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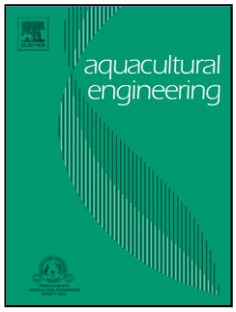

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Increased sulfate availability in saline water promotes hydrogen sulfide production in fish organic waste

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Highlights

- H_2S concentration and production rates increase with sulfate in saline water.
- H_2S measurement method was proven accurate for RAS water and waste
- Traditional sulfate reducing bacteria exist only in low salinities

Abstract

The risk of hydrogen sulfide (H_2S) production can be a challenge in marine land-based recirculating aquaculture systems (RAS). Hydrogen sulfide is a toxic gas that can cause massive fish mortality even at low concentrations, and in addition, serious odour problems in the surroundings. It is a bacterial by-product originating from the degradation of organic matter in sulfur-rich waters such as marine waters. In order to hinder H_2S production in marine land-based RAS, more information on the H_2S production conditions and the associated microbiology is needed. In this study, the production of H_2S from rainbow trout (*Oncorhynchus mykiss*) organic waste was examined using a novel H_2S measurement method under a range of salinities (0, 5, 10, 15, 25 and 35 g/L) in anaerobic mixed reactors, and the microbial communities as well as abundance of sulfate reducing bacteria (SRB) were characterized. The maximum H_2S concentration

increased from 23.1 ± 8.2 mg H_2S/L at 0 g/L salinity to 153.9 ± 34.1 mg H_2S/L at 35 g/L salinity. Similarly, the H_2S production rate increased from 5.6 ± 0.2 at 0 g/L salinity to 26.4 ± 12.7 mg of H_2S produced per day at 35 g/L salinity. The highest H_2S production was recorded after increased availability of volatile fatty acids, which were produced by fermentative bacteria from phyla Firmicutes and Bacteroidetes that dominated the microbial communities after day 5. The traditional sulfate reducing bacteria (SRB) were found only at 0 and 5 g/L salinity, while at higher salinities, H_2S production was carried out by novel unquantifiable SRB. The results demonstrate that H_2S can be a pronounced problem in marine RAS, although it can be controlled through preventing anaerobic conditions within the system.

Keywords: Hydrogen sulfide; Organic matter; Seawater; Sulfate; Sulfate reducing bacteria

1. Introduction

In the last few years, large-scale marine land-based recirculating aquaculture systems (RAS), producing e.g. Atlantic salmon (*Salmo salar*) and yellowtail kingfish (*Seriola lalandi*), have been constructed worldwide (Dalsgaard, 2017). Marine land-based RAS offer high level of bio-security and better control over environmental conditions as compared to the traditional sea cage production, (Martins et al., 2010), resulting in optimal fish growth as well as reduced environmental impact. They are, however, facing a potential challenge in the risk of hydrogen sulfide (H_2S) production within the system. Previously, elevated sulfide concentrations have been found below net cages (Chang et al., 2013; Holmer and Kristensen, 1992). Being a strong chemical asphyxiant, H_2S production poses even a more severe threat to fish health and welfare in closed aquaculture systems.

H_2S is a colorless flammable gas with a characteristic odor of "rotten eggs" (Arbison and Ourgeois, 2015). It has higher density than air, meaning that it accumulates in the bottom areas rather than dispersing easily in the air (Abdollahi and Hosseini, 2014). Recently, marine RAS facilities have experienced incidents

caused by H_2S, including mass fish mortality and severe odor problems in the surrounding areas (Dalsgaard, 2019). In fish, H_2S has severe consequences, preventing oxygen release, generating cellular anoxia and finally preventing ATP production (Kiemer et al., 1995). Toxicity of H_2S towards aquaculture-reared species has not been extensively reported, but LC_{50} values of 0.013 mg/L in 48 h have been found for walleye (*Sander vitreus*), 0.026 mg/L in 96 h for northern pike (*Esox Lucius*), 0.031 mg/L in 96 h for brook trout (*Salvelinus fontinalis*), 0.030 mg/L at 72 h for bluegill (*Lepomis macrochirus*), 0.007 mg/L at 96 h for fathead minnow (*Pimephales promelas*), and 0.025 mg/L at 72 h for goldfish (*Carassius auratus*) (Smith and Oseid, 1974). Atlantic salmon (*Salmo salar*) has been suggested to be more tolerant for H_2S, since a periodic exposure during 18 weeks to H_2S concentrations of 0.27 mg/L did not found to cause significant damage (Kiemer et al., 1995). However, a single acute dose of hydrogen sulfide between 0.75 and 0.99 mg/L induced considerable stress and gill tissue damage (necrosis), which was suggested to lead to progressive liver damage, reduced growth and greater susceptibility to diseases.

The main reason for H_2S being produced in marine RAS is the high abundance of sulfate in seawater, as marine water has a more complex chemistry with ion concentrations of 10 - 1000 times higher than in freshwater (Nazaroff and Alvarez-Cohen, 2001). Sulfate is consumed in a dissimilatory sulfate reduction process, where anaerobic sulfate reducing bacteria (SRB) utilize sulfate as electron acceptor for the decomposition of organic matter (Harada et al., 1994; van Loosdrecht et al., 2016). In general, SRB degrade fermentation products like acetate, propionate, butyrate, lactate and hydrogen, which are produced from complex organic molecules by fermentative bacteria. The-end products of sulfate reduction process are bisulfide (HS^-) and hydrogen sulfide (H_2S) (Gerardi, 2006). The quantity of H_2S, the form that escapes to the atmosphere, depends on the pH of the water, initial dissolved hydrogen sulfide concentration, and temperature (Eaton et al., 1995). The sulfide production rates of SRB depend on e.g. pH, temperature, sulfate concentrations, and organic matter bioavailability (Laanbroek and Pfennig, 1981; Muyzer and Stams, 2008; Plugge et al., 2011). Although sulfate reduction capacity is found within nine bacterial and archaeal phyla (Müller et al., 2015), the most commonly found SRB belong to ~23 deltaproteobacterial

genera (e.g. *Desulfobulbaceae*, *Desulfovibrionaceae*; Muyzer and Stams, 2008). SRB are abundant in sulfate-rich sea sediments, but are also common in wastewater treatment plants and in other engineered environments with high sulfate waters and organic matter concentrations (EPA, 1991; Hao et al., 2014; Zhang et al., 2008). Even though H_2S production has been observed below the fish cages (Chang et al., 2013; Holmer et al., 2005; Holmer and Kristensen, 1992), the identity and function of microbes involved in H_2S production in aquaculture environments has not yet been adequately described.

Despite the high operational and economic risks associated with H_2S in the aquaculture sector, the knowledge on the H_2S production and the causative microbiology is limited. The following study aims to: 1) test and evaluate the reliability of a H_2S measurement method for RAS water and sludge samples, 2) evaluate the production dynamics, rates and maximal values of H_2S obtained from fish organic waste at different salinities (0, 5, 10, 15, 25 and 35 g/L), and 3) examine the microbial community associated to the process.

2. Materials and Methods

2.1 Fish organic waste collection

Rainbow trout (*Oncorhynchus mykiss*) was reared in six different salinities (0, 5, 10, 15, 25 and 35 g/L) in 1 m^3 rectangular tanks under a flow-through system. The fish were stocked at a density of 20 kg fish m^3 and daily fed 1% of the biomass (200 g/d with a 12 h autofeeder), using Biomar Enviro 920 (Biomar A/S, Denmark), salinity was continuously monitored with a Seawater Refractometer (HI 96822, Hanna Instruments, US). Samples of fish organic waste produced during 24 h were collected at the bottom of 20 L swirl separators with a 2 L collectors and stored at 4°C. Samples from two consecutive days were pooled for the H_2S production experiments.

2.2 Experimental design and sampling

To evaluate the production of H_2S, the fish organic waste from the six different salinities were transferred into 2 L enclosed Blue Cap bottles (SCHOTT Duran, Germany) serving as anaerobic batch reactors. The reactors were kept at room temperature (19.6 ± 1.4 °C) with continuous magnetic stirring at 200 rpm (Big Squid, IKA, Germany). The bottles were sealed with screw caps with two ports for sampling purposes (cap GL, Duran Group, Germany), designed to avoid potential oxygen interference. The data was collected during two separate experiments, each lasting for 25 days. The first experiment examined salinities of 0, 15, 25 and 35 g/L, and the second experiment salinities of 0, 5, 10 and 35 g/L. Before starting the experiment all reactors were spiked with sodium nitrate (VWR, Denmark) to a final concentration of 50 mg NO_3^--N/L, to ensure that possible VFAs were consumed through denitrification as has been reported by Suhr et al. (2013). A sample (50 mL) for analysis of soluble chemical oxygen demand (sCOD), volatile fatty acids (VFAs), and total dissolved sulfide (TDS) was taken every two days. At the same time, pH and temperature were measured using a portable meter (Hach HQ40d, Hach Lange, Germany). Total chemical oxygen demand (TCOD) in each reactor was analyzed at the start of the experimental period (day 0). In experiment 2, an additional 1.5 mL sample was taken for microbiological analysis on day 0, 1, 3, 5, 10 and 20.

2.3 Chemical Analysis

Samples for sCOD and VFA were centrifuged at 4,500 rpm for 15 min at sample temperature, and supernatants were filtered with 0.2 µm syringe filters (Filtropur S, SARSTEDT, Germany). The filtered samples for sCOD, and VFA, as well as unfiltered samples for TCOD were subsequently preserved by adding 1% v/v of sulfuric acid (4 mol/L H_2SO_4, Merck Millipore, Germany), and maintained at +4 ^{0} C until analysis. VFAs were analyzed using a 930 Compact IC Flex 1 with a Metrosep A Supp 7 -250/4.0 column coupled with a 887 Professional UV/VIS detector (Metrohm, Sweden), and 0.1 M H_2SO_4 was used as suppressor and 3.6 mN Na_2CO_3 as eluent. The determination of TCOD and sCOD was performed using digestion vials LCK 514 and LCK 314, respectively (Hach Lange, Germany). Total dissolved sulfide

concentration (S^{2-}) was analyzed using a modified methylene blue method, "Sulfulla" (see Supplementary material). In addition, pH, temperature and salinity of the sample were recorded for estimating the H_2S fraction according to Eaton et al. (1995).

2.4 Microbiological analysis

Immediately after sampling, microbiological samples were frozen and stored at -20 ^{0} C. Before DNA extraction, samples were centrifuged at 10,000 g for 1 min, and supernatant was removed. DNA was extracted using the DNeasy PowerLyzer™PowerSoil DNA Isolation Kit (Qiagen, Germany) according to the manufacturer's instructions. PowerLyzer Homogenizer was applied once, 3,400 rpm for 45 s, during the extraction. The quantity of extracted DNA was measured with Qubit™ dsDNA HS assay and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, US).

In order to quantify the abundance of sulfate reducing microbes, qPCR quantifications were performed using primer pairs targeting the two key enzymes of sulfate reduction pathway: dissimilatory sulfite reductase (*dsr*A; RH1-dsr-F/RH3-dsr-R; Ben-Dov et al., 2007) and the dissimilatory adenosine-5′-phospho-sulfate reductase (*aps*; RH1-aps-F/RH2-aps-R; Ben-Dov et al., 2007). Both qPCR reactions included 5 ng of template DNA, 0.2 µM forward and reverse primers, and 1x Maxima SYBR Green/Fluorescein Master Mix (Thermo Fisher) in a total volume of 25 µl. The thermal conditions were as follows: initial denaturation 10 min 95 °C, then 40 cycles at 95 °C for 15 s, 60 °C for 60 s and 72 °C for 30 s. Amplification efficiencies were between 88-93 % for the qPCR assays. The quantification was performed using CFX96 qPCR thermal cycler (Bio-Rad, US).

Microbial community composition was studied using next generation sequencing targeting V4 region of 16s rRNA gene with primers 515F-Y (Parada et al., 2016) and 806R (Caporaso et al., 2011) and the analysis of gene sequences was done using mothur (version 1.39.5; Schloss et al., 2009) as in von Ahnen et al. (2019). Before calculating alpha and beta diversities, the data was normalized by subsampling to 15,866

sequences. Sequences have been submitted to NCBI Sequence Read Archive under BioProject PRJNA562807.

2.5 Statistical and data analysis

The statistical analysis was carried out using the open-source software R (version 3.5.3; R Core Team, 2019). Since data was normally distributed, a one-way ANOVA analysis followed by a Tukey-Kramer multiple comparison test was used to test for significant differences between the maximum H_2S concentration and normalized maximal H_2S production between salinity treatments.

H_2S production rates in the different salinities tested were estimated according to zero and first order kinetic reactions (Nazaroff & Cohen, 2001). Non-metric multidimensional scaling (NMDS) conducted with metaMDS function in "vegan" package (Oksanen et al., 2013), plots calculated based on Bray–Curtis distance matrix were used to visualize dynamics in the microbial community structure. Before NMDS, Wisconsin and square- root-transformations were applied to OTU abundance data. The relationships between microbial OTU abundance and H_2S concentration was studied using Kendall correlation analysis.

3 Results and Discussion

The amount of fish organic waste collected was rather constant between the tanks, with a final average of 30 ± 15 g TCOD/L inside the batch reactors (Table 1). However, the reactors at 0 g/L salinity in experiment 1 and at 35 g/L salinity in experiment 2, had higher TCOD concentration as compared to the other treatments. This can be explained by an increased amount of uneaten pellets found in the collectors, increasing the final collected TCOD concentration, even though fish were fed the same amount in all tanks.

Table 1. Organic matter concentration (total chemical oxygen demand (TCOD g/L; mean ± SD, *n*=3) in the anaerobic reactors at different salinities in the two experiments at day 0. Samples are based on subsequent pooling for two consecutive days.

3.1 Effect of salinity and organic matter in H_2S production

Based on the standard curve (see Supplementary material), the "Sulfulla" method showed high accuracy (R^2 = 0.999) for concentrations ranging between 0 and 1.2 mg S^{2-}/L, while samples with higher concentrations should be diluted with microfiltrated (MQ) water. The analysis is equally accurate for both RAS water and fish organic waste samples, the latter requiring pre-handling of the sample (see Supplementary material).

Production of H_2S was found at all salinity levels. In both experiments, H_2S production (concentration > 0.5 mg/L) was detected already at day 2. The zero or first order kinetics of H_2S production differed slightly between experiments, starting at day 5 in experiment 1 and at day 7 in experiment 2. In all reactors, H_2S accumulated until reaching a maximum concentration (Table 2) to further decrease with time (except 10 g/L salinity) (Fig. 1). The reduction of H_2S concentration could be due to; H_2S oxidation by bacterial sulfide oxidation, chelation in solution by fatty acids produced during the fermentation of the organic matter, precipitation with soluble metals e.g. cadmium, iron and zinc present in seawater, or diffusion to the gas phase (Dague, 1972; Gerardi, 2006; White et al., 1997).

Both the maximum H_2S concentration (r = 0.84, $p < 0.05$) and H_2S production rates (mg H_2S/d; r = 0.83, $p < 0.05$), calculated according to zero or first order kinetics, increased with increasing salinity (Table 2).

Table 2. Maximum H_2S concentration (mg H_2S/L) and H_2S production rate (mg H_2S/d) from fish organic waste at different salinities (0, 5, 10, 15, 25 and 35 g/L) (mean ± SD, n=3).

The correlation between salinity and total dissolved sulfide (S^{2-}) concentration (r = 0.74, p < 0.05) was slightly better than the correlation between salinity and H_2S concentration (r = 0.70, p < 0.05). This is due to S^{2-} including both end-products of bacterial sulfate reduction, H_2S and bisulfide (HS^-). At 35 g/L salinity in experiment 1, the maximum S^{2-} concentration (251.0 ± 23.5 mg/L) was higher than in experiment 2 (184.1 ± 65.4 mg/L of S^{2-}) (Suppl. Figs. 1, 2). However, the calculated concentration of the unionized form

(H_2S), was 7% higher in experiment 2 than in experiment 1, because the pH was lower in experiment 2 (6.0 ± 0.2) than in experiment 1 (6.8 ± 0.3). Leading to the higher proportion of S^{2-} being present as H_2S in experiment 2. This demonstrates that reporting H_2S values as S^{2-}, and not as calculated unionized H_2S, can lead to too small erroneous estimates, which can have severe consequences in RAS, since even H_2S concentrations as low as 0.02 mg/L can have negative effects on fish health (Adelman and Smith Jr., 1972; Kiemer et al., 1995; Oseid and Smith, 1974; Smith et al., 1976; Smith and Oseid, 1972).

As the initial TCOD i.e. organic matter used at the start of each experiment varied between reactors, the maximum H_2S concentrations were normalized with TCOD, expressing the amount of H_2S produced per gram of TCOD i.e. organic matter in each reactor (Fig. 2).

The maximum normalized H_2S concentration (0.68 mg H_2S/g TCOD) was lower at 0 g/L salinity (Tukey-Kramer, $p < 0.05$), while no significant differences were found between the higher salinities (10, 15, 25 and 35 g/L). The correlation between maximum normalized H_2S concentration and salinity ($r = 0.32$, $p < 0.05$) was weaker as compared to the one between non-normalized H_2S concentration and salinity ($r = 0.70$, $p < 0.05$). This indicates that salinity or the amount of sulfate present in water (ranging from 37.0 ± 8.4 mg/L SO_4^--S at 0 g/L salinity to 924.4 ± 61.9 mg/L SO_4^--S at 35 g/L salinity) had a stronger effect on H_2S production than the total organic matter (TCOD) present in the reactors. This result can be explained with TCOD not reflecting the amount of electron donors (VFAs), leading to the differences in the availability of electron acceptors and donors. While SO_4^- (the electron acceptor for SBR) is readily available, the concentration increasing with salinity, organic matter is present in complex forms that need to go through hydrolysis and fermentation processes to be available as electron donors for SRB (Henze et al., 1997; Ucisik and Henze, 2008). In RAS, fish organic waste, submitted to an anaerobic environment (nitrate depleted), will be hydrolyzed and fermented into formate, acetate, propionate, butyrate and valerate (Aboutboul et al., 1995; Letelier-Gordo et al., 2017), the highest solubilization rates being reached around 5 to 7 days (Conroy

and Couturier, 2010; Letelier-Gordo et al., 2015; Suhr et al., 2013, 2015). This means that once hydrolyzed and fermented, fish organic waste will provide electron donors (VFAs) for SBR, which will result in the production of H_2S and consumption of SO_4^--S. Indeed, in this experiment at 35 g/L salinity, VFA concentrations started to increase after 5 days, leading to the increasing H_2S and decreasing SO_4^--S concentration after 10 days (Fig. 3). This situation can eventually occur in rearing tanks with low removal of solids or in pipes with high biofilm growth, as has been observed in municipal sewers (Hvitved-Jacobsen et al., 2002; Lahav et al., 2004; Liu et al., 2015), or in biofilters, where organic matter can build up (Nootong and Powtongsook, 2012). In this study, the maximum obtained H_2S concentration was 7.21 ± 0.8 mg H_2S/g TCOD at 15 g/L salinity, meaning that one kg of organic matter submitted to anaerobic conditions has the potential to produce 7.2 g H_2S. When assuming that a marine land-based RAS uses 700 m^3 tanks, 1 kg of organic matter will in a worst-case scenario produce of 0.01 mg H_2S/L, a concentration that is already dangerous for fish.

3.2. Effect of salinity on the microbial community and sulfate reducing bacteria

The overall microbial community, measured in experiment 2, changed gradually in time and between salinities, the communities at 35 g/L being significantly different than communities at 0 g/L and the other tested salinities (Suppl. Fig. 3). In the beginning of the experiment, Proteobacteria was the most common phyla in all salinities, but the relative abundance decreased steadily during the experiment. This group disappeared completely at 35 g/L salinity between days 3 and 20 (Fig. 4). The most abundant proteobacterial families were facultative anaerobic gammaproteobacterial *Aeromonadaceae*, *Enterobacteriaceae* and *Vibrionaceae*, which probably originated from rainbow trout intestines (Kim et al., 2007) , and decreased when conditions became strictly anaerobic. The abundance of Bacteroidetes and/or Firmicutes increased with time in all salinities, representing >50% of microbial community after day 3. Both phyla include important obligatory anaerobes involved in the fermentation of organic matter, such as orders Lactobacillales, Clostridiales and Bacteroidales, that could thrive only when oxygen was depleted in the

reactors and be responsible for the VFA production observed after day 5. The common SRB from Deltaproteobacteria were found in significant abundances (0.1 - 6% of total reads) only in 0 g/L and 5 g/L salinity reactors (data not shown).

Altogether, the abundance of 23 OTUs correlated positively with H_2S concentration, but the correlation pattern was not equal between the salinities, reflecting the differences in the abundance of these OTUs (Suppl. Table 1). Only members of family *Ruminococcaceae* from Firmicutes (OTU27, 28, 39, 48) were found to correlate with H_2S at all salinities. This family has not been found to take part in sulfide production, but is a common fermenter that became abundant towards the end of the experiment when concentrations of VFAs also increased. At 0 and 5 g/L salinity, the abundance of the common SRB, deltaproteobacterial Desulfovibrio (OTU23), increased with H_2S, while being completely absent at the higher salinities. At low salinities (0, 5 and 10 g/L), the abundance of several OTUs assigned to genera *Bacteroides* and *Macellibacteroides* from phylum Bacteroidetes (OTU6, 11, 19, 33), as well as the abundance of gammaproteobacterial genus *Acinetobacter* (OTU18, 35) and of family *Lachnospiraceae* from phylum Firmicutes (OTU22, 25, 38) increased with H_2S concentration. Since both *Bacteroidetes* and *Lachnospiraceae* are considered as common fermenters, the correlation describes more increased fermentation than H_2S production, as the abundance of OTUs assigned to this phylum correlated also positively with VFA concentrations (data not shown). However, *Acinetobacter* has been identified to be involved in H_2S metabolism (Luo et al., 2013), suggesting that there was some sulfide consumption happening when the concentration increased. At 10 and 35 g/L salinity, high H_2S concentration coincided with the high abundance of *Fusobacterium* (OTU9). The physiology of this group is not well established, but it has been found to possess at least four enzymes related to the alternative H_2S production pathways, degrading aminoacids e.g. L-cysteine and peptides into H_2S (Basic et al., 2017) and to possess anaerobic sulfite reductase (asr) (Anantharaman et al., 2018) instead of traditional dissimilatory sulfite reductase (dsr) that was quantified in this study. At 35 g/L salinity, several OTUs not found in the lower salinities increased

with H_2S concentration: Firmicutes family *Peptostreptococcaceae* (OTU16), gammaproteobacterial *Marinobacterium* (OTU60) and *Marinobacter* (OTU133), and two OTUs with unidentified taxonomy (OTU90, 219). None of the identified taxa has known connection to sulfur metabolism, although *Peptostreptococcaceae* was recently found to be abundant in sulfidic streams (Hotaling et al., 2019), suggesting that it might participate to H_2S metabolism.

Total microbial abundance measured as the copy numbers of 16S rRNA gene increased steadily from the day 0 (Fig. 5a). In 35 g/L salinity reactors, the abundance was very high in the beginning, but decreased significantly already after day 1. Furthermore, the abundance stayed 10 - 100 times lower at 35 g/L salinity than in other reactors, although increasing towards the end of the experiment, reflecting the highly selecting conditions in the most saline reactors. The abundance of the two main marker genes for sulfate reduction, *dsr*A gene coding for sulfite reductase enzyme and *aps* gene coding for adenosine 5'-phosphosulfate reductase gene, increased towards the end of the experiment (Fig. 5b,c). The abundance of both genes was highest at 0 g/L and 5 g/L salinity treatments, although they exhibited lower H_2S production than 10 and 35 g/L salinity reactors. This result cannot explained with the lower total microbial abundance in the higher salinity treatments, as the relative abundances (*dsr*A or aps gene copy number normalized with total microbial abundance) exhibited a similar pattern (data not shown). Since the traditional SRB groups were not found in 10 and 35 g/L salinity reactors, it is possible that the SRB present represents novel groups that are not covered by the current primer sets or that they produce H_2S through alternative pathways with enzymes that were not quantified here. Based on the correlation analysis results, these novel unquantifiable SRB could belong to Fusobacteria that was abundant both in 10 and 35 g/L salinity reactors, or to Firmicutes family *Peptostreptococcaceae*, or be currently completely unidentified. This means that the SRB abundance cannot be reliable estimated within marine land-based RAS at the moment. Further microbiological research is needed to solve the main H_2S production pathways under high salinity in order to develop methods to prevent H_2S production when treating saline fish organic waste. When relating the abundance of SRB to H_2S produced, it was found that one SRB (one *dsr*A gene copy; Müller et al., 2015) could produce

$1.6*10^{-8} \pm 2.9*10^{-9}$ mg H_2S at 0 g/L salinity, and $2.6*10^{-8} \pm 8.3*10^{-9}$ mg H_2S *dsr*A at 5 g/L salinity during the maximum H_2S production.

4 Conclusions

This study fills up essential knowledge gaps, providing a reliable method to analyze H_2S and new information required for understanding H_2S production in aquaculture systems. Altogether, the results demonstrate that salinity or mainly the sulfate contained in it, increases H_2S production creating a potential problem in marine land-based RAS, but also in the bottom of marine sea cages. The microbiological results indicate that the problem is presumably manageable, since SRB are dependent on the readily available carbon sources produced by slow-growing fermentative bacteria, both groups requiring oxygen-free habitat conditions. This means that implementing frequent cleaning protocols for pipes, tanks, and biofilters, and an efficient system design to avoid organic matter accumulation could limit the production of suitable carbon sources, and thus growth of SRB, and hinder H_2S production. Once produced, H_2S concentration could also be reduced with nitrate addition (Torun et al., 2020), but it is not an environmentally sustainable approach, as the main effort of the marine land-based RAS technology is to reduce nitrogen discharge. However, in order to develop and test preventive and responsive measures to reduce the risks associated with H_2S in marine RAS, more studies on acute H_2S toxicity to fish, and the different microbiological H_2S production routes are needed.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure 1. Concentration of H_2S over time at different salinities (0, 5, 10, 15, 25 and 35 g/L).

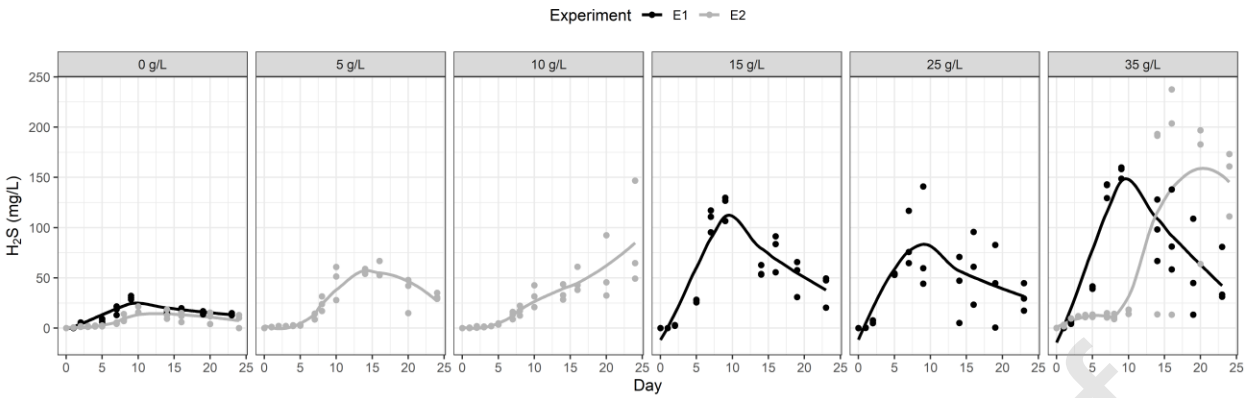

Figure 2. Maximum H_2S concentrations normalized with the amount of organic matter (H_2S/g TCOD) at different salinities (g/L). Letters denote significant differences (Tukey–Kramer, $p < 0.05$).

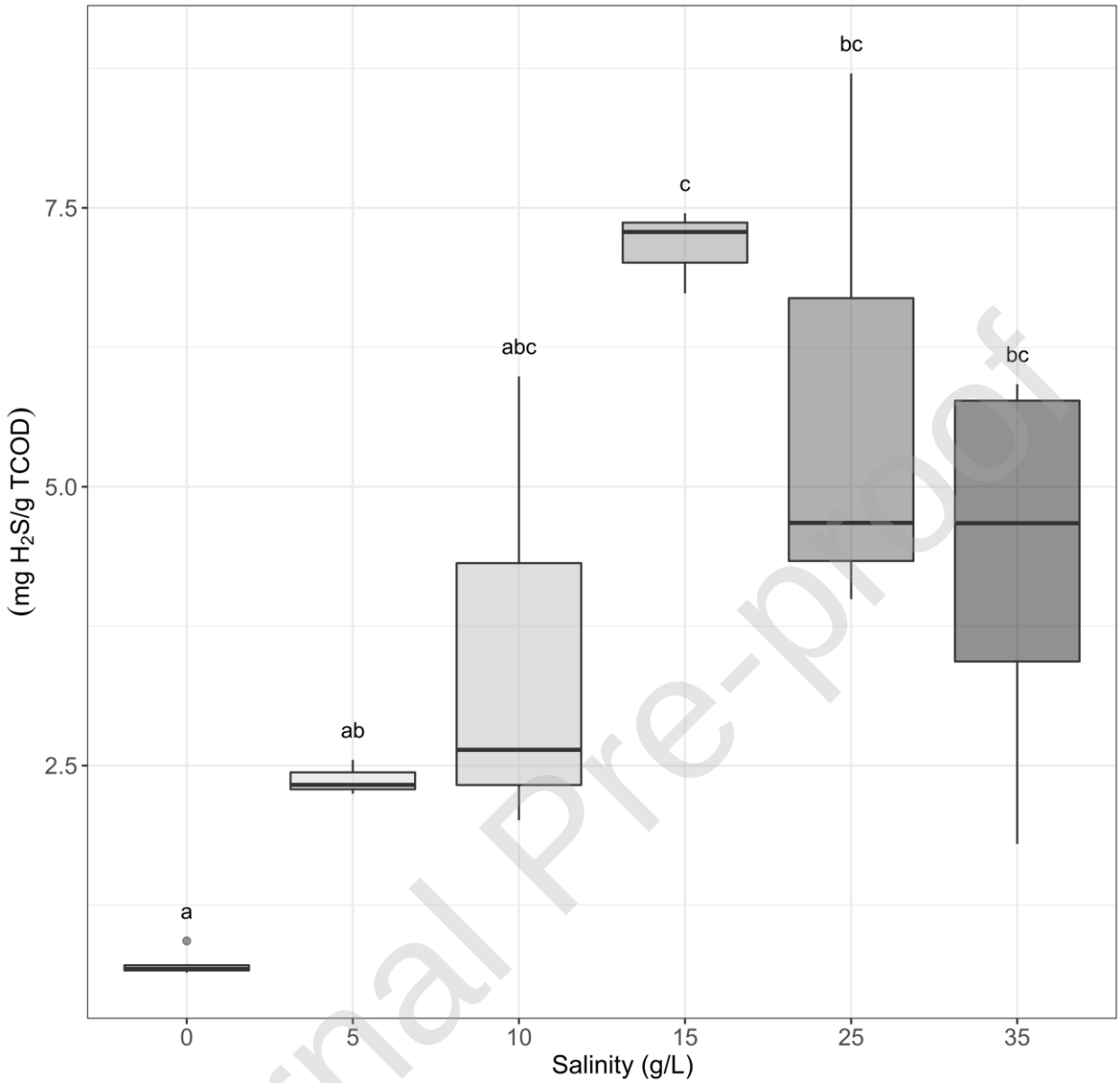

Figure 3. Sulfate, VFA dynamics and H_2S found in the 35 g/L salinity anaerobic reactor in the experiment 2.

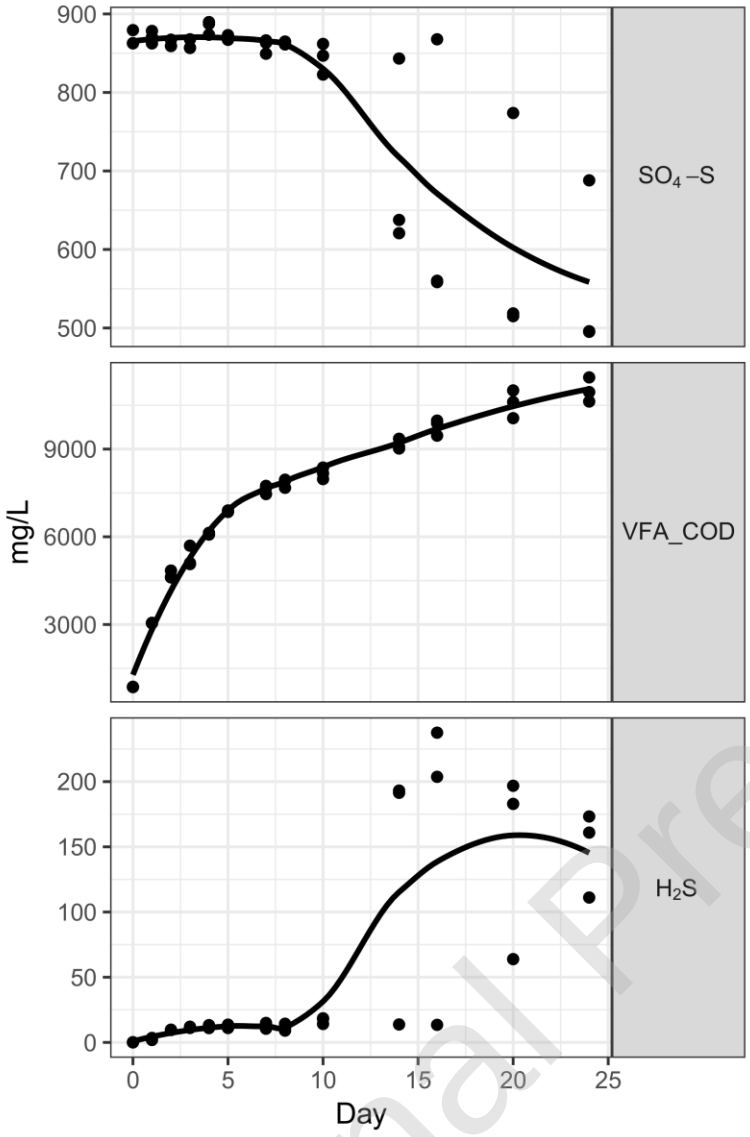

Figure 4. The relative abundance of bacterial phyla in reactors at different salinities during 20 days of experiment 2.

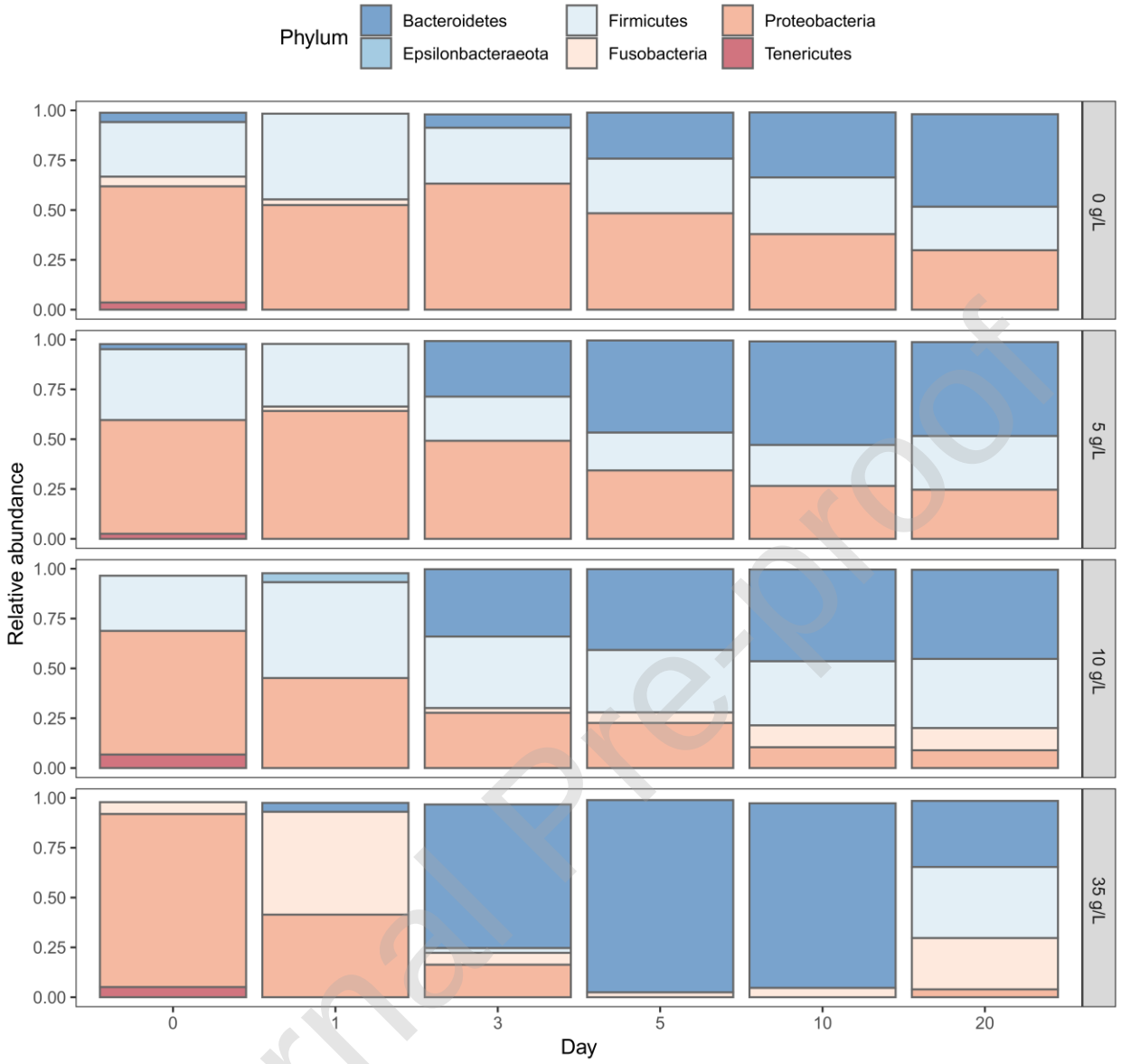

Figure 5. The abundance of a) all microbes (16S rRNA gene copies/L), b) microbes carrying dissimilatory sulfite reductase gene (*dsr*A gene copies/L), and c) microbes carrying APS reductase gene (*aps* gene copies/L) in reactors at four different salinities during 20 days of experiment. Notice different scale in panel a.

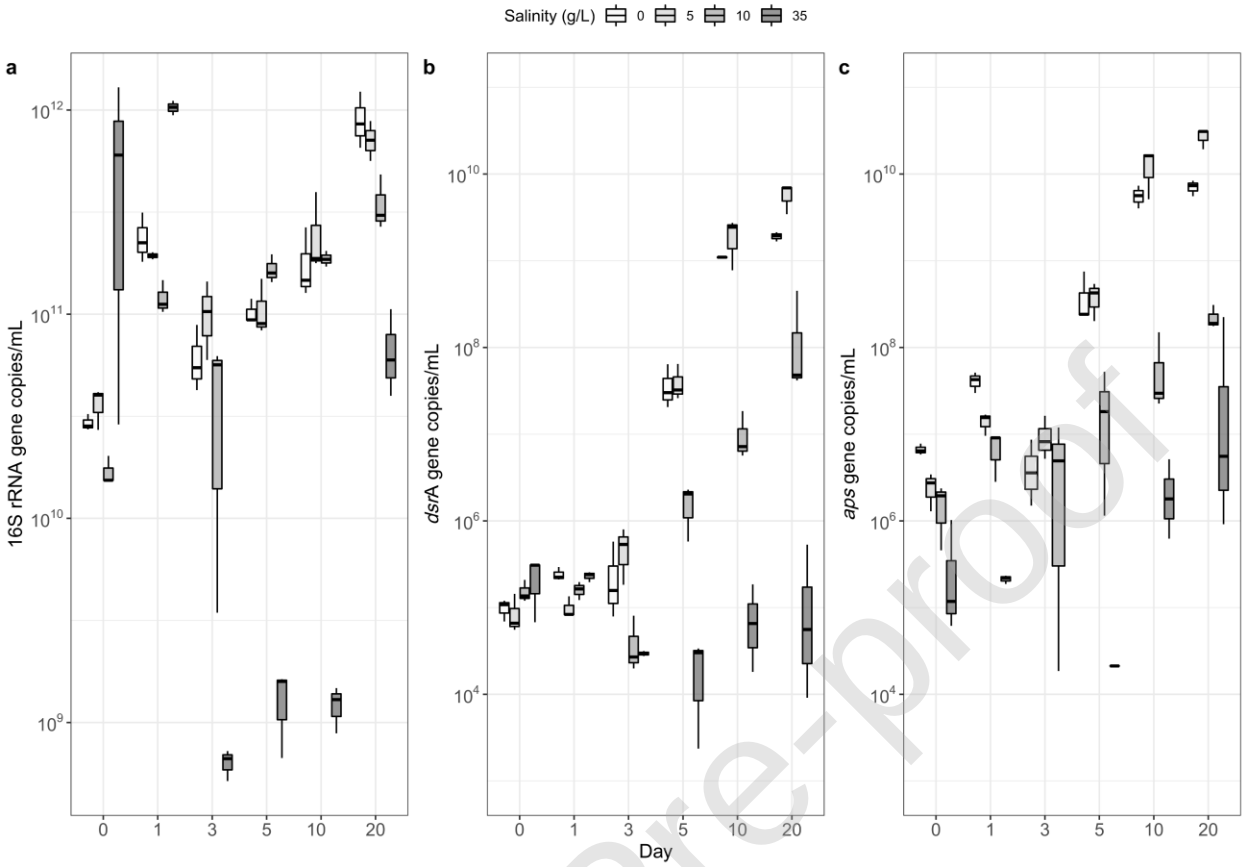

Table 1. Organic matter concentration (total chemical oxygen demand (TCOD g/L; mean ± SD, *n*=3) in the anaerobic reactors at different salinities in the two experiments at day 0. Samples are based on subsequent pooling for two consecutive days.

Salinity (g/L)		0	5	10	15	25	35
TCOD (g/L)	Exp. 1	44.6±1.1^{a}	--	--	17.4±1.1^{d}	16.2±0.8^{d}	27.0±0.6^{c}
	Exp. 2	23.4±0.7^{b}	26.2±1.4^{c}	24.5±0.4^{bc}	--	--	61.8±0.4^{e}

Values not sharing a common superscript letter were significantly different (Tukey–Kramer, $P < 0.05$).

Table 2. Maximum H_2S concentration (mg H_2S/L) and H_2S production rate (mg H_2S/d) from fish organic waste at different salinities (0, 5, 10, 15, 25 and 35 g/L) (mean ± SD, *n*=3).

	Salinity (g/L)					
	0	5	10	15	25	35
H_2S concentration						
Experiment 1 (mg H_2S/L)	30.3±1.7			124.5±6.5	93.8±41.2	148.8±10.9
Experiment 2 (mg H_2S/L)	15.9±3.2	55.5±2.4	70.3±39.3			159.1±52.0
H_2S production rate						
Experiment 1 (mg H_2S/d)	5.6±0.2[a]			21.0±2.3[b]	14.6±4.5[a]	26.5±1.3[b]
Experiment 2 (mg H_2S/d)	3.1±0.7[b]	7.0±2.2[a]	4.0±1.6[b]			26.4±12.7[a]

In superscripts, [a] denotes for zero order and [b] for first order production kinetics.