g dL^{-1})	8.05±0.88	6.40±0.75	7.08±1.15	5.10±0.34	7.48±1.89	6.45±1.85

Table 5 : Two-way ANOVA analysis of ORP, fish size and ORP* fish size on related hematological parameters

Indice	ORP				Fish Size				ORP*Fish Size			
	P1	P2	P3	P4	P1	P2	P3	P4	P1	P2	P3	P4
pH	+	+++	+++	+++	+	++	++	+	+	+++	++	++
pCO_2(mmol L^{-1})	+	+++	+++	+++	+	+	+	+	+	+	+++	+++
pO_2(mmHg)	++	+++	+++	+++	+	++	+	+++	+	+++	+++	+++
HCO_3(mmolL^{-1})	+	+++	+++	+++	+	++	+	+++	+	+++	+	+
tCO_2(mmol L^{-1})	+	++	+++	+++	+	+	+	++	+	+++	+	+
sO_2 (%)	++	+++	+++	+++	+	++	+	++	+	+++	+++	+
Na (mmol L^{-1})	+	+	+	+	+	+	++	++	+	+	+	+
K (mmol L^{-1})	+	+++	+++	+++	+	+++	+	+	+	++	+	+
iCa (mmol L^{-1})	+++	+	+	+	+	+	+	+	+	+	+	+
Glu (mg dL^{-1})	+	+++	+++	+++	+	+++	+++	++	+	+++	+++	+++
Hct (%PCV)	++	++	++	+++	+	+	+	++	+	+	++	++
Hb (g dL^{-1})	++	+++	++	+++	+	+	+++	+++	+	+	+++	+++

+: difference is not significant ($p>0.05$); ++: difference is significant ($0.01 \leq p<0.05$); +++: difference is extremely different ($p<0.01$)

3.3.2. P_2 period

At the end of the P_2 period, fish in RAS O_3 had a higher blood oxygen, lower blood CO_2, lower Hct and Hb compared with same size in RAS. Besides, fish in RAS O_3 showed a higher blood Na and Glu (Table 6).

Two-way ANOVA analysis revealed that statistically significant effects of ORP ($p < 0.05$) on most hematological parameters, including pH, pCO_2, pO_2, HCO_3, tCO_2, sO_2, K, Glu, Hct and Hb (Table 5). Fish size had significant effects ($p < 0.05$) on pH, pO_2, HCO_3, sO_2, K and Glu. In addition, fish size and ORP interaction had significant impact on pH, pO_2, tCO_2, sO_2, K and Glu concentrations.

Table 6 : Hematological parameters of three size classes in RAS and RAS O_3 during the P_2 period

Parameters	***S1***		***S2***		***S3***	
	RAS	***RAS O3***	***RAS***	***RAS O3***	***RAS***	***RAS O3***
pH	7.33±0.03	7.35±0.04	7.32±0.04	7.42±0.06	7.38±0.01	7.34±0.04
pCO_2(mmol L^{-1})	22.36±1.05	20.31±1.36	24.65±1.39	19.51±1.21	23.03±3.97	18.98±1.28
pO_2(mmHg)	46.38±3.78	53.25±1.90	40.17±2.86	74.67±2.88	53.00±2.31	85.75±2.63
HCO_3(mmolL^{-1})	11.91±0.56	11.26±0.80	11.88±0.70	13.10±1.27	13.30±0.73	11.50±0.77
tCO_2(mmol L^{-1})	13.00±0.53	11.75±1.03	12.33±1.21	13.33±1.37	14.00±1.15	12.25±0.96
sO_2 (%)	77.63±5.63	85.63±2.83	72.33±6.15	96.00±0.89	88.25±1.26	90.00±4.90
Na (mmol L^{-1})	161.88±2.10	164.13±2.53	162.00±3.10	163.17±2.40	159.5±1.29	162.25±3.40
K (mmol L^{-1})	4.59±0.31	4.45±0.30	4.61±0.51	3.81±0.16	4.00±0.24	3.70±0.08
iCa (mmol L^{-1})	1.53±0.05	1.52±0.02	1.48±0.06	1.50±0.05	1.50±0.02	1.47±0.02
Glu (mg dL^{-1})	64.75±3.41	69.13±4.32	67.00±4.00	100.33±5.43	77.25±13.07	84.50±13.20
Hct (%PCV)	23.00±1.85	22.88±1.34	22.00±2.82	19.67±2.07	24.50±0.58	20.75±3.30
Hb (g dL^{-1})	7.76±0.68	7.16±0.46	7.82±0.60	6.65±0.67	8.28±0.15	7.05±1.12

3.3.3. P_3 period

One day after ORP reached around 320–350 mV at the P_3 period, fish in RAS O_3 began to show a appetite depression. Three days later, mortality occurred. During the P_3 period, fish in RAS O_3 exhibited a ~~–t~~ired" behavior and responded slowly to stimulation. They could be divided into two populations: fish swimming in the upper water column and fish lying on the bottom of the tank. Once fish were lying down, they died 1–2 days later. Thus, only upper swimming ones were sampled for blood parameters including hematological parameters and plasma protein.

Compared with the RAS group, fish in the RAS O_3 S group continued to show a higher O_2, lower CO_2, Hct and Hb concentrations and had a higher Glu concentration compared with fish of the same size in RAS (Table 7).

Two way ANOVA revealed that ORP significantly ($p < 0.05$) impacted most hematological parameters, including pH, pCO_2, pO_2,HCO_3, tCO_2, sO_2, K, Glu, Hct and Hb (Table 5). Fish size had significant effects ($p < 0.05$) on pCO_2, Na, Glu and Hb. Fish size and ORP interaction had significant effects ($p < 0.05$) on pH, Glu, Hb, etc.

Table 7 : Hematological parameters of three size classes in RAS and RAS O_3 during the P_3 period

Parameters	***S1***		***S2***		***S3***	
	RAS	***RAS O3 S***	***RAS***	***RAS O3 S***	***RAS***	***RAS O3 S***
pH	7.38±0.03	7.38±0.02	7.36±0.04	7.47±0.03	7.30±0.04	7.*41*±0.05
pCO_2(mmol $L^{-1)}$	19.88±1.48	18.40±0.42	21.70±0.59	15.40±1.19	22.73±1.87	15.55±1.49
pO_2(mmHg)	47.25±2.22	53.00±6.78	44.50±3.70	53.50±4.20	44.75±7.41	69.75±11.79
HCO_3(mmolL^{-1})	11.93±1.14	10.63±0.17	11.95±0.73	11.28±1.13	11.28±1.61	9.80±0.92
tCO_2(mmol L^{-1})	12.50±1.00	11.50±0.58	13.00±0.82	11.75±0.96	12.00±1.83	10.00±0.82
sO_2 (%)	84.25±2.63	83.50±2.38	75.25±3.50	93.25±3.40	78.75±3.74	93.75±3.30
Na (mmol L^{-1})	153.50±6.56	158.75±1.50	160.00±4.32	161.00±0.82	162.50±3.11	161.25±3.95
K (mmol L^{-1})	4.20±0.83	3.80±0.16	4.13±0.35	3.50±0.22	4.23±0.31	3.53±0.24
iCa (mmol L^{-1})	1.48±0.07	1.42±0.09	1.45±0.10	1.52±0.03	1.49±0.06	1.49±0.06
Glu (mg dL^{-1})	65.25±3.59	76.50±3.32	64.00±3.16	123.00±17.90	74.75±4.57	90.75±15.72
Hct (%PCV)	23.50±1.91	20.75±3.40	23.25±2.63	13.00±3.56	24.00±2.83	20.00±7.07
Hb (g dL^{-1})	8.50±0.24	7.40±0.22	7.25±0.17	4.75±1.15	8.40±0.48	7.28±1.54

3.3.4. P_4 period

At the end of the P_4 period, all groups in RAS O_3 had lower PCO_2, HCO_3, and TCO_2 and higher PO_2 and SO_2 than the groups in RAS (Table 8). The increase in Glu concentration in RAS O_3 was higher with increasing fish size, and the level was higher in the S_2 and S_3 fish compared with the fish of the same size in RAS. Hb of S_2 and S_3 fish and Hct of S_2 fish in RAS O_3 were lower than those in RAS.

Two-way ANAOVA revealed that ORP had significant impacts on all hematological parameters except Na and iCa concentrations ($p<0.05$) (Table 5). Meanwhile fish size notably influenced pO_2, HCO_3, tCO_2, sO_2%, Na, Glu, Hb and Hct. Fish size and ORP interactions significantly impacted pH, pCO_2, pO_2, Glu, Hct and Hb.

Table 8: Hematological parameters of three size classes in RAS and RAS O_3 during the P_4 period

Parameters	***S1***		***S2***		***S3***	
	RAS	***RAS O3***	***RAS***	***RAS O3***	***RAS***	***RAS O3***
pH	7.39±0.06	7.38±0.03	7.45±0.03	7.41±0.03	7.34±0.01	7.46±0.05
pCO_2(mmol $L^{-1)}$	25.78±2.06	16.25±1.67	25.95±0.65	16.05±0.47	27.38±0.39	12.38±0.42
pO_2(mmHg)	45.00±6.27	61.25±8.00	54.00±2.45	88.75±2.99	47.25±3.59	94.00±1.63
HCO_3(mmolL^{-1})	16.78±0.43	10.08±0.51	15.03±1.31	9.60±0.80	14.18±0.24	8.88±1.11
tCO_2(mmol L^{-1})	17.50±1.73	10.50±0.58	15.75±1.70	10.50±1.00	15.50±0.58	8.75±0.96
sO_2 (%)	77.25±9.43	87.50±1.00	86.75±1.70	94.00±4.24	81.00±3.16	94.25±3.20
Na (mmol L^{-1})	158.75±4.35	160.75±1.70	162.25±0.96	160.25±4.1	164.50±2.38	163.00±1.83
K (mmol L^{-1})	3.88±0.26	3.48±0.19	3.95±0.19	3.68±0.22	4.08±0.26	3.28±0.24
iCa (mmol L^{-1})	1.50±0.03	1.49±0.05	1.46±0.02	1.49±0.03	1.50±0.03	1.50±0.03
Glu (mg dL^{-1})	76.25±8.88	79.75±4.11	70.5±2.38	88.75±2.99	70.25±1.50	105.75±13.33
Hct (%PCV)	23.75±2.75	20.50±2.52	23.75±2.87	14.00±2.45	23.50±2.38	21.00±2.16
Hb (g dL^{-1})	7.93±0.51	7.30±0.33	8.30±0.58	4.78±0.83	8.78±0.19	7.05±0.56

3.4. Protein concentration in plasma

At the end of P_3 period, RAS O_3 had a lower plasma protein concentration compared with RAS(Fig. 3).

The two-way ANOVA analysis showed that ORP significantly impacted (p<0.05) plasma protein concentrations of fish in RAS O_3 since the P_2 period. At the end of P_3 period, the significant influence of ORP (p<0.05) on the protein concentration continued. At the same time, fish size also had a significant influence (p<0.05) on the plasma protein concentration. While at the P_4 period, no significant impact (p > 0.05) of fish size and ORP on the plasma protein was found.

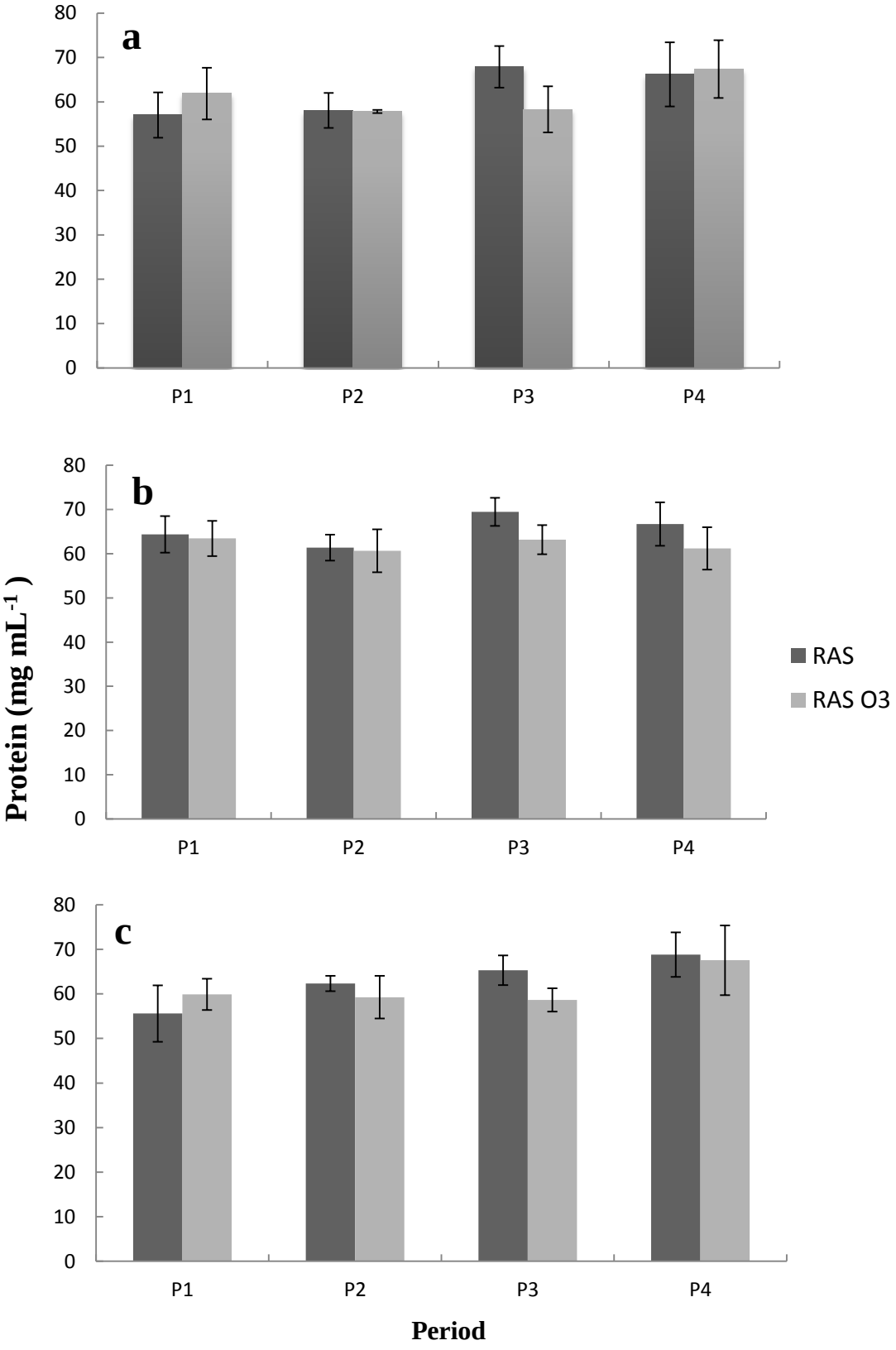

Fig. 3a-c : Plasma protein concentration (mg ml-1) of S_1: small size (a) and S_2: medium size (b) and S_3: large size (c) in RAS and RAS O_3 during four periods

Table 9: Two-way ANOVA analysis of ORP, fish size and ORP* fish size on plasma protein

Indices	P1	P2	P3	P4
ORP	+	++	+++	+
Fish Size	+	+	++	+
ORP*Fish Size	+	+	+	+

+: difference is not significant ($p>0.05$); ++: difference is significant ($0.01\leq p<0.05$); +++: difference is extremely ($p<0.01$)

4. Discussion

4.1. Water parameters

It is noticeable that ORP levels and TRO concentrations measured in this study were not consistent with those reported from previous studies, likely due to the use of different ORP probes and their locations.

In a freshwater rainbow trout RAS, the ORP measured in the fish tank drain using a platinum electrode was 155–200 mV, without ozonation (Davidson et al. 2011). It was 220 mV in a marine Atlantic halibut RAS without ozone (Tango and Gagnon 2003). In a marine sea bream RAS, ORP was ~240 mV without ozone in the foam fraction (Park et al. 2011). In a lobster hatchery without ozone, ORP was 350 mV (Jensen et al. 2011). In the current study, the ORP level measured in fish tanks of the RAS, using ODEON probes calibrated once a week on average was 250–270 mV. Thus, it is not easy to compare the ORP level among different RAS because of differences in seawater parameters, aquaculture operations, and probes (Liu et al. 2009). Moreover, it would take 2 h to obtain the ORP equilibrium value when salinity is 30 ppt (Wang et al. 2011). Overall, ORP measurement remains a challenging issue.

Ozone effectively oxidizes ammonia, and nitrite degrades yellow substances and modifies the biochemical oxygen demand and organic carbon concentration in freshwater or marine RAS (Davidson et al. 2011; Schroeder et al. 2011; Summerfelt et al. 1997; Summerfelt et al. 2009). In an ozonated marine Atlantic halibut RAS, when ORP reached 320–340 mV in the biofilters, it resulted in a 15% reduction of total organic carbon and a reduction in nitrite, color, and suspended solids compared to the control RAS with an ORP of ~220 mV (Tango and Gagnon 2003).

No significant benefit of ozonation on ammonia or nitrite reduction was detected in the current study. This was likely because the fish biomass was far from the carrying capacity of the biofilters. Water in both systems showed a suitable quality for sea bass. However, there was an increase in TSS in RAS O_3 during the P_3 period, and this was attributed to accumulation of uneaten feed in the system. TSS in RAS O_3 during the P_4

period decreased, likely because the fish exhibited decreased appetite. Parallel experiments, carried out on the microbial flora of both the rearing water and the biofilter media, revealed a moderate impact of ORP on bacterial abundance, activity and diversity (data not shown).

4.2. Mortality

Once ORP exceeded 320 mV during the P_3 period, fish appetite decreased. Three days later, first mortality occurred. After the ORP was decreased to 300–320 mV during the P_4 period, mortality continued. Most previous studies related to ozonation toxicity were carried out on fresh water species or focused on the acute response (Bullock et al. 1997; Fukunaga et al. 1992; Good et al. 2011). Rarely data are available for European sea bass. Reiser et al (2010) suggested concentrations of ≤ 0.06 mg L-1 as acceptable OPO levels for turbot juveniles. For white perch, gill cell damage was observed at an OPO concentration of 0.05 mg L^{-1} for 96 h, and LC-50 (lethal concentration leading to 50% death) for adult white perch was around 0.2 mg L^{-1} (Richardson et al. 1983). For *Paralichthys olivaceus*, 48 h LC-50 was around 0.13 mg L^{-1}(Jiang et al. 2001). Therefore, the mortality, which occurred two days after ORP reached 320-350 mV in the RAS O_3 fish tanks at the P_3 period is to be attributed to the ORP and not to TRO concentration.

In addition, the mortality continued at the P_4 period, even when the ORP level fell to the same level as it was at the P_2 period. It seems that the damage to fish health caused by too high ORP levels is not easy to be recovered.

In conclusion, even a short-term too high ORP in fish tanks should be avoided in aquaculture systems.

4.3. Hematological parameters

In this study, the hematological parameters was accordance with data reported from Sammouth et al. 2009. Hematological parameters are widely used to evaluate toxic and chemical stress and to determine the health status of fish (Saravanan et al. 2011). The alterations of iCa and K that were induced by ORP indicated an adjustment of metabolism in blood cells.

Hb is the protein in red blood cells that delivers oxygen to the organs, and Hct describes the percent of red blood cells. Hb and Hct were significantly affected by ORP levels over the P_1, P_2, P_3 and P_4 period. A decreased Hb and Hct and a blood gas balance modification (higher blood O_2 and lower CO_2) was measured in the RAS O_3 seabass compared with same size fish in the RAS. A two-way ANOVA analysis revealed that the ORP level significantly affected the gas balance in the blood. At the end of the P1 period, sO_2% was significantly influenced by ORP. During P2 period it affected significantly most blood gas parameters including pCO_2, pO_2, HCO_3, tCO_2,

and sO_2%. It seems that ORP above 300-320 mV would negatively influence the seabass hematological parameters. There is available information on the relationship between ORP levels and fish physiology. It seems that fish species react differently to ozonation. Silva et al (2011) reported the increased red blood cell and Hct concentrations in turbot juveniles exposed to ozonated seawater, should be regarded as adaptation to TRO rather than to toxicity. For rainbow trout in an ozonated RAS, Hb and Hct were slightly lower than in fish in an RAS without ozone, but the difference was not significant (Good et al. 2011). Fukunaga et al (1992, 1999) suggested that, the decrease of Hct and Hb in the red blood cells of fish exposed to ozone, corresponded to liquid peroxidation and hemolysis of the cell membrane. In this study, TRO remained lower than 0.02 mg L-1 at the P_1, P_2 and P_4 period, and the concentration of 0.03-0.05 measured at the P_3 period was below the toxic dose. Therefore the decreased Hb and Hct observed in our experiment corresponded likely to the long-term adaption of the fish to the modified ORP and not to the toxicity of TRO on red blood cell. Since the oxygen concentration of fish tanks in RAS and RAS O_3 were similar, the influence of ORP on the gas balance and the function of related blood cells in the fish need to be investigated further.

Acid–base regulation in fish is complex and involves a series of reactions for regulating HCO_3^- and H^+ concentrations in the blood. In this study, blood pH was also significantly affected by the ORP levels at the end of P_2, P_3 and P_4 periods, and corresponded to a gas balance modification in the blood.

Most of living organism's energy comes from carbohydrate transformation, which increases to meet energy demand during stress situations. It is well acknowledged that the blood glucose levels are closely correlated to the level of stress of fish (Endo et al. 2006). In this study, the glucose concentration was significantly influenced by the ORP levels since the P_2 period (ORP between 300-320 mV). Glu was higher in RAS O_3 than in the same size fish in RAS. Same findings were reported by Maricchiolo et al (2011) when seabass were stressed. As 300-320 mV ORP started to stress seabass, it seems that the blood glucose could be a good indicator for ORP stress.

4.4. Protein concentration

During the entire experimental period, the plasma protein ranged between 55 and 70 mg ml^{-1}, which is accordance with data reported from a previous study (Sammouth et al. 2009). At the end of P_2 and P_3 periods, protein concentration was significantly influenced by ORP, indicating that ORP around 300-320 mV started to stress seabass. During P_3 period when ORP rose to 320–350 mV in the fish tanks, fish appetite was depressed and mortality occurred. As a consequence, protein in plasma decreased due to the lack of nutrients and impaired health. The increase of plasma protein measured when seabass recovered from a hydrogen peroxide exposure (Roque et al. 2010) was not observed in our experiment.

5. Conclusion

The results of the present study showed that several hematological parameters were altered by increased ORP levels. Blood glucose and plasma protein concentration alterations indicated that ORP above 300-320 started to stress seabass. Short term exposure to ORP levels around 320-350 induced mortality. Therefore it is strongly recommended that that the ORP level, which is increased by the use of strong oxidants as ozone, should be maintained below 320 mV for European sea bass rearing in RAS.

Further studies are necessary to identify the best ORP level for European sea bass grow out, and to investigate how ORP alters the physiological fish parameters.

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