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Effects of sub-lethal CO_{2(aq)} concentrations on the performance of intensively reared gilthead seabream (*Sparus aurata*) in brackish water: Flow-through experiments and full-scale RAS results



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ARTICLE INFO

Article history:

Received 9 December 2012

Accepted 9 April 2013

Keywords:

Gilthead seabream
CO₂ sub-lethal effect
Growth rate
RAS
Deformations

ABSTRACT

The effects of sub-lethal CO_{2(aq)} concentrations were tested for the first time on gilthead seabream (*Sparus aurata*) juveniles (4–25 g; 64 growth days) and adult (~300–400 g; 71 d) fish, both in fully controlled pilot tests and the latter also as part of full-scale RAS (recirculating aquaculture system) operation. In the pilot experiments (concentration range 5.2–56.3 mg CO₂/L) the specific growth rate, mortality rate, and physical fish disorders were monitored. In the full scale experiment, two groups of fish, originally from the same batch, were exposed for 197 d to controlled (by NaOH dosage) and uncontrolled pH conditions, resulting in exposure of the fish to significantly different CO_{2(aq)} concentrations. The pilot results showed, as expected, that the seabream fish grew faster at the lower CO₂ concentrations and that the growth rate of both juveniles and adult fish was only minimally inhibited up to roughly 20 mg CO₂/L (compared to a previously published curve). Mortality rate was considerable only at the highest CO₂ concentration (~56 mg CO₂/L). Physical irregularities were not observed, apart from abnormally high absence of swim bladder at the highest CO_{2(aq)} treatment. The (statistically significant) results from the full-scale RAS operation showed that growing gilthead seabream for 197 d at roughly constant and relatively low (~16 mg/L) CO_{2(aq)} concentration resulted in fish with ~10% larger mean weight relative to the fish grown in ponds in which CO_{2(aq)} was not controlled and its concentration fluctuated daily between 19 and 37 mg/L.

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1. Introduction

Dissolved carbon dioxide (CO_{2(aq)}) accumulates in the water of recirculating aquaculture systems (RAS), particularly in pure O₂-supplied systems, due to the combined effect of high feeding loads and limited efficiency of CO₂ stripping devices. Chronic exposure to high CO_{2(aq)} concentrations is known to adversely affect intensively reared fish. CO_{2(aq)} is a highly soluble species, which penetrates the blood and body tissues of the fish by diffusion across the gills (Moran and Støttrup, 2011), resulting in several potential disorders such as reduced blood oxygen capacity (impaired hemoglobin binding), lowered brain pH (inducing anesthesia) and cardiovascular malfunction (Lee et al., 2003). Quite a few studies have described the detrimental physiological effects associated with high CO_{2(aq)} concentrations, as related to a variety of fish species,

which ultimately result in reduced growth rates (e.g. Danley et al., 2005; Moran and Støttrup, 2011; Fivelstad, 2013), worse feed conversion ratios (Bromley and Smart, 1981; Moran and Støttrup, 2011), and a lower general health condition (e.g. Fivelstad et al., 1998). The majority of recent publications addressing this issue focus on the conditions that develop in the open sea as a result of ocean-acidification (Fabry et al., 2008; Hwang et al., 2009).

Only a handful of studies (Danley et al., 2005; Good et al., 2010; Moran and Støttrup, 2011,) have focused thus far on the effect of high CO₂ concentrations that develop in RAS, and none have been published on the effect that CO_{2(aq)} has on the growth of gilthead seabream (*Sparus aurata*). Danley et al. (2005) and Good et al. (2010), who worked on CO₂ effects on rainbow trout (*Oncorhynchus mykiss*) arrived at almost similar conclusions. Danley et al. (2005) reported close to 100% survival of the fish (average size = 261.6 ± 24.7 g) exposed for 84 d to three CO₂ concentrations (22.1 ± 2.8, 34.5 ± 3.8 and 48.7 ± 4.4 mg/L) but significantly lower growth rates at the medium and high CO₂ concentrations tested. Good et al. (2010) reported that no significant difference was observed between fish grown for 158 d at 8 and 24 mg CO₂/L. In the latter work the fish were grown from an average size of ~50 g to a final weight of between 820 (low CO₂ concentration)

Abbreviations: FCR, food conversion ratio; GR, growth rate; NIST, National Institute of Standards; NBS, National Bureau of Standards; NPF, no pathogens found; SGR, specific growth rate; SG, splenic granuloma.

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and 850 g (high CO₂ concentration). Good et al. (2010) reported that growing rainbow trout in up to 24 mg CO₂/L did not significantly affect the overall fish performance. They thus concluded that there was no incentive to reduce CO_{2(aq)} concentrations to the conservative value of 10 mg CO₂/L. In contrast, Moran and Støttrup (2011), who investigated the effects of CO_{2(aq)} concentration on the performance of juvenile (15 to 80 g) Atlantic cod (*Gadus morhua* L.) grown at 20 ppt salinity (CO₂ concentrations of 2 ± 0.9, 8 ± 0.5 and 18 ± 0.2 mg/L) reported that although size variance and mortality rates were not different between the treatments, the growth rates of the fish reared in the medium and high CO₂ concentrations were much lower than in the low CO₂ concentration. As a result, the authors claimed that Atlantic cod was more sensitive to high CO₂ concentrations than any other marine fish examined thus far.

High head RAS supplied with pure-oxygen are particularly susceptible to the development of high CO_{2(aq)} concentrations. A typical example of such system is the RAS located in Ein Hamifratz (Israel), which is based on Dutch technology (HESY) and uses local brackish ground water for rearing gilthead seabream at high densities. The HESY system is described in Section 2. Throughout the years, under standard operational conditions (average fish density ~70 kg/m³) the CO_{2(aq)} observed concentration in the water was typically in the range 25–50 mg CO₂/L, conditions which presumably limited the growth rate and health of the fish and thereby the profitability of the system. Since the CO_{2(aq)} concentration in the makeup water fed to this particular RAS is also high (around 25 mg CO₂/L), the only practical method to reduce the CO₂ concentration is to elevate pH by dosing a strong base (NaOH) to the RAS water. In order to determine the operative “safe” CO_{2(aq)} concentration, a pilot experiment was first conducted with the aim of establishing the effects that various CO₂ concentrations have on the growth rate, mortality rate and health condition of gilthead seabream fish of different sizes.

This study was divided in two: first, the results of growing gilthead seabream at the pilot scale, in waters characterized by low, medium and high CO_{2(aq)} concentrations are reported for two fish sizes (juveniles and adults). Thereafter, the conclusions from the 1st part (considering the CO_{2(aq)} concentration in which growth is only slightly retarded) were applied to a full-scale HESY-type RAS, operated with and without NaOH dosing (for CO₂ control), with the aim of comparing full-scale growth performance at different CO_{2(aq)} concentrations.

2. Materials and methods

2.1. Pilot scale flow-through growth experiments at varying CO_{2(aq)} concentrations

Two flow-through growth experiments were conducted in parallel to the full-scale RAS test, one with juvenile fish (initial size: ~4 g) and the other with adult fish (initial size ~310 g). The aim of these experiments was to isolate CO_{2(aq)} as the dominantly different water quality parameter and to examine its effects on gilthead seabream performance from four aspects: growth rate, mortality rate, pathogen/parasite susceptibility and possible development of physical irregularities (only for the juvenile fish). The method used for attaining a stable CO_{2(aq)} concentration in the pilot tests was to maintain a constant pH value (either lower or higher than the ambient brackish-water pH) in each of the treatment tanks. Hence, the pH value varied between the treatments. However, since the alkalinity in the brackish water was high (approximately twice the value in seawater), the pH range to attain the desired CO_{2(aq)} concentration range (i.e. 6.7 < pH < 7.8) was sufficiently close to neutrality, corroborating the assumption that by itself the pH value

did not have an effect on the fish performance (Timmons and Ebeling, 2010).

2.1.1. Experimental setup

The pilot-scale experimental facility consisted of four 1.1 m³ tanks, operated in a flow-through fashion with hydraulic retention time of 2.3 h. The water was pumped from an underground brackish reservoir with a constant temperature of 21.7 °C. From the equalization tank the water was pumped separately to each fish rearing tank. Either NaOH or HCl was added into the supply pipe lines, shifting the carbonate system and generating the desired CO_{2(aq)} concentration. The system was designed to maintain the dissolved oxygen (>6 mg/L) and NH₃ (TAN < 1 mg/L) concentrations within the recommended values for aquaculture (Timmons and Ebeling, 2010). Each tank was individually pH-controlled to maintain the following pH values: 6.70, 7.00, 7.20 and 7.80 and the following CO_{2(aq)} concentrations: 56.3, 32.3, 20.5 and 5.2 mg CO₂/L respectively, in the juvenile fish growth experiment, and the pH values: 6.90, 7.17 and 7.70, resulting in CO₂ concentrations of 39.3, 22.2 and 7.2 mg CO₂/L, respectively, in the adult fish growth experiment.

2.1.2. Fish management

Juvenile fish growth experiment: 1700 gilt-head seabream (*S. aurata*) fingerlings were obtained from a commercial supplier at an average mass of 3.2 g and stocked in an equalization tank for 24 h of environmental adaptation, after which the fish were distributed into four 1.1 m³ tanks (375 fish in each tank). The remaining 200 fish were screened by mamography for physical irregularities. During the first week, the desired CO₂ concentrations were approached gradually. At the end of the first week the fish were weighed and the controlled experiment was initiated. During the experiment period, about 120 fish were weighed once a week to determine the mean weight in each tank. The fish were fed 4 times a day according to a commercial feeding chart, plus 10%, to ensure no under feeding. Six times during the experiment, two fish from each tank were tested for pathogens (skin, gills and feces). At the end of the experiment (after 64 d) 100 fish from each tank were X-rayed for final physical irregularities diagnosis.

Adult fish growth experiment: 255 adult fish (average weight ~250 g) were selected from a single rearing tank in the commercial RAS. The fish were stocked in three 1.1 m³ tanks, 85 fish in each tank. The fish were allowed to adapt for 30 d to the conditions in the ponds before the experiment started. During the growth experiment (which lasted 71 d), the fish were weighed every 10 d (about 50 fish from each tank), fed continually by an 8 h conveyor belt feeder according to a commercial feeding chart plus 10% (starting time 6 a.m.). One fish was taken from each tank, three times during the experiment for microscopic pathogen test (skin, gills and inner organs).

2.1.3. Measured values (juvenile and adult fish experiments)

Water quality parameters: DO was measured twice a day using an Oxyguard Handy meter. pH was controlled by a Eutech alpha 190 controllers and Cole Parmer alpha 100 controllers, either by the addition of a 25% NaOH solution to maintain pH levels higher than 7.30, or with 10% HCl (for pH < 7.30). pH values were verified twice a day with a Metrohm 826 manual pH meter. Alkalinity was measured, immediately after sampling, twice a week by the Gran titration method, using a Metrohm 775 titrator device. TAN was determined by the modified salicylate method (Willis et al., 1996). NO₃⁻ and NO₂⁻ were measured using an auto-analyzer (cadmium reduction). Phosphate was measured by the ascorbic acid method for soluble P (APHA, 1998). All chemical samples were filtered by Millipore 0.45 µm filter; samples were acidified to pH 2 and stored

at 4 °C before analysis. Analysis of metals was performed by ICP-OES Spectrometer (Thermo Scientific). X-ray tests, used for observing lack of swim bladder and skeleton deformities in the fish, were taken up in a finger exposition, 60 kV/4 mAs. Radiographic tests were done by a mammography unit at 22 kV/52 mAs. Fish skeleton deformities were interpreted using a medical image viewer software.

Fish performance parameters: absence of size distribution data or/and treatment replicates prevented the usage of normal distribution tests. All reported fish performance parameters are thus mean values of the whole examined population. Mortality was counted daily and presented as survival rate for the whole period. Other performance indicators were calculated using the following equations:

Specific growth rate: $SGR (1/d) = \{ \ln(W_f) - \ln(W_i) \} \times 100 (1/d)$

Food conversion rate: $FCR = F_T / (B_f - B_i)$

Growth rate: $GR (g/d) = (W_f - W_i) / N$

where $W_{f/i}$ is the final and initial mean weights, N is the number of days, F_T is the amount of feed supplied to each tank and $B_{f/i}$ is the final and initial tank biomass.

2.2. Full-scale commercial RAS comparative growth test

Commercial RAS description: The observation site comprised of two similar (but separate) water treatment sections (Sections C and D). The water treatment component in both sections included a 30- μ m drum-filter and a nitrification trickling filter with an active stripping effect, driven by fans pushing air upwards into the nitrifying bacteria attachment media. Each treatment section served ten 15 m³ rearing tanks. In Section D, an automated pH control system was installed to raise pH (by NaOH dosage) in order to control the CO₂ concentration at a predetermined value. Oxygen was supplied by dissolving pure O₂ into the recirculated stream, aiming for 100% saturation. Make up water was pumped from the same underground brackish reservoir as described before, at a flow rate of 90–240 m³/d, depending on the daily feeding load. The recirculation flow rate in each of the sections was ~280 m³/h.

pH control: A pH electrode was placed at the outlet stream of the pipe that collected the water from all the rearing tanks. An ABB AX400 pH controller operated a dosing pump, which injected NaOH 47% to a sump located under the trickling filter. The pH controller was set at 7.25, to provide ~16 mg CO₂/L in the water stream leaving the rearing tanks.

Fish management: 180,000 three g gilt-head seabream fingerlings were stocked in ten tanks in Section D (the pH controlled section). When the fish reached a weight of ~60 g, each tank was divided in two, half of the fish remained in Section D and the other half was transferred to a 'twin' tank in Section C (the section in which pH was not controlled). Every two weeks each of the 20 tanks was sampled (100–130 fish) to determine mean body weight and to decide on feeding quantity. Due to technical problems only 8 and 9 ponds, from Sections C and D respectively, were eventually monitored. Mortality count was performed daily. The facility was disease-monitored by a veterinarian on a weekly basis. The experiment lasted 197 d, during which the daily feed loads (normalized by the biomass load) in the two rearing units were similar. Periods with interferences, such as transfer events in a specific tank, were not considered in the fish performance data of a given tank, as suggested by Seginer and Ben-Asher (2011).

Data analysis: CO_{2(aq)} concentrations were calculated by PHREEQC using the Pitzer data base. Statistical analysis on growth data was performed using the statistical program JMP 10.0.

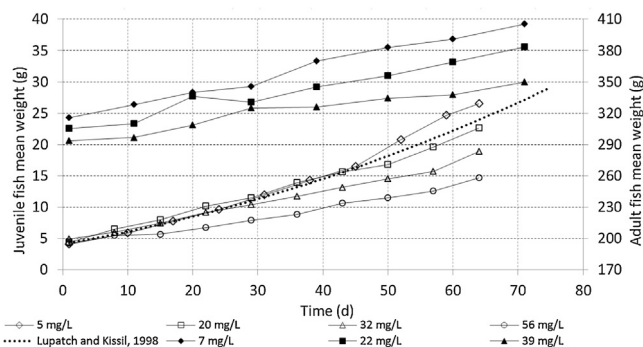


Fig. 1. Mean fish weight in the pilot-scale flow-through experiments, as a function of CO₂ concentration and time. Symbol legends refer to CO_{2(aq)} concentration treatments (empty symbols and left ordinate for juveniles ($n = 120$ fish); full symbols and right ordinate for adults ($n = 50$ fish)). Dotted line represents the growth curve developed by Lupatch and Kissil (1998), adjusted to 21.7 °C.

2.3. Calculation of CO_{2(aq)} concentrations

Several methods have been suggested over the years for quantifying the dissolved CO₂ concentration in saline water, ranging from simple calculation based on alkalinity and pH measurements with no adjustment for ionic strength, temperature, pH scale or complexation effects, through methods that are based on approximation of the carbonate system equilibrium constants in seawater and interpolation thereof (Millero et al., 2006) to direct CO₂ determination methods based on head space measurement methods (Pfeiffer et al., 2011). In this paper the CO_{2(aq)} concentration was calculated from alkalinity and pH measurements using the computer program PHREEQC by applying the Pitzer approach to account for ion interactions. For elaboration on this choice and the error associated with it the reader is referred to Appendix A.

3. Results and discussion

3.1. Flow-through pilot-scale growth experiments

3.1.1. Water quality

Temperature was measured daily and found stable at 21.7 °C during the two experimental periods. Other water quality parameters, shown in Table 1, were maintained within the range recommended for commercial fish growth (Timmons and Ebeling, 2010). Nitrite values were below detection level in all measurements. The observed standard deviations in measured pH in Table 1 coincide with predicted pH error associated with liquid junction potential effects (see Appendix A). Potential errors in the CO₂ concentration resulting from the deviations in pH measurements were in the range 10–14%, while the deviations in alkalinity contributed only ~5% to the error. For comparison with results appearing in related works, note that using empirical carbonic acid constants which are consistent with the seawater pH scale (Millero et al., 2006), but not with the NBS pH scale used in this work, result in CO₂ concentrations which are ~45% lower than the values shown in Table 1 (for elaboration see Appendix A).

3.1.2. Fish growth

The growth rate of the fish in the seven rearing tanks (4 juvenile fish tanks; 3 adult fish tanks) is shown in Fig. 1. The juveniles growth rates are compared with an empirical curve published for seabream growth in cages Lupatch and Kissil (1998). Statistical analysis of the curves was performed using JMP 10.0. A linear regression model was applied on the juvenile and adult fish growth curves. The test was aimed at assessing the probability of the hypothesis that higher CO₂ concentrations affected growth performance. This was done

Table 1

Water quality parameters (mean \pm STDV) recorded in the pilot growth experiments. Nitrite concentrations were at all times below detection level. Shown $\text{CO}_{2(\text{aq})}$ concentrations were calculated by PHREEQC. TDS, Cl^- , Ca^{2+} and Mg^{2+} concentrations in the supplied groundwater were $\sim 13,500$, 7689, 322, and 537 mg/L, respectively.

$\text{CO}_{2(\text{aq})}$ (mg/L)	pH	Alkalinity (mg/L as CaCO_3)	O_2 (mg/L)	TAN (mg N/L)	NO_3^- (mg N/L)
Juvenile fish					
5.2 \pm 0.5	7.80 \pm 0.04	293.4 \pm 4.7	8.42 \pm 1.77	0.73 \pm 0.15	0.75 \pm 0.07
20.5 \pm 2.3	7.21 \pm 0.05	261.4 \pm 5.6	8.35 \pm 1.37	0.86 \pm 0.29	1.44 \pm 0.20
32.3 \pm 2.5	6.99 \pm 0.04	246.8 \pm 4.1	8.45 \pm 1.35	0.91 \pm 0.19	1.36 \pm 0.18
56.3 \pm 7.3	6.70 \pm 0.06	215.7 \pm 5.4	8.50 \pm 0.92	0.97 \pm 0.41	1.86 \pm 0.58
Adult fish					
7.2 \pm 1.0	7.69 \pm 0.05	294.1 \pm 7.4	8.15 \pm 1.00	0.83 \pm 0.13	1.43 \pm 0.23
22.2 \pm 2.7	7.17 \pm 0.06	265.1 \pm 4.8	8.63 \pm 1.24	0.71 \pm 0.14	1.50 \pm 0.20
39.3 \pm 5.4	6.89 \pm 0.06	237.8 \pm 10.5	8.63 \pm 1.52	0.67 \pm 0.08	1.63 \pm 0.02

by including all four (juvenile) growth curves in the first test and three (adult) growth curves in the second test. A linear model was constructed based on the weight data, where the fish weight was the dependent variable and the explanatory variables were the length of the growth period and the CO_2 levels (defined as a categorical variable). Both linear models were found highly significant ($p < 0.0001$) with R^2 of 0.910 and 0.956 for the juvenile and adult fish, respectively. The explanatory variables coefficients were also found highly significant ($p < 0.0001$) in both models. The obtained linear model equations were:

$$\text{Juvenile fish weight}_{(\text{g})} = 6.42 + 0.252 \times \text{time}_{(\text{d})} - 0.154 \times L_{\text{CO}_2} \quad (1)$$

$$\text{Adult fish weight}_{(\text{g})} = 330.31 + 1.07 \times \text{time}_{(\text{d})} - 1.72 \times L_{\text{CO}_2} \quad (2)$$

where L_{CO_2} represented the CO_2 concentration, defined as a categorical variable.

Although growth curves are normally exponential (Lupatch and Kissil, 1998) or expo-linear (Seginer and Ben-Asher, 2011), since the fish weight range was relatively narrow, a linear assumption was reasonable and indeed found significant.

Water quality parameters such as NH_3 and NO_2^- concentrations, when exceeding their recommended chronic thresholds, have an adverse effect on the fish in a time-frame of days (El-Shafai et al., 2004; Lemarie et al., 2004). In contrast, Fig. 1 shows that a long exposure time (weeks) is required for gilt-head seabream growth to be impeded by CO_2 concentrations commonly observed in RAS aquaculture, assuming that 56.3 mg CO_2/L is the upper end of the practically attained range. The lag period at which the growth was unaffected by the $\text{CO}_{2(\text{aq})}$ concentration was relatively short in the fish grown at the high concentration (56.3 mg CO_2/L ; ~ 8 d lag period) and considerably longer (~ 43 d at 20.5 mg CO_2/L) at 20.5 mg CO_2/L , where the apparent lag period amounted to approximately 30 d. Note that the values in Fig. 1 do not contain standard deviation values because in order not to disturb the fish the mass in each pond was sampled once a week (~ 120 fish) and the average fish

weight was obtained by dividing the total weight by the number of sampled fish.

Table 2 presents fish-growth performance indicators for both the juvenile and adult fish experiments. The data show, as expected, that higher CO_2 concentrations slowed the fish growth rate. The food conversion ratio (FCR) values are somewhat exaggerated because of the intentionally applied over feeding, yet a consistent increase can be observed as a function of the CO_2 concentration. Both the 5.2 and 20.5 mg CO_2/L treatments (juveniles) showed satisfactory growth rates (and also good specific growth rate values), as compared to the growth prediction curve published by Lupatch and Kissil (1998). The adult fish experiment followed the same trend: the growth rate was the highest at the lowest CO_2 concentration (7.2 mg/L), decreased by 15% at the medium concentration (22.2 mg/L) and dropped significantly (by 38%) at the highest CO_2 concentration (39.3 mg/L). Survival rate was significantly adversely affected only in the 56.3 mg CO_2/L (juveniles) treatment.

It is noted that for technical reasons related to oxygen supply and ammonia accumulation the fish in the pilot experiments were stocked at a much lower density than those typically applied in RAS. This fact contributed to the isolation of the $\text{CO}_{2(\text{aq})}$ concentration as the main water quality parameter affecting fish growth and health.

3.1.3. Skeleton deformities and lack of swim bladder in the juvenile fish experiment

A common belief amongst fish growers links skeleton deformities with exposure to high CO_2 concentrations at the early fish development stages. To date, this hypothesis has not been confirmed. Table 3 lists three types of commonly encountered fish body irregularities: vertebrae fusion (Fig. 2a), lordosis (Fig. 2d) and lack of a swim bladder (Fig. 2e). Table 3 shows that for the examined CO_2 concentrations, only fish that were grown at the highest concentration (56.3 mg CO_2/L) appeared to differ from the norm with respect to lordosis and lack of swim bladder. The results indicate that if CO_2 is indeed responsible for such irregularities in gilthead seabream, it occurs (strictly speaking, in fish weighing less than 25 g) only at $\text{CO}_{2(\text{aq})}$ concentrations > 32.3 mg CO_2/L .

Table 2

Growth performance for both juvenile and adult fish in the pilot scale flow-through experiments (statistical data is based on $n = 120$ and $n = 50$ for juveniles and adults, respectively).

$\text{CO}_{2(\text{aq})}$ (mg/L)	Initial fish count	Initial and final fish weights (g)		Average growth rate (g/d)	SGR (1/d)	FCR	Survival rate (%)
Juvenile fish experiment							
5.2 \pm 0.5	370	4.1	26.5	0.36	2.96	1.15	100
20.5 \pm 2.3	362	4.4	22.7	0.29	2.60	1.52	99.4
32.3 \pm 2.5	357	4.9	18.9	0.22	2.14	1.80	97.8
56.26 \pm 7.3	363	4.2	14.7	0.16	1.99	2.25	87.9
Adult fish							
7.2 \pm 1.0	84	315.5	405.5	1.29	0.40	2.77	98.8
22.2 \pm 2.7	82	305.4	383.5	1.12	0.36	2.87	100
39.3 \pm 5.4	84	293.6	349.8	0.80	0.28	4.32	97.6

Table 3
Three deformity types, at the beginning and end of the pilot scale (64 d) fingerlings growth experiment. Values in the table represent number (%) of fish. Radiographic (start) and X-ray (end) analyses were performed by Philips DiagNet R2.3 medical viewer (software used for medical imaging analysis).

Observation	Start of experiment	End of experiment			
		5.2 mg CO ₂ /L	20.5 mg CO ₂ /L	32.3 mg CO ₂ /L	56.3 mg CO ₂ /L
Total number of fish sampled	199	100	100	100	100
Lordosis	2 (1%)	1 (1%)	2 (2%)	1 (1%)	5 (5%)
Vertebrae fusion	8 (4%)	4 (4%)	6 (6%)	3 (3%)	3 (3%)
Lack of swim bladder	0	0	0	0	3 (3%)

Gil-Martens et al. (2006), working with Atlantic salmon smolts (*Salmo salar* L.), arrived to closely similar results.

3.1.4. Presence of pathogens

Based on pathogen sampling after 50 d of growth the results in Table 2 suggest that the fish grown in the 5.2 and 20.5 mg CO₂/L treatments were in somewhat better health condition than the ones grown in 32.3 and 56.3 mg CO₂/L. Splenic granuloma presence, for example, indicates an adverse physiological effect. However, since no pathogens were found two weeks later (after 62 d of growth), the data should be considered inconclusive with respect to pathogens. The presence of parasites, also shown in Table 4, could not be ascribed to the different treatments. Tripartiella and Oodinium diagnoses were responded with treatment. In summary, no direct evidence was found that links the varying CO₂ concentrations with abnormal pathogenic/parasitic activity.

3.2. Full scale growth experiment

In contrast with the flow-through pilot-scale experiments, in which water quality conditions were maintained constant, un-controlled full-scale RAS operation is invariably characterized by a 24 h sinusoidal-like profile, corresponding with the feeding regime. Since the CO_{2(aq)} values fluctuate throughout a given day, comparing the conditions prevailing in a typical RAS to conditions prevailing in flow-through experiments or to conditions prevailing in a pH-controlled section in a commercial RAS is not

straightforward. Nevertheless, despite the obvious differences in operational conditions, a comparison was made in this work between pH controlled and uncontrolled sections of the tested RAS, based solely on fish growth performance.

In the full-scale experiment, fish originally from the same batch were subjected in one section of the plant (Section D, pH controlled tanks) to a roughly constant and fairly low CO_{2(aq)} concentration (~16 mg/L; pH 7.25), while in another section (Section C, without pH control) the CO_{2(aq)} concentration fluctuated on a daily cycle between ~19 and ~37 mg/L, as shown in Fig. 3, upper graph. For technical/economic reasons and also due to concerns related to surpassing the NH₃ comfort range, it was decided not to reduce the CO_{2(aq)} concentration in the pH-controlled tanks to the lowest level practiced in the pilot experiment (i.e. ~7.2 mg/L for the adult fish).

Water temperature during the whole observation period was 23.8 ± 1.5 and 24.1 ± 1.0 °C in Sections C and D, respectively. TAN and nitrite concentrations were monitored a couple of days after each change in feeding loads and found to be in a suitable range throughout the growth period. The same applied to oxygen concentration (O₂ > 90% saturation), which was constantly controlled and routinely monitored. The pH controller results were verified on a regular basis by a manual pH meter. Fig. 3 shows the daily fluctuations in CO₂ and pH (upper graph), and TAN and alkalinity (lower graph) for a particular (but representative) feed load (152 kg/d) applied to the uncontrolled section (Section C). At the highest TAN concentrations recorded (i.e. ~2.4 mg N/L) and pH 7.25,

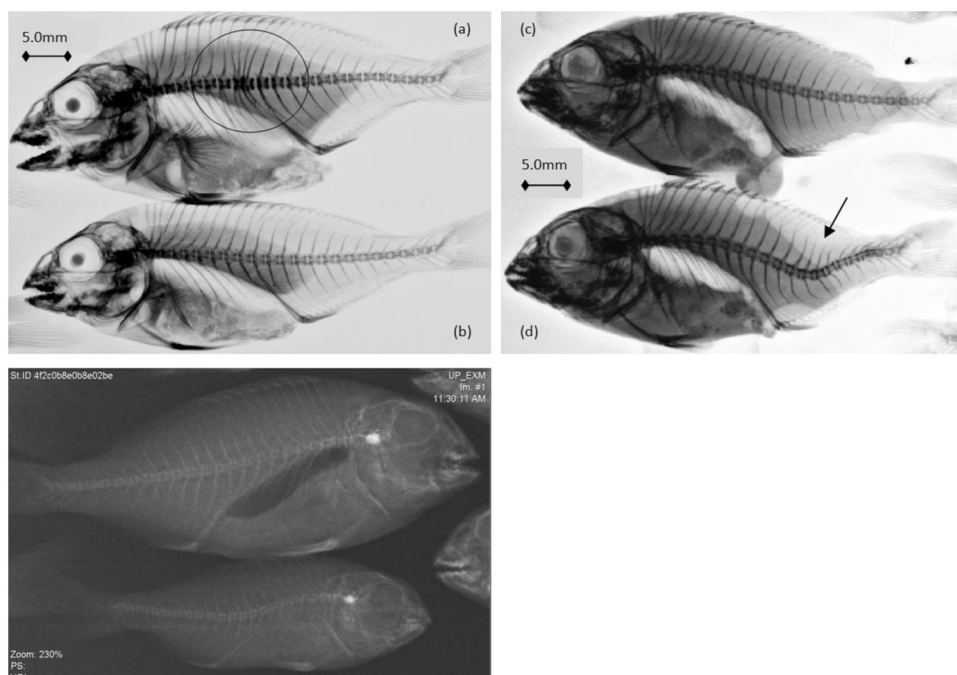


Fig. 2. Radiography photos of sea-bream fish (approximate weight: 4 g): vertebrae fusion (a; circled), normal spine cord (b,c) and lordosis (d; indicated by arrow). Fig. 2e presents x-ray analysis indicating absence of a swim bladder (bottom fish, grown in CO₂ concentration of 56.3 mg/L).

Table 4

Pathogenic findings in both juveniles (J) and adults (A) pilot-scale flow-through experiments. Findings are ranked as follows: + means low presence and ++ means high presence. NPF means “no pathogens found,” and SG stands for splenic granuloma. Positive diagnose for parasites which was responded with treatment is identified by the name of the parasite.

	CO ₂ (aq) (mg/L)	Day within experiment							
		6	27	34	41	48	50	62	
J	5.2 ± 0.5	NPF	NPF	NPF	NPF		NPF	NPF	
J	20.5 ± 2.3	NPF	NPF	+, Oodinium	NPF		NPF	NPF	
J	32.3 ± 2.5	NPF	+, Tripartiella	NPF	NPF		+, SG	NPF	
J	56.3 ± 7.3	NPF	+, Oodinium	NPF	NPF		++, SG	NPF	
A	7.2 ± 1.0			NPF		NPF			+, Tripartiella
A	22.2 ± 2.7			NPF		+, Tripartiella			NPF
A	39.3 ± 5.4			NPF		++, Tripartiella			NPF

the NH₃ concentration was roughly 0.02 mg NH₃ N/L, i.e. below the chronic threshold for seabream (Eshchar et al., 2006). The fluctuation in CO₂(aq) concentration shown in Fig. 3, along with the difference in the fish densities, demonstrates the difficulty in comparing the results derived from the commercial RAS with the pilot scale experiments.

3.2.1. Fish growth in the CO₂-controlled and uncontrolled RAS tanks

Fig. 4 compares the mean weight and mortality rate of the fish grown in the pH-controlled and uncontrolled tanks, as a function of time. Note that each curve represents the average weight of 90,000 fish grown in 8 (Section C) and 9 tanks (Section D), respectively. A statistical comparison of the mean weights as a function of time was performed (JMP 10.0). A linear model was constructed based on the weight data in the two treatments, where the fish weight was the dependent variable and the explanatory variables were the length of the growth period and the CO₂ levels (defined as a categorical variable). The model was found to be highly significant (p -value < 0.0001, meaning that the null hypothesis, i.e. that the

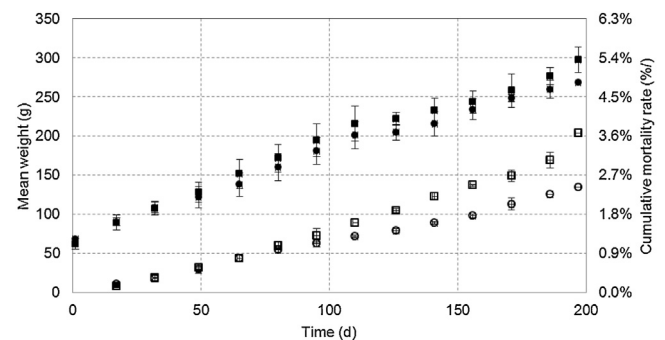


Fig. 4. Cumulative mean weight (full symbol) and mortality (empty symbol) data in Sections D (□) and C (○) during the 197 observation days. Error bars represent standard variation.

estimated parameter equals zero, was rejected) with R^2 of 0.916. Both explanatory variables coefficients were also found highly significant (p -value < 0.0001), the linear model equation (for both data sets) is shown in Eq. (3):

$$\text{Weight}_{(g)} = 76.7 + 1.07 \times \text{time}_{(d)} + \{5.82_{(\text{low CO}_2 \text{ treatment})} \text{ or } - 5.82_{(\text{high CO}_2 \text{ treatment})}\} \quad (3)$$

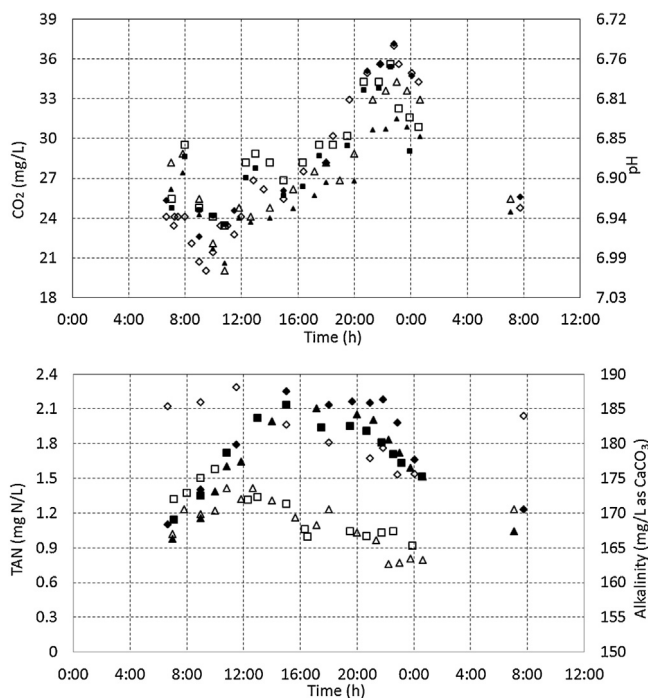


Fig. 3. Three 24-h profiles in the uncontrolled pH Section C (commercial RAS), representing fluctuations in water parameters following 152 kg/d of feed. A good correlation is observed between CO₂ (hollow symbols) and pH (filled symbols) in the upper graph. Bottom graph shows fluctuations in TAN (filled symbols) and alkalinity (hollow symbols). Similar symbol shapes represent a given sampling day.

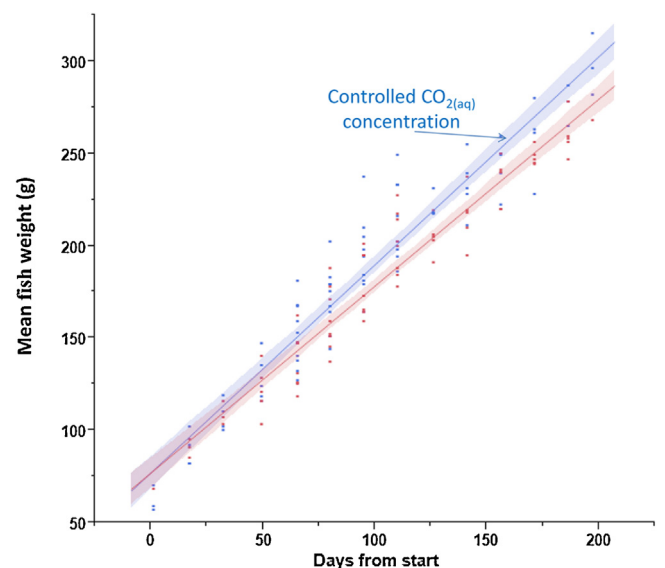


Fig. 5. Linear equations (plus 95% confidence level) predicting average fish weights vs. time of growth for the full scale RAS data.

Fig. 5 shows the prediction equations for the two data sets with 95% confidence levels. It can be seen clearly that the two prediction equations do not overlap from day 75 onward.

A significant 10% higher mean fish weight was observed at the end of the 197 experiment days in the pH controlled section. From a commercial growth perspective, the difference was substantial (a weight gap which is equivalent to ~25 d of growth). The analysis yielded a mean growth rate (\pm standard error) of 1.209 ± 0.068 g/d and 1.297 ± 0.063 g/d, in Sections C and D (pH controlled), respectively. The SGR values of the whole period were 0.70 and 0.80 1/d in Sections C and D, respectively. Contrary to expectation, a higher mortality rate was observed in the pH controlled section however, both absolute mortality values did not deviate from the norm in RAS operation.

From the economic perspective, under the conditions practiced in this experiment, the cost of increasing pH and lowering/stabilizing the CO_2 concentration was ~\$0.08/kg food supplied.

4. Summary and conclusions

- The effects of $\text{CO}_{2(\text{aq})}$ on the growth and health of *gilthead seabream* were quantified for the first time under both pilot (flow through) and full scale (RAS) conditions. Results from the controlled pilot experiments indicated that the growth rate of *gilthead seabream* juveniles was not inhibited at $\text{CO}_{2(\text{aq})}$ concentration of 5.2 ± 0.7 mg/L and only slightly retarded at 20.5 ± 2.3 mg/L, as compared to the growth curve suggested by [Lupatch and Kissil \(1998\)](#). At higher $\text{CO}_{2(\text{aq})}$ concentrations (32.3 ± 2.5 and 56.3 ± 7.3) growth was significantly inhibited (39% and 56%, respectively, relative to the growth at 5.2 mg/L) and at the highest concentration mortality rate was also considerable. The difference in growth rate between the different CO_2 treatments was found statistically significant.
- Similar findings (also statistically significant) were obtained in the adult fish pilot-scale experiments. The best growth rate (0.40 1/d) was observed at the lowest $\text{CO}_{2(\text{aq})}$ concentration (7.2 ± 1.0 mg/L). The growth rate dropped by 10% at 22.2 ± 2.7 mg/L and by further 22% at 39.3 ± 5.4 mg CO_2 /L.
- The hypothesis that high $\text{CO}_{2(\text{aq})}$ concentrations were responsible for skeleton deformations in juvenile fish was not confirmed in this study. The only observed irregularity was the absence of swim bladder in about 3% of the fish grown at $\text{CO}_{2(\text{aq})}$ concentration of 56.3 mg/L.
- No difference in presence of pathogens or parasites was found between the different treatments in the pilot experiment.
- The results from the full scale experiment showed that the fish grown in the pH-controlled, low $\text{CO}_{2(\text{aq})}$ concentration (~16 mg/L) tanks grew faster (a statistically significant difference of ~10% in mean weight after 197 d) than the ones who grew in much higher and fluctuating $\text{CO}_{2(\text{aq})}$ concentrations (19–37 mg/L).

Acknowledgments

This work was supported by a grant from the Israeli Ministry of Agriculture and Rural Development (RAS monitoring project). We are very grateful to Mr. Uzi Ben-Asher and Ein-haMifratz fishery personnel for the warm and helpful hospitality and to Mr. Hillel Amit for the technical support. We also wish to thank Prof. Eran Friedler and Mr. Yuval Alfia for the help in the statistical analysis.

Appendix A. Estimating $\text{CO}_{2(\text{aq})}$ concentrations in saline waters

The most widely used approach for determining $\text{CO}_{2(\text{aq})}$ concentration is via the indirect method (i.e. calculation via measured alkalinity and pH values) is. Accurate estimation of CO_2 by the indirect method requires very accurate measurements of pH and alkalinity and a reliable set of equations (or a model), through which the calculation of activity coefficients or apparent equilibrium constants can be obtained. Moreover, the measurements and the model have to be self-consistent (with respect to the pH scale used), in order to produce meaningful estimations. Alkalinity measurement by acid titration is relatively accurate in solutions dominated by the carbonate system, regardless of ionic strength (I) and ionic composition. In freshwater systems (i.e. $I < 0.1$ M), pH measurement is practiced according to the NIST (previously NBS) procedure, which includes the calibration of a glass electrode with standard buffer solutions. These buffers are traceable to primary pH standards determined by e.m.f. measurements of Harned cell and the Bates–Guggenheim convention (based on the Debye–Huckel approach) to determine the chloride ion activity ([Buck et al., 2002](#)). The NBS procedure, associated with a total error of 0.01 pH units, is consistent with the calculation of activity coefficients by the Davis equation, which is also based on the Debye–Huckel approach. When calculations are carried out in saline waters characterized by $I > 0.1$ M, the procedure used for freshwater gives rise to errors, which increase with salinity (S). pH measurements by glass electrodes are subject to errors resulting from the difference in the liquid junction potential (LJP) between the standard and the measured solution. An error of 0.017 pH units is associated with 1 mV of residual LJP ([Covington and Whitfield, 1988](#)). [Bagg \(1993\)](#) calculated LJP for seawater and estuarine waters and reported discrepancies ranging from $\text{pH}_{\text{NBS}} - \text{pH} = -0.03$ for low temperature (0°C) and salinity ($S = 10$ ppt), to $\text{pH}_{\text{NBS}} - \text{pH} = -0.08$ for high temperature (40°C) seawater ($S = 35$ ppt). Since the difference in LJP deviates when different glass electrodes are used, these presumed errors should be considered as rough estimates rather than a correction factor. The Davis equation is considered accurate up to $I = 0.5$ M, though inaccuracies can appear at lower ionic strength, depending on the ionic composition. Over the years, marine scientists have developed a procedure which enables the calculation of CO_2 in seawater and estuarine water with similar total accuracy as the NBS procedure has in dilute water ([Marion et al., 2011](#)). This procedure is based on the use of synthetic sea (and estuarine) water pH standards, buffered with TRIS acid (2-amino-2-hydroxymethyl-1,3-propanediol). The pH for these standards is assigned by accurate e.m.f. measurements ([Dellvals and Dickson, 1998](#)). The pH assigned to TRIS saline buffers (~ 8.1) is in the concentration scale, taking into account the complexation of H^+ with sulfate (and sometimes also with fluoride), thereby avoiding the need to calculate the Cl^- activity by convention and the need to calculate the apparent dissociation constants of HSO_4^- and HF. pH values measured according to the seawater scale were used to experimentally determine the apparent dissociation constants of carbonic acid ([Millero et al., 2006](#)) and boric acid ([Dickson, 1990](#)) as a function of temperature and salinity. Confusion between different pH definitions is rife and results in considerable error in pH and consequently in the calculated $\text{CO}_{2(\text{aq})}$ concentration. In standard seawater, for example, the pH in the seawater scale is ~ 8.09 , while the activity of hydrogen ion is ~ 8.32 ([Marion et al., 2011](#)) and the pH value measured by the NBS procedure is ~ 8.24 . Moreover, Marion et al.'s method is valid only at the range of pH 7.6–9.4 ([Marion et al., 2011](#)), due to the calibration with only one pH standard, which is not commercially available and rather complicated to prepare and maintain. For the reasons listed above, it is recommended that the use of the seawater procedure remains limited to accurate

measurements of CO₂ in the oceans, to be performed by marine scientists. Empirical constants are available with respect to the NBS pH scale, which can be used for pH calculations in seawater and solutions having a similar relative ionic composition (Piedrahita and Sealant, 1995). The accuracy of this method is limited by the accuracy of the pH NBS measurements in saline waters, as discussed above. For other applications involving saline water with varying compositions, a better approach toward the calculation of CO_{2(aq)} concentration from pH and alkalinity measurements is to use an accurate chemical equilibrium model. The most commonly used models for calculating activity coefficients in high ionic strength solutions are SIT (Bronsted–Guggenheim), which is valid up to $I=3$ M and the more accurate Pitzer model, which is valid up to $I=6$ M (Elizalde and Aparicio, 1995). Applying these models manually can be time consuming (especially in the case of the Pitzer approach which necessitates solving simultaneously dozens of equations). Fortunately, computer software packages which apply these models are freely available from the internet. These programs consider the full solution equilibrium, including complexation (e.g. ion pairing with CO₃^{2−}), reactions which have a direct effect on the CO_{2(aq)} concentration. This approach, which was used in the current work, still lacks a consistent pH measuring procedure; however this problem is more profound in seawater and in more concentrated brines (e.g. seawater desalination brines) For the brackish water used in this work ($T=21$ °C, $S=13$ g/kg) the error in pH resulting from the calibration in dilute NBS buffers is in the range of 0.05–0.06 pH units (Bagg, 1993). For example, such deviation would manifest itself in an error of ± 4 mg/L in the CO_{2(aq)} concentration (at pH 7.0 and alkalinity concentration of 200 mg/L as CaCO₃).

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