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RESEARCH ARTICLE

Effect of water salinity and dietary supplementation with *Microchloropsis gaditana* on growth, health, quality and microbiota of Barramundi *Lates calcarifer*

[version 1; peer review: 1 not approved]

Giovanni Marco Cusimano[1], Pablo Sánchez Cueto[2,3], Salvador Lladó[4], Simona Paolacci[1], Jui-Che Chiang[1], Natalie E. Panasiak[1], Radu Ghemis[3], Montse Bosch[3], Marcos Parras-Moltó[3], Dorinde Kleinegris[5], Florian Nagel[6], Simeon Deguara[1], Freya Robinson[1], Tamás Bardócz[1]

[1]Central Complex, Naggar Street, Targa Gap,, AquaBioTech Ltd, Mosta, Malta
[2]Biotechnology PhD, Faculty of Pharmacy and Food Sciences, Av. Joan XXIII 27-31,, University of Barcelona, Barcelona, Spain, 08028, Spain
[3]C/de la Innovació, 2,, LEITAT Technological Center, Terrassa, 08225, Spain
[4]Department of Genetics, Microbiology and Statistics, Av. Diagonal 643,, University of Barcelona, E-08028 Barcelona, 08028, Spain
[5]Thormøhlensgate 53,, NORCE Norwegian Research Centre AS, Bergen, NO-5006, Norway
[6]Hafentörn 3,, Aller Aqua Research GmbH, Busum, 25761, Germany

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Abstract

Background

Climate change has been associated with fluctuations of environmental parameters such as salinity, which can affect growth rates, quality, and overall health of farmed organisms. Consequently, farming euryhaline fish can be a solution to build resilience of aquaculture to climate change. Euryhaline species can undergo notable physiological and behavioural changes in response to fluctuations in salinity levels and a dietary supplementation with microalgae extract has the potential to foster the salinity tolerance thanks to its antioxidant properties.

Methods

The trial was performed on a well-known euryhaline species, *Lates calcarifer*, reared at two different salinities, namely seawater (SW) and brackish water (BW), within a recirculating aquaculture system (RAS). The effect of supplementation with the microalga *Microchloropsis*

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1. **Zelia Velez**, University of Algarve, Faro, Portugal
Centro de Ciencias do Mar, Faro, Portugal

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gaditana liquid extract 3% w/v top coated on the feed, was also investigated.

After ten weeks, the growth performance, the fillet quality, and the microbiome profile, were compared between four treatments (SW, SW+*M. gaditana*, BW, BW+*M. gaditana)*.

Results

The FCR and feed intake showed a significantly higher value in fish reared in SW. The viscerosomatic index (VSI) was also higher in the SW treatment compared to the BW. There were no significant differences in growth between fish fed with *M. gaditana* supplement and fish without algal supplementation. Moreover, there were no significant differences in body colour intensity. The fatty acid profile was also assessed for the four treatments. The algal supplementation resulted in higher MUFA and TFA, and lower SAFA of fish reared in SW compared to BW.

The analysis of the microbiome showed that changes in salinity strongly influenced the community composition in faeces and system compartments.

Conclusions

Overall, the results of this study indicate that barramundi can be reared successfully at different salinities without experiencing significant impact on growth, and should be considered to increase resilience of the aquaculture sector in a changing environment.

Plain language summary

The study conducted focused on evaluating the growth of barramundi, *Lates calcarifer* reared in two different type of water, seawater and brackish water. As an euryhaline fish species, barramundi can adapt to a wide range of salinity and this can represent an advantage in a context of climate change, where environmental parameters such as salinity are excepted to vary due to natural phenomena.

The adaptation to a different environment might be facilitated by the use of a potential antioxidant extracted from a microalgae named *Microchloropsis gaditana*. The experiment was performed during a ten weeks period in a recirculating aquaculture facility, AquaBioTech Group, based in Malta.

The experiment was structured in a growth phase, where fish were fed with the experimental diets, which included the algal extract in a specific percentage, and in a sampling phase when specific parameters such as total body weight, weight of the viscera, nutritional composition and coloration of the fillet were recorded and

evaluated prior to a mathematical analysis. The results were finally used to evaluate whether the algal extract had an effect, either positive or negative, on the growth and other parameters and if the growth in brackish and seawater was comparable among the different diets.

The outcomes of this study highlighted that barramundi can be reared successfully in different water type without affecting their growth, suggesting that this species should be considered as a valuable resource to promote the aquaculture sector, especially in a historical period when the climate is rapidly changing and only resilient species will adapt to it.

Keywords
Aquaculture; asian seabass; microalgae; adaptation

This article is included in the Adaptation to Climate Change collection.

Corresponding author: Giovanni Marco Cusimano (gmc@aquabt.com)

Author roles: Cusimano GM: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Sánchez Cueto P**: Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Lladó S**: Conceptualization, Data Curation, Methodology, Supervision, Writing – Review & Editing; **Paolacci S**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Chiang JC**: Investigation, Writing – Review & Editing; **Panasiak NE**: Investigation; **Ghemis R**: Formal Analysis, Supervision, Writing – Review & Editing; **Bosch M**: Formal Analysis, Supervision, Writing – Review & Editing; **Parras-Moltó M**: Formal Analysis, Supervision, Writing – Review & Editing; **Kleinegris D**: Methodology, Writing – Review & Editing; **Nagel F**: Methodology, Supervision, Writing – Review & Editing; **Deguara S**: Investigation, Methodology, Supervision, Writing – Review & Editing; **Robinson F**: Project Administration, Writing – Review & Editing; **Bardócz T**: Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing

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Introduction

Climate change is radically altering aquatic ecosystems modifying the distribution and productivity of marine and freshwater species (Cubillo *et al.*, 2021). Consequently, the impact on fisheries and aquaculture is cause for concern. The combination of climatic variables, including ocean acidification, rainfall variation, global warming, floods, droughts, sea-level rise, and salinity, poses a growing threat to the entire aquaculture production (Ahmed *et al.*, 2019). Farmed species' tolerance to environmental stressors will protect the aquaculture sector from the consequences of climatic fluctuations (Jaume, 2008; Zaksek *et al.*, 2009). Among the environmental parameters, salinity plays a crucial role for aquatic organisms, including fish. The changes in water salinity are caused by a number of climate change-related processes, including ice melting, river runoff, rainfall variation and increased evaporation (Barange & Perry, 2009) that have a dramatic effect on aquaculture productivity, especially in coastal aquaculture (De Silva & Soto, 2009). In this scenario, stenohaline organisms, which tolerate a small range of salinity, will be heavily affected, whereas euryhaline fish, which tolerate a broader salinity range due to osmoregulatory plasticity, can rapidly adapt to the new environmental conditions (Kultz, 2015).

Barramundi (*Lates calcarifer*) is an euryhaline species attracting an increasing interest from the aquaculture sector (Vij *et al.*, 2020). This is a carnivorous fish native to the Indo-Pacific region (Boonyaratpalin, 1997; Boonyaratpalin & Williams, 2002) that thrives in warm water, and it is known for its ability to live in environments characterized by different salinities. This species has established a strong market presence in countries such as Australia, Indonesia, Malaysia, Philippines, Taiwan, China, and Thailand (Anil *et al.*, 2010; Venkatachalam *et al.*, 2018) as it can be farmed in freshwater, marine, and brackish environments (Ganzon-Naret, 2013; Sorphea *et al.*, 2019). This adaptability also creates opportunities for integrated farming systems, where this species can be combined with other aquaculture species or utilized in multi-trophic aquaculture systems, leading to resource efficiency, diversification and production sustainability.

However, limited information is available about the effect of salinity changes on growth and other parameters of barramundi, particularly during the grow-out stage. Salinity can affect growth performance and feed conversion efficiency of Barramundi (Dunstan, 1959; Sorphea *et al.*, 2019). Moreover, it can affect important quality attributes, including flesh texture, flavour and pigmentation (Howgate, 2004; Percival *et al.*, 2008). The variation of salinity, in conjunction with factors such as hypoxia, pH fluctuations, and nutrient loads, can potentially have detrimental impacts on fish health, well-being, and growth rate (Birnie-Gauvin *et al.*, 2017; Chowdhury & Saikia, 2020; Lushchak, 2011). Salinity can also affect the gut bacteria and the microbiota community of water.

Salinity is one of the most determining factors also for aquatic microbial composition (Herlemann *et al.*, 2011). Although microbial biodiversity is increasingly being considered a prerequisite for resilience and resistance in sustainable aquaculture (Kim *et al.*, 2022; Tarnecki & Guttman, 2023), little is known about the interplay between osmoconformer fishes, such as barramundi, and their surrounding or digestive tract microbial diversity. To the best of our knowledge, only one study has been published characterizing the microbial composition of barramundi digestive tract under freshwater and seawater conditions (Zheng *et al.*, 2019). Otherwise, one recently related study went a step further in Asian Sea bass aquaculture, finding correlations between changes in intestinal microbiota, salinity and cytokine gene expression (Morshed *et al.*, 2023)

Potential negative effects of changes in salinity could be counter-balanced by supplementation of natural antioxidants in the diet like phytochemicals, which exhibit a wide array of beneficial effects such as growth-promoting, antioxidant, immune stimulating and anti-inflammatory effects (Lillehoj *et al.*, 2018; Terzi *et al.*, 2021). The pigments contained in microalgae have often antioxidant properties, and the vitamins and immunostimulants in their cells can contribute to the health of aquatic species (Prabha *et al.*, 2020; Zhou *et al.*, 2019). The use of algae-supplemented diets has been positively tested in barramundi to reduce the effect of environmental stressors, transportation and handling (Van Vo *et al.*, 2020), however, the antioxidant activity of microalgae on fish experiencing osmotic stress has not been largely documented and requires further research. Moreover, no combined studies have been carried out to test the effect of algae supplementation on fish reared at different salinities.

In line with these considerations, the present study aims to compare the growth, body quality traits and microbiome of barramundi reared in Recirculating Aquaculture System (RAS) at two different salinities. Additionally, the study aims to assess the ability of *Microchloropsis gaditana* liquid extract supplemented in fish diet to reduce the negative effects of osmotic stress.

The study contributes to the knowledge of growth and quality parameters of barramundi cultured at different salinities, providing information about the resilience of this species to osmotic changes, and investigating the potential mitigation of stress provided by algal supplementation.

Methods

Experimental set up and rearing conditions

Barramundi (*Lates calcarifer)* post-larvae, weighing approximately 0.2 g, were obtained from the hatchery Nam Sai International Co.,Ltd, Thailand and transported to the experimental facility, at AquaBioTech Group, Mosta, Malta. The fish were initially reared in freshwater until the target size was reached.

After two months, a gradual acclimation process to brackish water (BW, 14±2 ppm) was initiated. After one week, half of the fish were transferred to seawater (SW, 38±2 ppm). After two weeks, the fish were fully acclimated to their respective environments (BW and SW), marking the start of the experiment.

The study was conducted in two independent Recirculating Aquaculture Systems (RAS), one with BW and one with SW. Each system was composed of 6 tanks of 500L each, three used to the test of the effect of the algal supplementation and three for the control. The systems included a protein skimmer (Clarity™ CL 3), a drum filter (Ratz Base Drum 15), an UV sterilization (Bio UV TTPE 870 HO), ozone sterilization and biofilter. Throughout the trial, a light regime of 12 hours light and 12 hours darkness (12:12 L/D) was maintained; water temperature was 28.1±1.3°C; pH was 7.35±0.23, and dissolved oxygen was kept above 6.5 mg/l. NH_4^+, NO_2^- and NO_3^- in the water were monitored daily.

Fish of 64.9 ± 0.1g were randomly divided into tanks at an initial density of 50 animals per tank (initial stocking density of 6.4 kg/m3; final stocking density of 26.4 kg/m3).

In both systems, fish were fed four times a day (9:00, 12:00, 14:30, and 16:30) until satiation, and any uneaten pellets were removed and counted. The feed (Aller Nova ex Float, 4.5 mm) was provided by Aller Aqua A/S. A diet supplemented with *Microchloropsis gaditana* liquid extract (ALG), top coated at 3% (w/v) and a control diet (CTRL) without algae supplementation were tested in triplicates in each of the two systems (BW and SW system). The composition of the microalgae extract is presented in Table 1.

Statement of ethical considerations and welfare measures

The experiment was conducted in line with the license granted to AquaBioTech Group by the ministry for Agriculture, Fisheries, Food and animal rights, Animal welfare Department in Malta, in date 07/2021 based on Legal Notice 161 of 2017.

We confirm that all efforts were made to minimize the suffering of fish during the trial, in accordance with ethical guidelines and best practices for animal welfare.

Table 1. Declared proximate composition of the diet tested at both salinities.

	Aller Nova ex Float
Crude protein (%)	51.0
Crude Fat (%)	12.0
NFE (%)	19.70
Ash (%)	9.80
Fibre (%)	2.50
P (%)	1.40
Gross energy (MJ)	20.60
Digestible energy (MJ)	17.40

To achieve this:

- Humane Handling – Fish were handled with care to minimize stress, using appropriate nets and techniques to reduce physical injury.
- Optimal Environmental Conditions – Water quality parameters, including temperature, oxygen levels, and pH, were carefully monitored and maintained within species-specific tolerances.
- Minimization of Stress – Handling time was minimized, and fish were acclimated before the trial to prevent undue stress.
- Monitoring and Intervention – Fish were regularly observed for signs of distress, and any individuals showing undue suffering were promptly removed and humanely euthanized if required.

By implementing these measures, we ensured that fish welfare was a priority throughout the trial.

Microalgae extract

The microalgae supplement extracted from *Microchloropsis gaditana* was provided by the Norwegian Research Centre (NORCE) as broken cells (90% disruption efficiency by bead milling, powder form). The composition of the antioxidant supplement in liquid form is showed in Table 2.

Table 2. Nutritional composition of the liquid antioxidant supplement extracted from *M.gaditana* powder.

	M. gaditana liquid extract
Nutrition values	
Moisture (%)	*91.64*
Crude protein (%)	*4.23*
Crude Fat (%)	*<0.10*
NFE (%)	*2.10*
Ash (%)	*1.53*
Fibre (%)	*0.50*
P (mg/kg)	*1845*
Gross energy (Kcal/100g)	*26*
Sugars (%)	
Fructose	*<0.50*
Glucose	*1.30*
Sucrose	*<0.50*
Maltose	*<0.50*
Lactose monohydrate	*<0.50*

	M. gaditana liquid extract
Minerals and heavy metals (mg/kg)	
S	*674*
Cd	*<0.005*
Ca	*238*
Co	*0.20*
Cu	*<0.50*
P	*1845*
Fe	*8.20*
Mg	*682*
Mn	*5.50*
Ni	*<0.50*
Pb	*<0.005*
K	*2354*
Na	*3134*
Zn	*<0.50*
Amino acid (%)	
Aspartic acid	*0.33*
Glutamic acid	*0.40*
Serine	*0.14*
Histidine	*0.01*
Glycine	*0.17*
Treonine	*0.18*
Arginine	*0.18*
Alanine	*0.26*
Tyrosine	*0.11*
Valine	*0.21*
Methionine	*0.01*
Phenylalanine	*0.13*
Isoleucine	*0.13*
Leucine	*0.25*
Lysine	*0.15*
Hydroxyproline	*<0.04*
Proline	*0.55*
Tryprophan	*0.04*
Total	***3.33***

Growth data, bio-morphometric and body quality traits analysis

At the end of the 10-week trial, Fish was euthanised using an overdose of anaesthesia (800 mg/l; tricaine methanesulfonate MBW22, Sigma-Aldrich, St. Louis, MO, USA) and the total weight of the full biomass of all replicates was measured. The growth data obtained were used to calculate performance indexes (n=5/replicate) as follow:

Feed conversion ratio (FCR) = total feed supplied (g)/WG (g)

Specific growth rate (SGR, % day^{-1}) = [(lnFBW − lnIBW)/number of days] × 100

Survival rate (%) = 100 − [(number of dead fish/number of fish at start) × 100]

Feed intake (FI) = total amount of feed consumed (g)

Daily feed intake (DFI, %biomass day^{-1}): = total amount of feed consumed (g, DM)/((final number of fish + initial number of fish)/2)/days

Additionally, various health and biometric parameters including the condition factor (K), hepatosomatic index (HSI), viscerosomatic index (VSI), spleen somatic index (SSI), and fillet yield (FY), were determined as follow:

K = [fish weight (g)/(body length)3 (cm)] × 100

HSI (%) = [liver weight (g)/fish weight (g)] × 100

VSI (%) = [gut weight (g)/fish weight (g)] × 100

SSI (%) = [spleen weight (g)/fish weight (g)] × 100

FY (%) = [fillet weight (g)/fish weight (g)] × 100

Furthermore, the intensity of the fillet colour was assessed on the skinless left fillet on three different sections: close to the head, midway between head and tail and close to the tail. The measurements were recorded above the lateral line using a Minolta Chroma Meter (CR-400 Minolta, Japan). The colour intensity was expressed as the mean of nine reading per fish in terms of L* (lightness; 0 = black, 100 = white); a* represents the intensity of the red colour, and b* represents the intensity of the yellow colour.

Additionally, the fatty acid profile in terms of SAFA (saturated fatty acids), MUFA (monounsaturated fatty acids), and PUFA (polyunsaturated fatty acids) of the fillet was determined using a combined direct transesterification and solid-phase extraction method. Fatty acid methyl esters were separated and quantified by a high resolution gas chromatograph (Shimadzu GC 2010 Plus; Shimadzu, Kyoto, Japan) equipped with a flame-ionisation detector, and a CP-Sil 88 capillary column (100m×0.25 mm ID, 0.20 m film thickness; Varian Inc., Palo Alto, CA, USA). Injections were made in on-column mode and

the injection volume was 0.5 L. The temperatures of the injector and the flame-ionisation detector were maintained at 250 and 280°C, respectively. The column temperature was held at 45°C for 5 min, then raised 20°C min−1 up to 195°C and maintained for 65 min. Peaks were identified by comparing retention times to pure standards (Restek Corporation, Bellefonte, PA, USA) and by comparison with published chromatograms (Alves *et al.*, 2008). Quantification was assessed by using heptadecanoic acid (C17:0) as internal standard. All analytical determinations were performed in triplicate.

DNA extraction for microbiota characterization

Samples of fish digesta (n=71; called faeces, gut or digesta indifferently across the study), sludge (n=30), surrounding and inlet water (n= 30 each), and the biofilter (n=18; called biomedia across this paper) were collected at the beginning of the trial (T0), after 5 weeks (T1) and at the end of the experiment (T2) to evaluate the extent to which the microbiota is influenced by environmental condition and feed supplement. Faecal samples (1g of faecal matter from three fish for each treatment and timepoint) were collected through stripping and preserved using 1ml RNA later (RO901, Sigma Aldrich, St. Louis, MO, USA), in sterile 2mL Eppendorf vials. Water and sludge related samples (150 ml of each tank in triplicate) were collected and filtered using nitrocellulose filters (0.2 µm pore size). Biomedia pieces were vortexed at maximum speed for 1 minute in PBS (Phosphate buffered saline, P4474 Sigma Aldrich, St. Louis, MO, USA) 1X solution, and the resulting cells pellet was processed. All samples, were stored at -20ºC and sent to Leitat (Barcelona, Spain) for DNA extraction.

DNA extraction and Sequencing library preparation

The total DNA was extracted from samples using a commercial DNA extraction kit (D4300, ZymoBIOMICS DNA Miniprep, Zymo Research, Irvine, Canada), following manufacturer's indications. Once the DNA was extracted, it was quantified with Qubit™ 4 Fluorometer and the kits Qubit™ dsDNA Quantification Assay (Q32854, Invitrogen). DNA amplicon libraries were generated using the primer pair 341F (5'- CCTACGGGNGGCWGCAG -3') and 805R (5'- GGACTACHVGGGTWTCTAAT- 3') to amplify the V3V4 hypervariable region of the 16S rRNA gene. The sequencing was performed using Illumina MiSeq PE300 (© 2024 Illumina, Inc. All rights reserved) platform by Genome Québec Inc. (Centre d'expertise et de services Génome Québec, Montréal (Québec), Canada), following the recommandations of Illumina Inc. Sequencing.

The bioinformatic software QIIME2™ 2 version 2021.2 28 was used to process raw sequencing data (Bolyen *et al.*, 2018). In short, pair-end reads were merged using fastq-join (Aronesty, 2013). Chimeric sequences detection and deletion, followed by Amplicon Sequence Variant (ASV) assignment was performed with DADA2. This error model software package incorporates quality information, quantitative abundances, and infers identical sequences by processing each sample independently (Callahan *et al.*, 2016). Taxonomy was assigned at a 99% similarity level using the q2-feature-classifier QIIME2™ plugin, with the SILVA 132 database (version 2019.10.0) (Quast *et al.*, 2013), using the Multinomial Naive-Bayes algorithm. In short, It calculates the probability of each sequence for a given sample and then gives the taxonomy with the highest probability as output. Amplicon sequencing run resulted in a total of 1,225,346 paired-end quality checked reads which were clustered into 21,859 amplicon sequence variants (ASVs). The rarefaction analysis based on observed features (ASVs), confirmed that all samples reached a plateau between 1,000 and 2,500 counts, thereby the sequencing run sufficiently captured the bacterial diversity in each sample type.

Statistical analysis

The results are presented as mean ± SD. A two-way ANOVA was performed to analyse the effect of salinity and type of feed, and their interaction, on all the parameters observed in the study. The results were analysed using RStudio Desktop (64-bit). Statistical significance was considered when $p < 0.05$.

For the microbial community study, all statistical analyses and plot visualizations were conducted using R software (Version 4.1.2), based on the normalized ASV abundances table. Estimates of alpha diversity were calculated from the using the *diversity* function from the vegan R package, which quantifies different aspects of diversity in a community, such as species richness and evenness, based on species abundance or count data (Oksanen *et al.*, 2020). Statistical significance differences in Shannon diversity index values, which combine species richness and evenness with higher values indicate a more diverse community., were obtained using ANOVA tests with TukeyHSD adjustment from the stats R package. For beta-diversity analysis, the Bray-Curtis dissimilarity index was calculated using the *vegdist* function from the vegan R package (Version 2.6-4), based on a dissimilarity (or distance) matrices calculation from the species - ASVs abundance table. Multivariate statistical significance analysis was performed using one-, two-, and three-way PERMANOVA (Permutational Multivariate Analysis of Variance, adonis2) and pairwise PERMANOVA (pairwise.adonis) with 999 permutations (Anderson, 2001; Martinez Arbizu, 2020). These are non-parametric statistical test that evaluates differences between groups based on any distance or dissimilarity measure, often used for ecological or community composition data. To gain a deeper understanding of the taxonomic composition, phylum and genus relative abundances were represented using bar plots under the microeco R package (Version 0.20.0, Gao *et al.*, 2021). Differential abundance test to determine community differences at phylum and genus level across salinities and diets, were carried out with the random forest and non-parametric ADEx2 test, implemented boththrough microeco R package and based on An *et al.*, 2019. The first is a machine learning algorithm based on an ensemble of decision trees. useful to identify key taxa that distinguish between sample groups. Meanwhile the ADEx2 test is a compositional data analysis tool that uses Bayesian methods to infer technical and statistical error.

Working at the highest resolution possible in terms of microbial entities within this dataset, the most dominant ASVs by sample type (>20% of total abundance) that best correlated with sample disposition into the two main axes of Principal Coordinates Analysis (PCoA) were calculated using the envfit

R package. This is a correlation-based test done between the of environmental variables with the ordination axes and finds directions in the ordination plot where the environmental variables vary the most.

To investigate the relationship between salinity, microbiota composition and fish productivity, we first performed a causal mediation approach (Sohn & Li, 2019), considering the salinity as the exposure variable, the final fish weight as the response, and the faeces microbiota structure (two principal components of the PCoA) as the mediator. For this we applied the following formulas in R:

fit.total effect= lm(weight~Salinity,df)

fit.PCoA= lm(PCoA~Salinity)

fit.weight= lm(weight~Salinity+PCoA,df)

results = mediate (fit.PCoA, fit.weight, treat='Salinity', mediator=PCoA, boot=T)

Secondly, feed conversion ratio (FCR) and the relative abundance of ASVs of faeces, sludge and water, after 5 (T1) and 10 (T2) weeks by each tank, were correlated through Spearman´s rank test (Zar, 2014) using the *corr.test* function from the psych R package (Version 2.2.5). The Spearman correlation measures the strength and direction of association between two ranked variables (FCR and ASV abundances, in this case), and without assume linear relationships, which is optimal for this microbiome data. Correlations were considered significant when the p-value < 0.05, and moderate or strong when rho > 0.5 or rho > 0.7, respectively (Schober *et al*., 2018).

Results

Growth data, biometrics and body quality traits analysis

At the end of the trial, the average weight of the fish was assessed. Throughout the four treatments, the fish weight varied between 257.83±7.57 (BW, CTRL) and 272.3±9.21g (SW, CTRL) (Table 3). No significant interaction between salinity and type of feed was highlighted by the two-way ANOVA, for any of the parameters observed.

The analysis of the single main effects did not highlight differences between final weights of fish reared at different salinity. The SGR varied between 1.94±0.04 (SW, CTRL) and 2.02±0.05 (BW, CTRL) $\%\cdot d^{-1}$ and, similarly to final weight, the treatments were not significantly different. The survival rate of the fish was above 99% for all the treatments.

Salinity affected the FCR, with fish reared in SW having a significantly higher (p<0.001) FCR (0.93 ±0.02 and 0.94±0.01 in the CTRL and ALG diet respectively) than treatments reared in BW (0.89±0.01 and 0.90± 0.01).

The feed intake was also affected by salinity with fish reared in SW having a higher (p=0.001) feed intake (kg) (9.62a±0.25 and 9.53a±0.47) than fish reared in BW (8.59b±0.28 and 8.94b±0.13).

Detailed values for HSI, SSI, K, L, a and b are reported in Table 3. No statistically significant differences were found among treatments for these parameters. However, the VSI of fish reared in SW had a significantly (p=0.001) higher value (10.19±0.82 and 10.14±0.48) than fish reared in BW (8.92±0.42 and 8.98±0.47).

The analysis of fatty acid composition in the fillet was also assessed (Table 4). Fillets from fish reared in SW had a content of SAFA (%) significantly lower (p=0.05) than fish reared in BW (CTRL= 28.70a±0.36 and ALG= 28.8±0.2 versus CTRL= 29.13±0.2 and ALG=29.3±0.35). MUFA (%) content was also affected by salinity (p=0.05) with the content being higher in SW (36.3a±0.35 and 35.87a±0.67, CTRL and ALG respectively) than in BW (34.94b±0.65 and 34.7±1.48, CTRL and ALG respectively).

Trans FA (%) of fish reared in SW were also significantly higher (p<0.01) than in fish reared in BW (1.10a±0 and 1.07a±0.06 versus 0.87b±0.06 and 0.83b±0.06). PUFA (%) ranged between 34.97 and 36.03.

The salinity and the type of feed did not significantly affect the content of ω3, ω6, ω9, as well as the ω3/ω9 ratio, DHA and EPA. The values for these parameters can be found in detail in Table 4. However, DHA/EPA was significantly higher (p=0.05) in fish reared in BW (2.78±0.19 and 2.83±0.21) than in fish reared in SW (2.39±0.08 and 2.64±0.24).

Bacterial diversity across the system

The alpha diversity assessment of the bacterial communities within the system pointed out no general significant differences between sample type, salinities, or diets. However, samples of faeces from SW at T0 or T1 of fish fed the algae supplemented diet, showed high variability between individuals and were significantly less diverse than the rest. The alpha diversity assessment of the bacterial communities within the RAS system showed no significant differences between sample type, salinities, or diets. This is true for all the conditions except for the faeces from SW at T0 and T1 under algae supplemented diet, which showed high variability between individuals and were significantly less diverse than the rest. Despite the alpha diversity results, significant differences were observed between the most dominant bacterial taxa found in the fish gut compared to the rest of sample types (Figure 1A). At phylum level, Firmicutes (57 – 29%) and Proteobacteria were consistently dominant in faeces (32 – 20%) samples across timepoints and salinities, while Proteobacteria (66 – 34%) and Bacteroidetes (32 – 13%) dominated the inlet and tank waters, the tank sludge and the biomedia (Figure 1A). Moreover, the higher presence of Chlamydiae (12 – 2%) in faeces compared to other system parts (<1%), contrasted with the arguably high relative abundance of Verrucomicrobia (17-2%) and Chloroflexi (24 – 2%) in within the system, except faeces (<2%). At a higher taxonomic resolution, the microbial communities studied were majorly dominated by the genera *Clostridium sensu stricto 7* in faeces and *Muricauda*, *Ruegeria*, *Nautella* and *Nitrospira* in the rest of sample types, with variations depend on the condition.

Table 3. Trial performance of fish reared in seawater (SW) and brackish water (BW).

	CTRL (SW)	ALG (SW)	CTRL (BW)	ALG (BW)
Initial weight (g)	64.93 ± 0.12	64.93 ± 0.08	64.91 ± 7.57	64.93 ± 0.12
Final weight (g)	272.30 ± 9.21	268.51 ± 9.28	257.83 ± 7.57	263.19 ± 4.75
[1]SGR (%/day^{-1})	2.02 ± 0.05	2.00 ± 0.05	1.94 ± 0.04	1.97 ± 0.02
[2]FCR	0.93^{a} ± 0.02	0.94^{a} ± 0.01	0.89^{b} ± 0.01	0.90^{b} ± 0.01
Feed intake (kg)	9.62^{a} ± 0.25	9.53^{a} ± 0.47	8.59^{b} ± 0.28	8.94^{ab} ± 0.13
[3]DFI (%biomass day^{-1})	1.88^{a} ± 0.02	1.89^{a} ± 0.05	1.73^{b} ± 0.02	1.78^{b} ± 0.02
Survival (%)	99.33 ± 1.15	99.33 ± 1.15	99.33 ± 1.15	99.33 ± 1.15
[4]K	1.46 ± 0.08	1.43 ± 0.06	1.34 ± 0.04	1.34 ± 0.07
Fillet Yield %	43.26 ± 0.95	44.58 ± 0.18	45.37 ± 2.12	43.42 ± 2.07
[5]VSI	10.19^{a} ± 0.82	10.14^{a} ± 0.48	8.92^{b} ± 0.42	8.98^{b} ± 0.47
[6]HSI	1.91 ± 0.24	2.07 ± 0.18	1.78 ± 0.10	1.62 ± 0.11
[7]SSI	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
[8]L*	42.58 ± 0.74	42.51 ± 0.92	42.30 ± 1.59	42.87 ± 2.25
[9]a*	0.19 ± 0.41	0.08 ± 0.17	0.19 ± 0.84	0.43 ± 0.50
[10]b*	3.77 ± 0.95	4.12 ± 0.78	3.33 ± 0.83	3.90 ± 1.00

All values are given as the mean ± standard deviation (SD). Different letters highlight significant differences within rows.

[1]SGR: specific growth rate (%)

[2]FCR: feed conversion ratio

[3]DFI: daily feed intake

[4]K: condition factor

[5]VSI: viscerosomatic index

[6]HSI: hepatosomatic index

[7]SSI: spleen somatic index

[8]L*: lightness

[9]a*: red colour

[10]b*: yellow colour

Table 4. Fatty acids profile of fish reared in seawater (SW) and brackish water (BW).

Treatment	CTRL (SW)	ALG (SW)	CTRL (BW)	ALG (BW)
[1]SAFA	28.70^{a}±0.36	28.80^{a}±0.20	29.13^{b}±0.21	29.30^{b}±0.35
[2]MUFA	36.30^{a}±0.35	35.87^{a}±0.67	34.94^{b}±0.65	34.70^{b}±1.48
[3]PUFA	34.96±0.70	35.33±0.81	35.90±0.82	36.03±1.33
Trans FA	1.10^{a}±0	1.07^{a}±0.06	0.87^{b}±0.06	0.83^{b}±0.06
Omega 3	23.60±0.61	24.30±0.92	24.87±0.86	25.20±1.65
Omega 6	10.50±0.20	10.17±0.23	10.40±0	10.23±0.42
Omega 9	26.27±0.32	26.13±0.58	25.90±0.30	25.73±0.85

Treatment	CTRL (SW)	ALG (SW)	CTRL (BW)	ALG (BW)
Omega3/6	2.25±0.07	2.40±0.14	2.39±0.08	2.47±0.25
[4]DHA	12.60±0.26	13.6±0.92	14.27±0.96	14.67±1.70
[5]EPA	5.27±0.29	5.17±0.40	5.13±0.12	5.17±0.23
DHA/EPA	2.39[a]±0.08	2.64[a]±0.24	2.78[b]±0.19	2.83[b]±0.21
PUFA/SAFA	1.22±0.04	1.23±0.04	1.23±0.04	1.23±0.04

All values are given as the mean ± standard deviation (SD). N=3, data = mean ± standard deviation, different letters highlight significant differences within rows.

[1]SAFA: Saturated fatty acids

[2]MUFA: Monounsaturated fatty acids

[3]PUFA: Polyunsaturated fatty acids

[4]DHA: Docosahexaenoic acid

[5]EPA: Eicosapentaenoic acid

Impact of salinity and diet on the bacterial diversity of the RAS systems

At the community structure level, multivariate analysis of beta diversity confirmed the sample type as most strong driver of microbial community composition under our study. Thus, considering only timepoints where both diets and salinity are included (T1, T2), three-way PERMANOVA analysis of beta diversity revealed a significant impact of salinity in all the samples ($p = 0.001$). It can be noted the higher variability explained by salinity in environmental related samples, from 28% of microbial variability in sludge to 43% in the Biomedia, compared to the 8% observed in faeces related samples. Salinity effect was dependent on timepoint in all sample types ($p < 0.01$), with no significant impact by diet implementation independently of salinity or timepoint ($p > 0.05$; explaining 1%).

The sample ordination of the Bray-Curtis dissimilarity matrix through PCoA, shows the clustering of the bacterial communities of the different samples according to salinity, and the high overlap between diets under the same salinity (Figure 1A). Moreover, the impact of time on microbial structure varies depending on the sample type and salinity. Faeces and sludge are less clearly affected by time, whereas inlet, outlet, and biomedia samples under brackish water (BW) conditions show more dispersion between times along the second axis of the PCoA. To understand main taxa that explain the variation observed across the two main axis of the PCoA, dominant ASVs (total rel.abundance >20%) were correlated with the ordination disposition by by sample type. Consistent across fish and environment, except for biomedia samples, the ASV13745 belonging to the genus *Ruegeria* was key to ordinate the BW samples. Focus on the sludge, water tank, and inlet water related samples, the differentiation was mainly influenced by the abundance of ASVs belonging to *Nautella* and *Postechiella* in SW, and several unclassified ASVs at the genus level in BW, apart from the previously mentioned from *Ruegeria* genus. Particularly in the Biomedia, ASVs from *Nitrospira*, *Nitrosomonas*, and *Muricauda* fitted into the SW samples, while one unclassified ASV fit under BW at T1, and one from *Pseudorhodobacter* into BW at T2 (Figure 1B).

Dynamics of potential pathogenic bacteria genera

Following with the taxonomic analysis, several amplicon sequence variants (ASVs) belonging to potential pathogenic genera were found under both types of salinities across the system (Figure 2), except for the biofilter (Relative abundance < 0.1%). Although their abundance appears to be displaced by the previously mentioned most dominant genera it can be observed the prevalence and abundance *of Mycobacterium (0.1-11.5%, 63 out of 71)*, *Legionella (0.1 – 4.32%, 47 out of 71), Pseudomonas (0.1 – 4.12%, 68 out of 71) and Streptococcus (0.1 – 1.64%, 56 out of 71)* in faeces, and *Vibrio (0.1 - 4.3%, 83 out of 90 samples)* within the RAS system (Figure 2B). Meanwhile the other genera found such as *Aeromonas*, *Tenacibaculum* and *Francisella* remained marginal across conditions (Relative abundance < 0.5%). Similar faeces related samples, an increase of the genera *Mycobacterium* and *Legionella* under BW compared to SW was observed in environmental-related bacterial communities (tank/inlet water, sludge), but with no statistical significance between conditions. Otherwise, an increase of *Vibrio* genus was observed at the end of the experiment in faeces from SW and ALG diet compared to the rest of the conditions. Inlet, tank, or sludge-associated microbiota showed no statistical differences between potential pathogenic genera.

Links of microbiota composition across the system and fish performance

After confirming salinity impacts on microbial community structure, we assessed the potential mediating effects on individual fish weight. Using a mediation approach based on sequential linear modelswe found that significant changes in beta diversity (Axis1 and Axis2 of PCoA) did not impact fish weight. Thus, salinity-induced changes in barramundi microbiota did not lead to weight loss.

Moreover, to increase the understanding of potentially relevant bacteria in the FCR outcome by tank, relationship between the relative abundance of each microbial entity identified across this RAS system (ASVs) and fish performance were assess through Spearman correlations. Correlations were

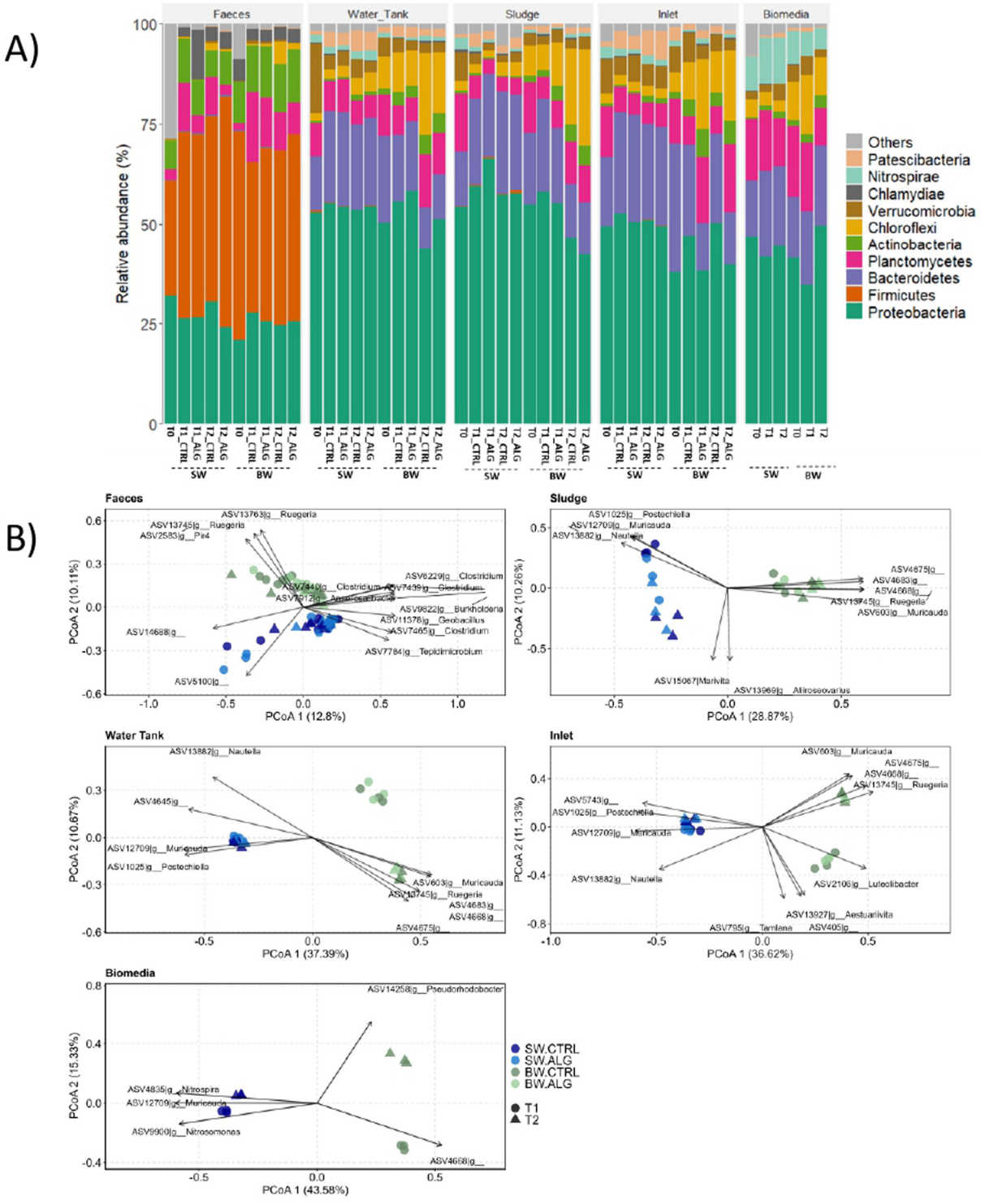

Figure 1. Global composition analysis of microbial communities under different salinities, timepoints and diets. Legend: **A**) Taxonomical composition of the microbial communities at phylum level. Average relative abundance of top 15 most abundant phyla by sample type, timepoint, salinity and diet. Bars have been colored depending on corresponding taxa. **B**). Salinity and diet effect at beta diversity level. Beta diversity Principal Coordinate Analysis (PCoA) computed upon the Bray-Curtis index, divided by sample type. From left to right: faeces, sludge, water tank, inlet and biomedia. All samples have been colored and grouped by the interaction of salinity and diet. Shape of samples is dependent on the timepoint. Significant correlated ASVs ($r > 0.5$; p.adjusted fdr < 0.1) with PCoA scores were represented by arrows.

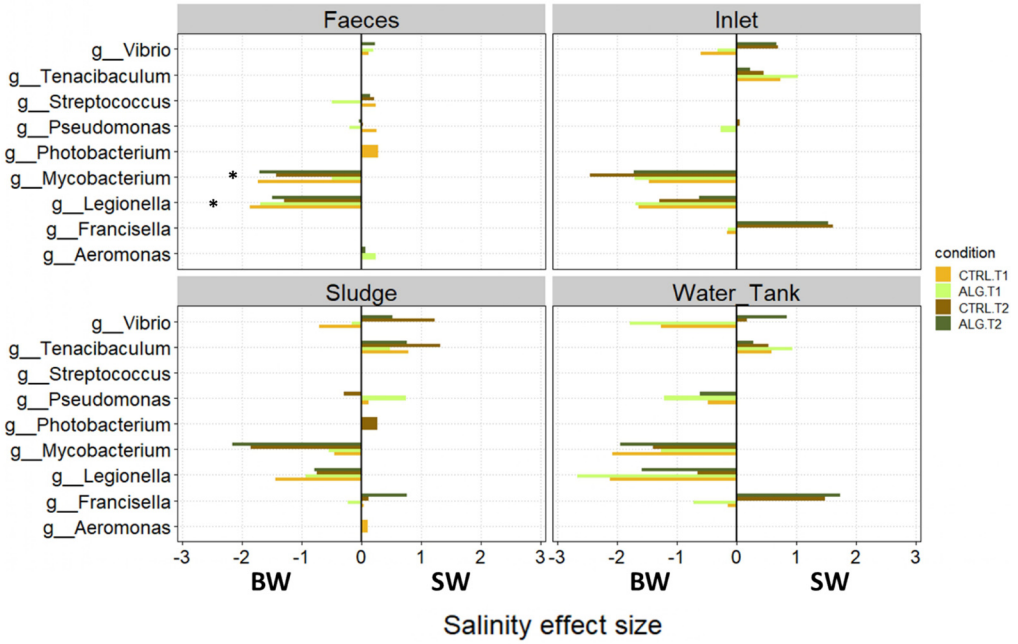

Figure 2. Dynamic of potentially pathogenic genera on the barramundi gut across conditions. Legend Barplots for each potential pathogenic genera prevalence across sample type, salinity, timepoint and diet are shown (biomedia was removed because of low prevalence of these genera - < 0.1% relative abundance). Mean effect of salinity was calculated byADEx2 test. Conditions and showed using different colours. Significance effect by salinity is showed with an asterisk (p value >< 0.05).

computed independently by salinity and led to a total of 1, 20 and 14 ASVs in faeces, water tank, and sludge, respectively, with strong significative correlations (Figure 3, r= |0.8 - 0.9|, p value < 0.05). In general terms, no significative correlations were found between barramundi associated ASVs and FCR by tank, except for one ASV belonged to *Hyphomicrobium* which was negatively correlated with FCR under SW. As for the environmental related samples (sludge and water tank) under SW, several ASVs belonged to *Vibrio*, *Leucothrix*, *Postechiella*, *Marinomonas*, *Bauldia*, *Muricauda*, Caldilineaceae (family) and Micavibrionaceae (family) had a strong positive correlation with FCR. It can be noted the opposite trend followed by several ASVs from *Roseibacillus*, *Taeseokella*, *Novispirillum*, *Portibacter*, *Amylibacter*, *Kordiimonadales* (order) and Candidatus Kaiserbacteria (order). In Commonly between sludge and sludge, two ASVs belonged to *Vibrio*, one form *Leucothrix* and another from Candidiatus Kaiserbacteria (order) showed similar significative correlations with FCR among both environmental related samples. In terms of BW condition, ASVs belonged to *Pir4 lineage*, *Fluviicola*, *Amphilicatus*, Caldilineaceae (family), Microtrichaceae (family), Rhodobacteraceae (family) and Saprospiraceae (family) were found positively correlated with FCR. On the other hand, one ASV from *Rhodobacter* and one unclassified at genus level from *Bradymonadales* order showed negative strong correlations.

Discussion

Euryhaline species such as barramundi, have the physiological ability to move between waters of various salinities and is often exposed to rapid changes in ionic gradients that may alter acid–base homeostasis (Weakley *et al.*, 2012). Water salinity may influence the stimulation of osmoregulatory hormones linked to growth, leading to different growth rates at different salinities (Canosa & Bertucci, 2023).

However, the present study demonstrated that the reduced salinity of fish reared in brackish water did not affect the final mean weight and the SGR in barramundi. Sen *et al.*, 2019 found that Asian seabass fingerlings reared at 10 ppm displayed higher body weight at all weighing intervals compared to fish reared at 20 and 30 ppm.

The results of the present study do not align to those observed in other euryhaline species, such as European seabass (Eroldogan & Kumlu, 2002; Eroldogan *et al.*, 2004; Goda *et al.*, 2019), gilthead seabream (Laiz-Carrion *et al.*, 2005), golden-line sea bream (Woo & Kelly, 1995), black sea bream (Kelly *et al.*, 1999), red seabream (Vargas-Chacoff *et al.*, 2011), and turbot (Imsland *et al.*, 2001). In those studies, the use of salinity ranging from 10 to 20ppt enhanced the growth through the reduction of the metabolic demand for osmoregulation and potentially directing more energy toward growth.

In the present study, a reduced feed intake (FI) at lower salinity (BW), resulting in a lower FCR, was also observed. The higher FCR recorded in SW compare to BW aligns with the findings reported for barramundi juveniles by Mozanzadeh *et al.*, 2021 and in black bream (*Acanthopagrus butcheri*) by

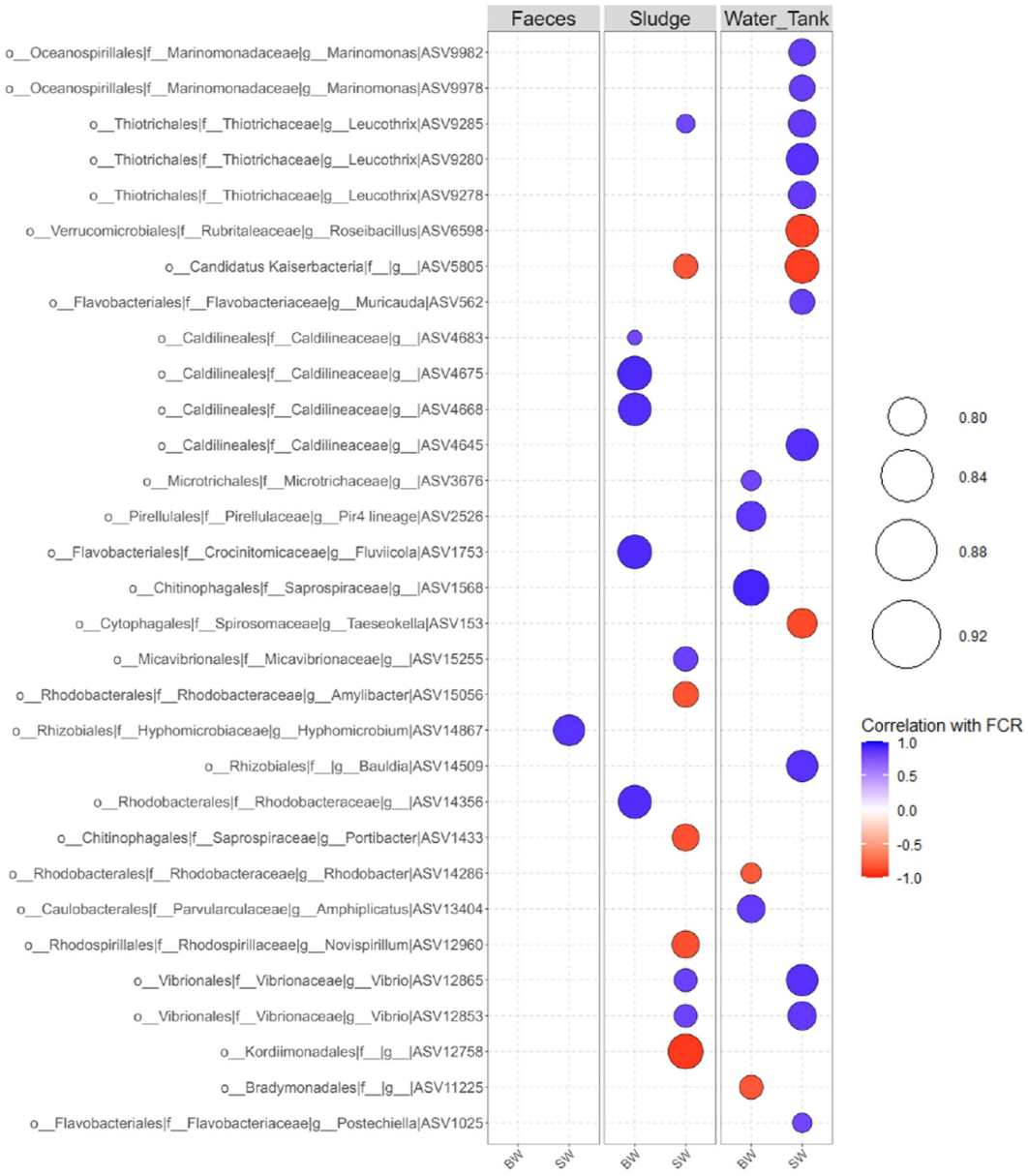

Figure 3. Main strong correlations between ASVs abundance and FCR, divided by sample type. Legend: Circle heatmap plotted from Spearman ranks correlation tests. From left to right: faeces, sludge and water tank. Each circle represents the significative strong correlations ($p < 0.05$, $rho > 0.7$), with the size proportional to the strength of the correlation coefficient (rho). Warm colors representing negative correlations and cool colors representing positive correlations.

Partridge and Jenkins, 2002. This can be attributed to changes in the evacuation rate, low retention of nutrients, and high excretion of metabolites in hyperosmotic environments (Barman *et al.*, 2005; Lambert *et al.*, 1994).

The results observed in the present study do not align with the outcomes presented for juvenile cobia, Atlantic cod and black bream by Chen *et al.*, 2009; Lambert *et al.*, 1994, and Partridge and Jenkins, 2002 respectively. However, these findings are in agreement with the results of Klaoudatos and Conides, 1996 and Lambert *et al.*, 1994, where gilthead sea bream showed a higher food intake and better feed conversion at 28 ppm and Atlantic cod exhibited improved conversion efficiency at 14 ppm but showed no differences in food intake across salinities.

Regarding the somatic indexes, they were not significantly affected by reduced salinity, with the exception of the VSI,

which was significantly higher in fish reared in SW. This contrasts with the findings of Mozanzadeh *et al.*, 2021, where juveniles of barramundi reared at different salinities did not show any difference in the somatic indexes among the treatments.

The overall survival rate observed in the present study, was not affected by salinity, exceeding 99% in all treatments. This aligns with the data reported by Boonyaratpalin, 1997, where Asian seabass adapted well to seawater and lower salinity once they reached a size of 13.9 – 17.7 mm.

As part of this study, the effect of a microalgae extract on fish growth and health was investigated at both salinities. The inclusion of microalgae in aquafeed in previous studies showed a beneficial effect due to its capacity to enhance growth, improve physiological activity, stress response, disease resistance, and flesh quality (Abdel-Tawwab *et al.*, 2008; Ansari *et al.*, 2021; Guroy *et al.*, 2007). However, in the present study, the inclusion of a *M. gaditana* liquid extract top-coated at 3% w/v did not enhance the growth performance, in line with the findings of Ayala *et al.*, 2020, where a low inclusion level of *M. Gaditana* at 2.5% and 5% in the diets did not affect the growth, and daily feed intake of juvenile gilthead seabream. Conversely, Sørensen *et al.*, 2016 and He *et al.*, 2018 found that the inclusion of *Phaeodactylum tricornutum* at 3% and *I. Galbana* at 4.8% had a positive effect on the growth performance of Atlantic salmon and *T. Ovatus*, respectively. In these two studies mentioned, the supplementation of microalgae was done by inclusion of either powder or paste into the feed extrusion process, while in the present study a liquid solution was sprayed on the feed already extruded. The lack of effect of the microalgal extract might be due to a lack of absorption of the solution into the feed, leading to the leaking of the extract into the water and to a reduced quantity adsorbed by the fish.

Fatty acids profile

The fatty acid profile of muscle was found to be similar in all diets, confirming previous findings in certain species where the tissue fatty acid profile largely reflects the dietary fatty acid profiles (Bell *et al.*, 2001; Francis *et al.*, 2007; Jobling *et al.*, 2008; Xue *et al.*, 2006).

However, the impact of salinity on fatty acid composition has been investigated by Tocher *et al.*, 1994, 1995, revealing effects on saturated fatty acids (SAFA), docosahexaenoic acid (DHA), DHA/EPA ratio, and trans fatty acids. Other studies have also demonstrated that salinity influences the tissue fatty acid profile (Bautista *et al.*, 1991; Haliloglu *et al.*, 2004). In certain migratory fishes (*Plecoglossus altivelis* and *Oncorhynchus masou*), differences in the ω6/ω3 fatty acid ratio in response to salinity changes have been observed (Ota & Tagaki, 1977; Ota, 1976). Our study revealed a significantly higher DHA/EPA ratio in BW compared to SW, which contrasts with the findings of Kheriji *et al.*, 2003 in mullet. Additionally, EPA did not show a significant difference, yielding different results compared to Xu *et al.*, 2010; Steffens, 1997, and Hunt *et al.*, 2011, where EPA and DHA contents were higher in SW than at lower salinities in Japanese seabass (*Lateolabrax japonicus*) and European seabass (*Dicentrarchus labrax*).

However, the TFA was higher in the fish reared in SW. The authors of the present study did not find similar studies on barramundi to compare the results observed. Comparison was only possible with similar studies with different species. Fallah *et al.*, 2013, for example, observed that the content of total saturated fatty acids (SFAs) in *C. damascina* was not significantly different with increasing salinity, while EPA and DHA were significantly increased by salinity.

The final coloration of the fillets was also not affected by salinity, as the lack of significant difference in the parameters L^*, a^* and b^* suggests. A similar coloration of the fillets is a positive observation as differences in colour could be associated to reduced consumers' acceptance. The present study did not assess the organoleptic characteristics of fish reared at different salinities. These should be assessed in future studies to confirm the quality of this species produced in BW.

Bacterial diversity in barramundi RAS systems and salinity effects

As a consequence of the raising awareness of the microbial diversity role in a more resilient and sustainable aquaculture industry (Tarnecki & Guttman, 2023), salinity impacts on microbial biodiversity have been extensively discussed across a wide variety of captive species and systems. These go from salmonids in laboratory tanks (Morales-Rivera *et al.*, 2022) or flow through commercial hatcheries (Dehler *et al.*, 2017) to juvenile shrimps in commercial farms (Chaiyapechara *et al.*, 2022). Under our study, we contribute to this field offering a thorough characterisation of bacterial communities dynamics in barramundi RAS systems, evaluating the diet effect under different salinities. Overall, our aquaculture system presented similar high bacterial alpha diversity across all types of samples but different bacterial composition. In line with previous studies in the barramundi gut microbiota (Morshed *et al.*, 2023) or other fish species (Sánchez-Cueto *et al.*, 2023; Schmidt *et al.*, 2015), our results reinforce the view of the fish gut as a selective habitat. There a series of conditions allow more adapted bacteria to proliferate and dominate this space, Clostridiales in our case, despite others being the most dominant in the surrounding environment, such as Rhodobacterales, Flavobacteriales or Nitrospirales in our case. This identification of care members and its dynamics, in turn, should enhance our ability to isolate microbes that can thrive under varying environmental pressures, such as temperature and salinity, while also competing for niche space within the host's mucosal areas. Linked to our study, Zheng *et al.*, 2019 reported no changes in microbial alpha diversity of barramundi gut due to changes in water salinity. This is in line with our results, where we found similar alpha diversity values for samples from different water salinities. Albeit some studies described higher values of alpha diversity by being associated with a healthy status of fish species (Miyake *et al.*, 2020; Mougin & Joyce, 2023; Wang *et al.*, 2018; Xavier *et al.*, 2024), other contradict these statements (Bozzi *et al.*, 2021;

Mondal *et al.*, 2022; Vasemagi *et al.*, 2017). In our study, we do not link this index with any health parameter. However, the observation of stable alpha diversity, among both salinities and sample types, suggests the maintenance of microbial equilibrium when shifting from seawater to brackish water.

Although this alpha diversity stability between salinities can contradict the previous observations in different RAS compartments (Bakke *et al.*, 2017) or in the gut of catfish juveniles (Hieu *et al.*, 2022), our results reinforce the framework of salinity as a strong environmental driver of microbial community composition. Impacts at beta diversity levels and taxa distribution are even clearer for the environmental related samples (tank/inlet water, sludge and biomedia) compared to the barramundi digestive tract. Which can be attributed to the more protected niche that encompass euryhaline fish digestive tract, where physiological changes keep the plasm and intestinal fluids salinity keep relatively constant ~7 ppm no matter external salinities (Scott *et al.*, 2008).

Unravelling the effect of microalgal supplementation on gut microbiota, no significant differences were observed in terms of bacterial community composition under both salinities. Although other feeding test experiments in barramundi enhanced the abundance of several gut genera such as *Clostridium*, *Lactobacillus*, and *Ruminococcus* (Chaklader *et al.*, 2021), the absence of significant changes by new ingredients in the diet may indicate a harmless effect of *M. gaditana* on barramundi gut microbiota, providing room for testing higher concentrations.

Bacterial dynamics on the water and sludge

The strong shifts in sludge, water tank and inlet bacterial communities structure were mainly mediated by an increase of *Ruegeria* and unclassified ASVs from *Caldilineaceae* family under BW tanks, being concomitantly with the decrease of *Nautella* and *Muricauda* genus compared to SW ones. These genera belong to Rhodobacteriaceae and Flavobacteriaceae families, which are two of the most prevalent prokaryotic families in the water column of RAS system, (Moschos *et al.*, 2022). Both *Nautella* and *Ruegeria*, are closely related genera from the Roseobacter clade, a widespread group of marine bacteria encompassing several strains proposed as potential probiotics in aquaculture (Prado *et al.*, 2009; Sonnenschein *et al.*, 2021). In addition, members from *Muricauda* genus were found in other aquaculture RAS systems under different salinities (Hüpeden *et al.*, 2020). Species from this genus have been linked with the fish innate immune responses activation and protection against potential pathogens such as *Vibrio* (Tang *et al.*, 2015) or to the nitrification and denitrification process in the environment or aquaculture systems (Hu *et al.*, 2012; Marchant *et al.*, 2018). On the other hand, the Caldilineaceae family (Chloroflexi phylum) literature in aquaculture system is scarce, with a recent association of this family with antibiotic resistance genes transfer (ARGs) (Deng *et al.*, 2023) or to be significantly increased in the intestines of tilapia under a beta-glucan supplemented diet with lower salinity (Xu *et al.*, 2020). Overall, our results suggest that these bacteria groups may have a competitive advantage in the water or sludge against the other microbes as the salinity fluctuates. In addition, previous findings in literature suggest these genera as beneficial for both salinities systems functioning, without putting at risk the barramundi production.

Bacterial dynamics on the gut: special attention on potential pathogens

The same dynamics of *Ruegeria* genus between salinities in the faces (as in water/sludge), highlights the interrelationship between host and surrounding environment microbiota. Moreover, the potential pathogenic genera *Mycobacterium* and *Legionella*, suggest a favorable niche developed under BW conditions, consistent with patterns observed in the sludge, inlet and tank water samples. Previous studies in vitro have been shown that higher salinity concentrations reduced their ecological niche in host-adapted strains (Asmar *et al.*, 2016) or their pathogenicity (Catrenich & Johnson, 1989), but no clear associations have been made regarding aquaculture systems. These genera which are associated with pathogenic strains for both fish and humans (Hashish *et al.*, 2018; Wang *et al.*, 2021), were not documented to cause outbreaks in barramundi. This may indicate that the presence of *Mycobacterium* and *Legionella* in the system not necessarily pose a hazard to fish health.

Besides these two genera, we show the dynamics of another 8 potential pathogenic genera previously found in aquaculture systems (Haenen, 2017). Among all, the most damaging documented for barramundi aquaculture were: *Vibrio spp.* (such as *Vibrio harveyi* and *Vibrio anguillarum*) because of its capacity to cause vibriosis and account for about 7% of barramundi production costs (Mohd Yazid *et al.*, 2021), Streptococcus (*Streptococcus iniae*) characterized by septicemia, meningitis (Agnew & Barnes, 2007), *Photobacterium* as responsible of pasteurellosis and high mortality rate in barramundi (Korun & Timur, 2005) and *Aeromonas* which can cause motile aeromonad septicemia (Al Khaziri *et al.*, 2019). The results of the present study indicate that *Streptococcus* and *Pseudomonas* are present in the microbiota of faeces under all conditions studied (0.1–1.8%). Meanwhile *Vibrio* is the genus with higher prevalence across the other sections of the RAS sampled (appeared 83 out of 90 samples, 0.04% to 4.32% relative abundance), except for the biomedia which remained free of potential pathogens. Although these genera have been widely identified in other disease-free individuals (Siddik *et al.*, 2022; Zheng *et al.*, 2019), monitoring these potential pathogens over time is crucial for controlling dysbiosis and preventing disease outbreaks.

Bacteria dynamics on the biofilter: special attention on nitrifiers

In terms of the biofilter, an opposite trend was noted for bacteria involved in nitrogen/ammonia removal processes depending on the salinity. Specifically, there was a significant increase in the *Pseudorhodobacter* genus, a denitrifying phosphorus bacteria group (Chao *et al.*, 2020), and a decrease in *Nitrospira*, which is a key group of aerobic nitrifying bacteria. This

suggests that salinity may impact the denitrification process carried out by the biofilter. Previous studies have reported that abrupt salinity changes significantly affect nitrifying bacteria diversity and ammonia/nitrite oxidation steps of the nitrification process (Kinyage *et al.*, 2019). Therefore, it is crucial to further understand biofilter biological dynamics to optimize biofiltration at different salinities.

Relevant bacteria from a productivity point of view

In the current context where the aquaculture industry is facing increasing pressure to meet the animal protein demands under a sustainable way, there is growing evidence that bacterial dysbiosis is associated with decreased productivity (Infante-Villamil *et al.*, 2021; Wang *et al.*, 2021). Despite both systems in our study maintaining acceptable productivity levels, we have provided a promising proof of concept that the relative abundance of specific bacterial entities (ASVs) within the system can be linked to one of the main productivity indexes measured in the aquaculture industry, the FCR.

The consistently strong positive relationship observed between members belonging to *Vibrio* and *Leucothrix* genera suggests them as negative environmental bacterial biomarkers for productivity under SW conditions. While *Vibrio* and *Leucothrix* include opportunistic pathogenic strains in host guts (Dahle, 2022; Sanches-Fernandes *et al.*, 2022), this study highlights the possibility of an association between less productive systems and their abundance, even in the absence of pathogenic events. On the other hand, members from *Roseibacillus* and *Candidatus Kaiserbacteria*, or *Rhodobacter*, are suggested to be positive biomarkers under SW or BW systems, respectively. These taxa have been described in various aquaculture systems and are sensitive to environmental factors such as water temperature, light exposure, salinity, or organic matter content (Jiang *et al.*, 2020; Kyritsi *et al.*, 2023; Wang *et al.*, 2020). Moreover, members from *Rhodobacter* have been widely used in ecological restoration to remove pollutants from the environment and have been discovered to play a role in boosting the immunity of aquatic animals (Luque-Almagro *et al.*, 2017). Therefore, we propose members of this genus and *Roseibacillus*, to track in environmental-related samples. Only one member from *Hyphomicrobium* was positively correlated with FCR in gut microbiome under SW. This was primarily related to denitrification processes (Ju *et al.*, 2014), with healthy zebra fish (Chen *et al.*, 2018), differential metabolites in the gut microbiota (Wang *et al.*, 2020), but no previous information was collected on the role of this genus in barramundi fish guts. Overall, further studies should be conducted to fully understand the relationship between environmental/host-related microbial communities and system optimization, potentially involving daily or weekly sampling to capture bacterial diversity fluctuations more precisely, including in productive vs. unproductive systems.

Conclusions

The present study indicates that farming barramundi in BW is an efficient alternative to SW rearing. The reduced salinity does not lead to a reduced productivity and, overall, the quality of the meat produced in SW and BW is comparable. Moreover, BW led to lower feed intake and FCR.

The dominance of well-known bacterial taxa previously observed in other healthy aquaculture systems under all sampling points, and the absence of diversity decrease under both salinities suggest that the growth of barramundi under different salinities is viable also from a microbiological point of view. However, the presence of *Mycobacterium*, *Legionella*, and *Nitrospira* genera should be further monitored when barramundi is produced in low salinity waters. Albeit our sample size is limited, members from *Vibrio* and *Leucothrix* are suggested not only as potential pathogens, but also as potential negative indicators for productivity reduction under this study. Otherwise, members from *Roseibacillus* and *Roseobacter* as positive ones. Dynamics between probiotics and potential pathogenic strains in waters with widely varying salinities should be conducted to ensure the safety of culturing euryhaline species.

Data availability

[Zenodo]: [iFishIENCi, Intelligent Fish feeding through Integration of IFN01_RawDataset Lates calcarifer [Data set]. Zenodo. https://doi.org/10.5281/zenodo.14389073 (Cusimano (2024)). The projects contain the following underlying data: checklist for study using laboratory animals (ARRIVE) and Excel raw data.

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication) (http://creativecommons.org/publicdomain/zero/1.0/).

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✗ **Zelia Velez**
[1] University of Algarve, Faro, Portugal
[2] Centro de Ciencias do Mar, Faro, Faro District, Portugal

The objective of the present work was to evaluate the adaptability of *Lates calcarifer* to different salinities without compromising the productivity and quality of this species in aquaculture. This is especially important in the climate change scenario where it is expected that changes in salinity, pH and temperature may have strong negative impacts on the cultivation of many species.

The work is of scientific interest, however the way in which it is written does not emphasize the applicability of the study. In general, in my opinion, the manuscript needs to be thoroughly revised so that it is easier to read, and the objectives, results and conclusions are clear and highlighted.

Below I present my main comments regarding each part.

Abstract
Background - To make it easier to understand the following sections, the objectives of the work should be presented here. Besides, I am not sure if the authors mean that 'supplementation with microalgae extract has the potential to' or may has the potential to. If it known that microalgae have that potential, there is no need to teste.

Methods: This part needs to be rewritten; it has information that is not necessary. Suggestion: The study was carried on a euryhaline species, Lates calcarifer, reared in sea water (SW) and brackish water (BW), in a recirculating aquaculture system (RAS). The effects of salinity and pellet supplementation with microalgae was assessed after ten weeks by the analysis of growth performance, fillet quality (color, fatty acid profile) and microbiome profile.

Results: define FCR.
Resume the results avoiding repetitions. FCR, feed intake and the viscerosomatic index are significantly higher in SW than in BW. Changes in salinity also influenced the microbiome composition in faeces and system compartments.

There are no differences in growth depending on pellet supplementation with microalgae extract, neither on the colour of the fillets. However, microalgae supplementation induced changes in fatty acid profile. MUFA, TFA and SAFA should be defined.

Methods:

Climate change may lead to a decrease in salinity (due to rainfall or thawing) or an increase in salinity due to periods of drought. In this study, fish were transferred from freshwater to saltwater or brackish water, but the opposite was not analysed. The objective of this type of study is to analyze the ability of species to cope with changes; physiologically, the impacts of moving from freshwater to saltwater and from saltwater to freshwater are different. Therefore, for the conclusions to be more robust, fish that were in salt water and transferred to fresh and brackish water should also have been used.
Growth data, bio-morphometric and body quality traits analysis - Is the name of the anaesthetic correct (MBW22)? I only know MS222

I do not find the definition of WG, FBW, IBW.

The definition of a* and b* is not clear, it took me long time to get it.

Results - The results section should only present the results, without discussing them or mentioning the methods used. The results section is very long and difficult to follow because the authors have added information that should not be in this section.
e.g. 'At the end of the trial, the average weight of the fish was assessed' this information was given in methods. The statistical test used is also methods

Discussion - The discussion is very confusing; the authors should organize the discussion in a more schematic way. For example: -Effects of salinity/-Effects of supplementation with microalgae extracts. The discussion is too fragmented, and therefore there is no good integration of the results.

Several references are made to previous studies with other euryhaline species whose results are divergent, and no hypotheses are raised to justify these divergences. Most euryhaline species, despite being able to withstand large fluctuations in salinity, prefer freshwater or saltwater habitats. The preference of each species can lead to different results; this was not discussed and is extremely important. To cope with climate change, euryhaline species with high adaptability to low salinity should be cultivated where salinity is expected to decrease; and euryhaline species with high adaptability to high salinity where salinity is expected to increase.

The authors do not mention characteristics of the species studied that constitute evidence that this species is better able to cope with climate change than other euryhaline species such as sea bream and sea bass. It is also unclear whether supplementation with microalgae extracts is beneficial. Supplementing feed with these extracts has costs; does the benefit obtained justify these costs?

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Effects of environmental factors such as climate change and pollution on fish physiology. In recent years my team has focused on the effects of temperature, salinity and acidification on teleost fish and sharks. The effects of salinity focus especially on sensory capacity and impacts on reproduction and foraging.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.