1 **Nitrate amendment to control sulphide accumulation in shrimp ponds**

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Abstract

The production of shrimp is often performed in earthen outdoor ponds in which the input of feed and faeces on the bottom results in deterioration of the water quality that results in a negative impact on the aquatic animals and the environment.

Nitrate application in shrimp ponds could improve pond water and sediment quality by oxidizing anaerobic zones of the sediment. This study was conducted to evaluate the possible benefits of nitrate treatment in combination with probiotics application to decrease sulphide formation in shrimp ponds, which is a crucial problem during the growth phase of shrimp. A batch system including shrimp feed and faeces, representing the bottom waste produced during 90 days of shrimp culture, was used to test the effect of nitrate addition under anaerobic conditions that mimic the dead zones in shrimp pond bottoms where high amounts of organic matter accumulate. Nitrate addition acted as a curative solution for hydrogen sulphide (H_2S) accumulation, rather than preventive. Nitrate amended treatments ($12.5 \text{ mg/L.day NO}_3$) decreased the accumulated H_2S in the headspace of incubation bottles by $93 \pm 3\%$ compared to the controls. Nitrate addition increased pH and decreased organic acids accumulation to trace amounts. The use of a denitrifying strain did not improve the beneficial effect of nitrate, indicating that intrinsic capacity for anoxic conversion of waste by the present community was sufficient. In conclusion, nitrate treatment showed a curative potential to control H_2S accumulation in the shrimp ponds that is a critical problem during the growth of shrimp.

Keywords: nitrate treatment, probiotic, shrimp pond, sulfide, water quality

1. Introduction:

Fish and shrimp are an essential source of proteins for humans. About 50% of all aquatic animal protein originates from aquaculture, a number that should increase with an additional 10% by 2030 to keep on feeding the growing human population (FAO, 2018). The production of shrimp is usually performed in outdoor ponds with a surface area of a few thousand m² up to several hectares. The shrimp grow in the pond for a period of about 3 months until they reach harvest size of around 15 g or more (Hung and Quy, 2013). These ponds are constructed either as earthen ponds, with a direct continuous contact between culture water and bottom sediment, or as lined ponds where a plastic type material (*e.g.*, high density polyethylene) is placed above the earthen bottom, thereby minimizing contact between the culture water and the soil (Pralitwilaikul et al., 2006). In both earthen and lined ponds, there is a high input of organic material (OM) to the pond bottom from uneaten feed and digested feed (faeces) in combination with dead material (*e.g.*, shrimp shedding). This dense layer of organic matter accumulation can reach up to 7.5 – 10 cm per growing season (Avnimelech and Ritvo, 2003).

The organic-rich conditions on the pond bottom in combination with the typical temperatures of 25-30 °C in shrimp ponds promote microbial activity, which substantially influences water quality and overall shrimp health. Under aerobic conditions, the mineralisation of OM and the release of carbon dioxide, ammonia and other nutrients stimulates phytoplankton growth, which is advantageous for shrimp growth, since they serve as the main food for zooplankton and benthic organisms, which, in turn, become the food of shrimp (Burford et al., 1998).

Aerobic microbial activity at the pond bottom, however, can become so high that the rate of oxygen consumption exceeds the rate of oxygen transfer from pond water to pore water of the sediment, and oxygen gets depleted at the water-sediment interphase (Boyd, 2003). Anaerobic processes then take over, leading to release of potentially toxic metabolites, such as organic acids, reduced organic sulphur compounds, reduced manganese and hydrogen sulphide (Avnimelech and Ritvo, 2003).

Hydrogen sulphide (H₂S) and iron sulphide create a bad odour and black colour of the sediment, respectively, and pose a toxicological risk for the shrimp that naturally dwell and feed on the pond bottom (Boyd, 1998; Avnimelech and Ritvo 2003). Sulphide toxicity depends on the H₂S concentration (*i.e.*, pH dependent), and varies between the different species and growth phase of the shrimp (Vismann,

1996; Panakorn, 2016). For toxicity testing, a 48 h LC50 value of 0.0087 mg/L H₂S was reported for Pacific white shrimp (*L. vannamei*) post-larvae, while for juveniles this was 0.0185 mg/L (Federal Register, 2010) and for black tiger shrimp (*P. monodon*) it was 0.033 mg/L (Chen, 1985). Suo et al. (2017) found that chronic exposure to sub-lethal levels (1/10 of 96-hour LC50) could lead to a damage of the gut structure, stimulate the response of the inflammatory and immune systems, and change the structure of gut microbiota in *L. vannamei*. Sulphide also inhibits nitrification (Joye and Hollibaugh, 1995), and, thereby, increases ammonium accumulation in the pond.

Current approaches to control waste accumulation and its negative effects in ponds are limited. In most cases, mechanical manipulation is used to remove and/or oxidize the sludge, a labour-intensive approach, which is to be applied in between shrimp grow-out cycles during a dry period in the ponds. A number of probiotic products are suggested to improve water and sediment quality, yet, so far no high-quality scientific proof is available on their potential (Antony and Filip, 2006; Martinez Cruz et al., 2012; Boyd, 2014).

Among the approaches applied, sodium nitrate has been widely used by farmers as a fertilizer, water quality conditioner and bottom soil oxidant in pond culture of *L. vannamei*. Nitrate application could improve pond water and sediment quality by enhancing denitrification and the composition of phytoplankton communities. Nitrate is the preferred electron acceptor for bacteria under oxygen deficient conditions. Nitrate addition may control sulphate reduction by replacing sulphate reducing bacteria (SRB) with denitrifying bacteria through interspecies competition (Hubert and Voordouw, 2007), inhibition of dissimilatory sulphite reductase with the nitrite produced through nitrated reduction (Greene et al., 2006) or a metabolic switch of certain SRB that prefer nitrate over sulphate as terminal electron acceptor (Schwermer et al., 2010). Nitrate application to the aquaculture ponds was reported to stabilize the redox potential, oxidizing anaerobic zones that might lead to the production of hydrogen sulphide and other potentially toxic microbial metabolites (Avnimelech and Zohar 1986, Chainark and Boyd, 2010). Yet, despite the nitrate treatment for wastewater studies in recirculating marine aquaculture systems (Tal et al., 2009; Schwermer et al., 2010; Christianson et al., 2015), to best of our knowledge, no peer-viewed study described the direct impact and mechanism of nitrate treatment to control sulphide accumulation in traditional shrimp aquaculture ponds.

The objective of this study was to investigate the capacity and the mechanism of curative and preventive nitrate amendment, including its application together with a probiotic, to control H₂S accumulation at the shrimp pond bottom. The probiotic strain, *Bacillus licheniformis* was used for its documented use in *L. vannamei* culture and for its denitrification capacity (Kim et al., 2005; Li et al., 2007).

2. Materials and Methods:

2.1. Feed and faeces collection and storage

Shrimp faeces were collected directly from the flush outlet of shrimp tanks where *L. vannamei* at post-larvae phase were fed with CreveTec grower 2 (CreveTec, Ternat, Belgium), at the facilities of the Aquaculture and *Artemia* Reference Center, Ghent University, Belgium. For each experiment, new and fresh batches of faeces were used to avoid degradation during storage. The pH and conductivity of the faeces solution was measured directly upon arrival in the lab. Samples for total solids (TS) and volatile solids (VS) measurements were stored at 4 °C until use. The CreveTec grower 2 was also used as dosed feed, and stored at -20 °C until addition.

2.2. Experimental set-up and operation

2.2.1. Dual dose nitrate treatment

The addition of three different nitrate concentrations (50, 100, 200 mg/L NO₃⁻) as sodium nitrate (Carl Roth GmbH & Co.KG, Karlsruhe, Germany) to improve water quality in the shrimp ponds was evaluated under anaerobic conditions. Glass Schott bottles (Duran Group GmbH, Mainz, Germany) with a total volume of 500 mL, closed with airtight lids using rubber stoppers, were used for this experiment for the pond model batch set-up. The headspace of the bottles was flushed with nitrogen gas to replace air for ensuring anaerobic conditions. The presence of the shrimp and accumulated waste was represented by supplementing feed and faeces in the batch culture. Each treatment consisted of three biological replicates, including the controls (N-0) without any NO₃⁻ supplementation, and received 392 ± 1 mg feed and 12.9 mL (4% dry weight) faeces in a final volume of 300 mL artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH, USA) to achieve the final concentration of 2.87 g/L total waste as feed and faeces (Table 1). The salinity of the artificial sea water was adjusted to 20 g/L. The amount

of feed and faeces were calculated based on dry weight of the cumulative waste of 90 days of shrimp culture at a stocking density of 50 shrimp/m³ using feeding tables (Supplementary Information, Table 1). It represents a growth cycle in shrimp grow-out ponds, assuming all the waste accumulated in the lower 10 cm water column. About 25% of cumulative input feed was assumed to be accumulating in the pond bottom, with 15% considered digested feed (faeces), while 10% was uneaten feed. All treatments and the control were incubated for 66 days in a temperature-controlled room at 28 ± 1 °C without shaking.

The NO₃⁻ was supplemented on day 0 and day 14 in three different concentrations, *i.e.*, 50 (N-50), 100 (N-100) and 200 (N-200) mg/L NO₃⁻, using a sterile syringe and needle. The overhead gas pressure was measured every 2-4 days with an UMS-Tensiometer (Infield 7) device (UMS, Munchen, Germany), using a needle pierced through the rubber septum, and reported at standard temperature and pressure (STP, 273K and 101,325 Pa) conditions. Gas and liquid samples were collected through the rubber septum of all replicates. The gas samples were analysed immediately, while liquid samples were collected from the same data points, filtered over a 0.20 µm Chromafil® Xtra filter (Macherey-Nagel, PA, USA), and stored in the fridge (4 °C), prior to analysis for nitrate, nitrite and sulphate concentrations. Organic acids samples were evaluated immediately after sampling at the end of the experiment.

2.2.2. Multiple dose nitrate treatment

Three different treatments with lower concentrations of nitrate at 12.5 (N-12.5), 25 (N-25), 50 (N-50) mg/L NO₃⁻ were tested in the same anaerobic set-up with addition of the respective concentrations of NO₃⁻ every 4 days in contrast to the previous test where only dual dose applied. Each treatment consisted of three biological replicates, and was supplemented with 391 ± 1 mg feed and 13.87 mL faeces (dry weight 3.7%) in a 300 mL instant ocean solution to achieve the final concentration of 2.87 g/L total waste (see section 1.2.1). Treatments and the control were incubated for 56 days under anaerobic conditions in a temperature-controlled room at 28 ± 1 °C without shaking. Gas and liquid samples were collected and analysed in the same way as the previous test (section 2.2.1).

2.2.3. Microbially assisted nitrate treatment

For this experiment, a nitrate treatment at 50 mg/L NO₃⁻ with and without addition of *Bacillus licheniformis* spores at 10⁷CFU/mL as a probiotic strain was tested in the same set-up as section 2.2.1

and 2.2.2. *Bacillus licheniformis* was provided by INVE Aquaculture and is the strain used in the probiotic product Sanolife PRO-W. Similar to previous tests, each bottle contained 392 ± 1 mg feed and 11.34 mL faeces (4.6 % dry weight) in a total volume of 300 mL of instant ocean to achieve final concentration of 2.87 g/L total waste (same as previous tests), and was incubated under anaerobic conditions in a temperature controlled room at $28 \pm 1^\circ\text{C}$ for 44 days until the stability of the system was observed. The test was conducted in three biological replicates and included the treatment with only *Bacillus licheniformis* without NO_3^- addition (B), the treatment with only 50 mg/L NO_3^- without *B. Licheniformis* addition (N), the treatment with 50 mg/L NO_3^- and *B. Licheniformis* addition (B+N) and a control without any *B. licheniformis* or NO_3^- addition (C).

The NO_3^- was supplemented every 4 days in a similar operation to the previous test with multiple dose NO_3^- treatment. Gas and liquid samples were collected and analysed in the same way to previous tests (section 2.2.1 and 2.2.2).

2.2.4. Comparison of nitrate and sodium hydroxide treatments

To compare the impact of nitrate and base treatments on pH increase and H_2S production and to better understand the impact of pH on sulphur speciation, the same set-up that included 390 ± 1 mg feed and 13.23 mL faeces (3.9% dry weight) in a total volume of 300 mL (2.87 g/L total waste) was used. The test bottles were incubated under anaerobic conditions in a temperature controlled room at $28 \pm 1^\circ\text{C}$ for 8 days. The test was conducted in three biological replicates and included treatments with 200 mg/L NO_3^- and treatments with 1 M sodium hydroxide (NaOH) addition until pH reached above 8 (final concentration to reach target pH was 320 mg/L NaOH). The NaOH and NO_3^- treatments were supplemented only on day 5 when an accumulation of H_2S was measured. Gas and liquid samples were collected and analysed in the same way to previous tests.

2.3. Analytical techniques

The TS and VS were determined according to Standard Methods (Greenberg et al. 1992). The pH was measured with a pH meter (Methroohm, Herisau, Switzerland). The conductivity was measured with a conductivity electrode (Consort C532, Turnhout, Belgium) at the start and the end of the experiment. The headspace gas composition was analysed on a compact GC (Global Analyser Solutions,

Breda, the Netherlands). In the first channel, O₂, N₂, CH₄ and H₂ (Porabond Q pre-column and Molsieve 5A column) were measured, while in the second channel (Rt-QS-bond column and pre-column) CO₂, N₂O and H₂S were determined with a thermal conductivity detector that has a lower detection limit of 100 ppmv for each gas component. The concentrations of nitrate, nitrite and sulphate were determined with ion chromatography (930 Compact IC Flex, Metrohm, Herisau, Switzerland), equipped with a Metrosep A supp 5–150/4.0 column and conductivity detector, after diluting the samples 1:50 using ultra-pure water (Milli-Q, Millipore Corporation, Burlington, MA, USA). The detection limit was of 0.05 to 200 mg ion/L. Organic acid concentrations, *i.e.*, formate, acetate, propionate, butyrate and isobutyrate, were determined with organic acids chromatography (930 Compact IC Flex, Metrohm, Herisau, Switzerland), equipped with a Metrosep Organic acids 250/7.8 (61005200) column, inline bicarbonate removal (MCS) and an 850 IC conductivity detector with a detection limit of 0.5-2000 mg/L.

2.4. Statistical analyses

One-way analysis of variance (ANOVA) was used to determine the significant differences in water quality parameters among treatments, following the verification of homogeneity of the data (Levene's test) using IBM SPSS Statistics 25 software. If the assumption of homogeneity of the variances is found not tenable, a Welch test is used. The ANOVA and Welch tests were followed by Tukey's post-hoc comparison when significant differences were observed. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Dual dose nitrate treatment

The addition of three different NO₃⁻ concentrations (50, 100 and 200 mg NO₃⁻/L, further indicated as N-50, N-100, N-200, respectively) was evaluated in comparison to a control without any supplemented NO₃⁻ (N-0), all tested under anaerobic conditions. The NO₃⁻ addition for the respective concentrations was conducted on day 0 and 14 during the test. After the first dose of NO₃⁻, all the supplemented NO₃⁻ was consumed in the first 2 days, yet it did not prevent H₂S accumulation except delaying H₂S production for the N-200 treatment (Fig 1A and 1C). Four days after the second dose of nitrate addition (day 18), 23 ± 4 %, 53 ± 2% and 86 ± 6% less accumulated H₂S was measured for the

N50, N100 and N200 treatments, respectively, as compared to the control ($p<0.0001$). When the NO_3^- was consumed, H_2S accumulated again, and at the end of the incubation time (day 66), in the N-50, N-100 and N-200 treatments, $10 \pm 1\%$, $19 \pm 0\%$ and $33 \pm 7\%$, respectively, less accumulated H_2S was measured compared to the control. According to Tukey test, compared to the control, this difference was significant for N100 and N200 treatments ($p=0.04$ and $p<0.0001$, respectively). A similar trend was observed in CO_2 concentrations after the second dose of NO_3^- on day 14, with a decline in accumulated CO_2 . On day 18, in the N-50, N-100 and N-200 treatments, 15%, 44 $\pm$ 3% and 74 $\pm$ 9% less CO_2 was observed compared to the controls (Figure 1B). Likewise, after all NO_3^- was consumed, CO_2 started to increase again.

At the beginning of the experiment, until all NO_3^- was consumed (Figure 2A), the concentration of nitrite measured was higher than the safe limits (approximately 6 mg/L $\text{NO}_2\text{-N}$) indicated for culture of shrimp in ponds, however, the concentrations dropped after day 4 (Supplementary Information, Figure 1). Sulphate concentrations showed a decreasing profile for all the treatments during incubation. A slightly lower reduction in sulphate concentrations was measured in the N-200 treatment (Figure 1D), but at the end of the incubation time no significant difference could be reported ($p=0.220$). Although there was a H_2S decline in N-200 treatment between day 14 to 18 (Figure 1A), this change was not observed in the sulphate concentrations.

Similarly, by the end of incubation time, compared to the control, 18 $\pm$ 9%, 59 $\pm$ 4% and 80 $\pm$ 12% less total organic acids were measured in the N-50, N-100 and N-200 treatments, respectively (Table 2). A slight pH increase was observed in treatments in comparison to the controls ($p<0.0001$) (Table 2). The initial pH was 7.05 $\pm$ 0.01 for all the treatments and the controls. At the end of the incubation time, the N-200 treatment had the highest pH (7.40 $\pm$ 0.02), while the control pH remained similar to the initial pH. The conductivity of N-50 treatments and the control did not show a significant difference, but there was a slight increase for the N-100 and N-200 treatments ($p=0.017$ and $p=0.001$, respectively according to Tukey test compared to the control) (Supplementary Information, Table 2).

3.2. Multiple dose nitrate treatment

This test was conducted to overcome the limitation of NO_3^- as the electron donor, since all added NO_3^- was consumed within first 4 days in the dual dose test. Supplementation of three different NO_3^- concentrations every 4 days at lower concentrations than the previous test (N-12.5, N-25, N-50) were evaluated in comparison to a control (N-0). A curative effect was observed after 20 days with frequent supplementation of NO_3^- every 4 days (Figure 2A). Since there was no NO_3^- limitation in the treatments, by the end of the incubation time they yielded $20 \pm 0\%$, $41 \pm 9\%$ and $95 \pm 1\%$ of less H_2S in the headspace compared to the control. Hence, the treatment with 50 mg/L NO_3^- was the most effective dose to control H_2S accumulation (Figure 2A). In parallel to the dual test, the CO_2 concentrations had showed a similar trend as the H_2S concentrations (Figure 2B).

The supplemented NO_3^- was consumed in first 2 days in the beginning of the experiment (Figure 2C). This was similar to the dual dose test. The NO_3^- uptake stopped around day 52 for N-50 treatment and NO_3^- started to accumulate. Nitrite concentration remained in most cases under detection limit, but the N-50 treatment had over 6 mg/L $\text{NO}_2\text{-N}$ on day 52 (Supplementary Information, Figure 2). There was no significant difference in SO_4^{2-} concentrations between the treatments and the control ($p=0.372$).

With frequent supplementation of nitrate, the removal of organic acids was also enhanced. By the end of the incubation time, $54 \pm 9\%$, $97 \pm 2\%$ and $98 \pm 1\%$ less total organic acids were measured in the N-12.5, N-25 and N-50 treatments, respectively, compared to the control (Table 3). The rise in pH in the treatments was significantly higher ($p < 0.0001$) (Table 3). The initial pH of the treatments was 7.05 ± 0.01 . At the end of the incubation time, N-12.5, N-25 and N-50 treatments had pH of 7.69 ± 0.01 , 7.90 ± 0.06 , 8.78 ± 0.03 respectively, while the control had a pH of 7.45 ± 0.05 . The conductivity at the end of the experiment was very similar between the treatments but they were slightly larger than the control ($p=0.03$) (Supplementary Information, Table 3).

3.3. Microbially assisted nitrate treatment

A curative effect for H_2S accumulation was observed after 20 days with frequent supplementation of 50 mg/L NO_3^- every 4 days (same as section 3.2). There was no significant impact of sole *B.licheniformis* treatment to control H_2S accumulation (Tukey HSD, comparison between control and

B treatment, $p=0.836$). The 50 mg/L NO_3^- treated incubations (N and N+B treatments) yielded 3.8 ± 0.8 and 3.4 ± 0.7 mg S/L H_2S in the headspace of the treatment vials by the end of the incubation time, while controls and sole *B.licheniformis* treatment had 56.2 ± 0.8 and 57 ± 1.6 mg S/L H_2S , respectively (Figure 3A). The CO_2 concentrations followed a similar trend as the H_2S concentrations (Figure 3B).

Similar to previous experiments, the added NO_3^- in the beginning was consumed in the first 2 days, and despite the frequent NO_3^- addition every 4 days, no detectable NO_3^- accumulation was measured in the liquid samples until day 36 when the system reached the saturation of the NO_3^- uptake (Figure 3C). Sulphate concentrations did not show a significant difference ($p=0.828$) between the treatments and the control (Figure 3D).

The NO_3^- supplemented treatments decreased total organic acids accumulation to trace amounts of butyrate, while formate, acetate and propionate went below detection limit ($p<0.0001$). Initial pH was 7.41 ± 0.02 for all incubations. At the end of the incubation time, an increase in pH was measured with NO_3^- supplemented treatments when pH reached 8.09 ± 0.03 and 8.08 ± 0.03 for N and N+B treatments, respectively, while the control and only probiotic added treatments retained a pH around 7.26 ± 0.03 and 7.22 ± 0.02 (Table 4). There was no significant difference in conductivity between the treatments and the control ($p=0.276$) (Supplementary Information Table 4).

3.4. Comparison of nitrate and sodium hydroxide treatments

A short test was performed to compare the impact of a base and nitrate addition on pH change and H_2S production. On day 5, when H_2S production reached 30.1 ± 2.7 mg/L, NO_3^- and sodium hydroxide (NaOH) addition was performed (Supplementary Information, Figure 4). While the control continued producing H_2S (on day 9, 41.7 ± 0.7 mg/L H_2S), NaOH supplemented treatments showed a stable H_2S concentration (29.7 ± 0.5 mg/L), but NO_3^- supplemented treatment resulted in a significant decline, and reached 3.5 ± 1.3 mg/L H_2S ($p<0.0001$).

The initial pH was 7.02 ± 0.02 for all incubations. Just after addition of the treatments, the pH of NaOH supplemented treatment reached 8.66 ± 0.02 but for NO_3^- supplemented treatment, this value

was 7.29 ± 0.08 . At the end of the experiment, the NaOH treatment had a pH of 7.59 ± 0.01 and the NO_3^- treatment had a pH of 8.02 ± 0.08 , while controls remained at 7.25 ± 0.01 .

4. Discussion

Nitrate treatment was studied in different concentrations and treatment frequencies through three different experiments. We also evaluated the combination of nitrate with a probiotic strain. Nitrate addition was shown to be a better curative solution to control H_2S accumulation, rather than a preventive one. Nitrate amended treatments ($12.5 \text{ mg/L.day NO}_3^-$) decreased accumulated H_2S in the headspace for about 90% compared to the controls. Adding denitrifying bacterial strains for anoxic degradation of organic matter did not have an additional impact, since supplemented nitrate was quickly removed by the present microbial community.

4.1. Frequent nitrate addition could decrease accumulated H_2S in the pond as a curative solution

Denitrification occurs due to development of anaerobic/anoxic zones in the pond. The denitrification rate in a densely stocked fish pond was reported to be $0\text{--}0.05 \text{ mg NO}_3\text{-N/L.h}$ during the first 3 months after stocking, yet, later this rate increased to $0.2 \text{ mg NO}_3\text{-N/L.h}$ (thus $21.8 \text{ mg NO}_3^-/\text{L.day}$), despite the continuous surface aeration with paddlewheel aerators (Avnimelech and Zohar, 1986). In a recent study, a similar denitrification rate was reported with the fish waste as the electron acceptor, at $4.5 \text{ g N/m}^3\text{.day}$, hence, about $20 \text{ mg NO}_3^-/\text{L.day}$ in a marine recirculating aquaculture system (RAS) microcosm (He et al., 2018). In the present study, a simulation of anaerobic zones in the pond was induced through high input of organic material and replacement of headspace air with nitrogen gas.

Addition of nitrate had only a transient effect to control sulphide accumulation, since its production quickly recovered when all supplemented nitrate was removed. Similar results were obtained by Okabe et al. (2003) and Schwermer et al. (2010), where the *in situ* sulphide production quickly came back to the initial levels following NO_3^- depletion. High amounts of nitrate requirements in the anaerobic zones also confirm that in the current study, despite the frequent addition of nitrate through every 4 days pulses, was insufficient until the majority of sulphate was reduced. It might be that SRB activity continued despite nitrate addition, because although the preferential use of nitrate by SRB is possible in many

systems since nitrate reduction is thermodynamically more favourable, some species of SRB such as *Desulfovibrio desulfuricans* use sulphate over nitrate when both of the electron acceptors are present in excess (Dalsgaard and Bak, 1994; Marietou, 2009). For this metabolic preference, Sousa et al. (2017) proposed that *D. desulfuricans* activates additional pathways involving amino acid and/or protein biosynthesis to adapt to nitrate medium, whereas in the presence of sulphate the bacterium expresses predominantly the proteins required for its constitutive energy metabolism. For the current study, we speculate that the addition of nitrate did not kill SRB, but rather induced interspecies competition and after the majority of the sulphate was consumed, it enhanced the oxidation of produced H_2S as alternative electron donor.

Nitrate addition was shown to induce interspecies competition for common carbon source (*i.e.*, acetate) between SRB and heterotrophic nitrate reducing bacteria in numerous studies mainly targeting microbial corrosion problems (Eckford and Fedorak, 2002; Okabe et al., 2003; Hubert and Voordouw, 2007; Gittel et al., 2009). Apart from toxicity of organic acids to the aquatic environment (Richardson and Gangolli, 1992), these short chain fatty acids also serve as primary substrates for SRB activity. In this study, nitrate addition decreased accumulated acetate and propionate to trace amounts compared to the control. Enhancement of denitrification might have oxidized these short chain fatty acids avoiding their further uptake by SRB to produce H_2S .

Besides organic acids, the produced H_2S could also serve as electron donor for the denitrifiers. The NO_3/S^{2-} ratio might play a role on formation of intermediate and final degradation of products for the chemolithotrophic reaction (Cardoso et al., 2006). In this study, since in most cases nitrate appears to be limiting for the ratios of NO_3 consumed / H_2S consumed, it was consistent with the denitrification linked to partial oxidation of H_2S to elemental sulphur (S^0) rather than sulphate. During the decrease of accumulated H_2S , it was observed with a yellowish colour with colloidal precipitate turbidity in the liquid phase of the incubation vials. Also increasing pH in the nitrate treated samples indicated an incomplete oxidation of sulphide to S^0 rather than SO_4^{2-} (pH neutral) (Sayama et al., 2005; Kamp et al., 2006). Later, at the end of the incubation time, this colour disappeared without any significant increase in sulphate concentrations, possibly to form polysulphides.

Yet in real pond conditions, natural accumulation of waste is much slower, since the input feed gradually increases depending on the growing shrimp size. Then the formation of anaerobic zones in the pond would be also slower and should require less nitrate addition in practice. This might make preventive measures more effective, since added nitrate would be sufficient for oxidizing organic matter at least in the first stages after stocking.

4.2. Nitrate addition increases pH and might lead to sulphur speciation

Nitrate addition stimulates the denitrification process in which OH^- ions are produced in proportion to amount of nitrate reduced, thereby resulting a higher pH. This was also reported in previous fish pond studies (Pavek, 1998; Chainark, 2008). In this study, higher pH values in nitrate treated samples were recorded. Pulse dose treatments above 50 mg/L NO_3^- resulted in pH values above 8, while the controls remained around pH 7.2-7.4. These values were similar to recommended levels, since the optimum pH range for shrimp culture is 7.5 to 8.5 (Roy et al., 2011).

After H_2S accumulated and reached steady state values, the supplemented nitrate partially removed the produced H_2S through sulphur speciation, due to the increased pH resulting from denitrification. The H_2S might have been also partially removed through oxidation of produced H_2S to elemental sulphur (Cai et al., 2017). This was also confirmed by an additional test where the impact of nitrate was compared to NaOH addition after H_2S accumulation occurs. In this comparative test, NO_3^- addition showed a greater decrease in produced H_2S than NaOH addition, thus surpassing the impact of sulphur speciation through pH change.

4.3. Addition of extra denitrifying strains did not improve the water quality parameters

Bacillus species have been used in dietary supplements and for disease control in shrimp aquaculture for over decades (Hong et al., 2005; Wang, 2007; NavinChandran et al., 2014; Sha et al., 2016; Llarío et al., 2019). The use of probiotics to prevent pond bottom deterioration has been suggested in literature, but so far no high quality scientific proof is available on their potential (Martinez Cruz et al., 2012., Boyd, 2014). Due to its denitrification potential, we tested *B. licheniformis* at 10^7 CFU/mL in combination with a nitrate treatment to enhance anoxic conversion of the pond bottom waste. However, the addition of this probiotic strain did not enhance removal of accumulated H_2S or organic acids more

than the sole nitrate treatment. Since supplemented nitrate was quickly removed from the system during all of the tests, this was probably because intrinsic capacity of denitrification in this study was high enough.

It is also possible that addition of the probiotic strain in such a harsh environment with high organic material and anaerobic conditions did not work for the waste management. Addition of probiotic strains also did not cause further increase in pH apart from the impact of nitrate treatment. This also supports the study of Hostins et al. (2017) in which probiotic application did not change water quality parameters, such as pH, water temperature, dissolved oxygen, salinity or alkalinity.

5. Conclusion

Nitrate amendment in simulated shrimp aquaculture pond system showed a potential to control H₂S accumulation that poses a lethally toxic risk during the growth of the shrimp. The mechanism of the nitrate treatment was shown to work better as a curative solution to control H₂S accumulation, rather than preventive, and frequent addition to provide 12.5 mg/L.day NO₃⁻ was observed to be a better strategy. Moreover, addition of the probiotic did not show an additional impact to control H₂S accumulation than sole nitrate addition.

The results of this study can contribute to understand the capacity and mechanism of curative and preventive nitrate treatment to control H₂S accumulation in the shrimp ponds, which is currently considered a critical problem in the shrimp culture.

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 494

496 Table 1. The overview of experiments conducted

Experiment name	Amount of nitrate tested (mg/L)	Timing of nitrate addition	Other treatments used in comparison or combination	Waste input (In total volume of 300 mL instant ocean)
Dual dose nitrate treatment	50, 100, 200	On day 0 and 14	-	392±1 mg feed+12.9 mL faeces (4% TS, hence 0.516 mL)
Multiple dose nitrate treatment	12.5, 25, 50	Every 4 days	-	392±1 mg feed+13.87 mL faeces (3.7% TS, hence 0.514 mL)
Microbially assisted nitrate treatment	50	Every 4 days	<i>Bacillus licheniformis</i> at 10 ⁷ CFU/mL	392±1 mg feed+ 11.34 mL faeces (4.6 % TS, hence 0.521 mL)
Nitrate treatment in comparison with NaOH	200	On day 5	NaOH at 320 mg/L	392±1 mg feed+ 11.34 mL faeces (4.6 % TS, hence 0.521 mL)

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Table 2. The pH and concentrations of the organic acids after incubation time for the dual dose nitrate treatment. Mean values and standard deviations of the biological replicates are reported.

Treatments	pH	Formate (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Total organic acids (mM)
N-0	7.07±0.01	0.04	2.92±0.26	0.58±0.08	0.04±0.03	3.58±0.37
N-50	7.16±0.01	0.05	2.64±0.25	0.22±0.03	0.03±0.02	2.94±0.35
N-100	7.27±0.01	0.06	1.24±0.12	0.12±0.01	0.03±0.02	1.45±0.15
N-200	7.40±0.02	0.08	0.59±0.39	0.03±0.01	0.03±0.03	0.73±0.43

Table 3. The pH and concentrations of the organic acids after incubation time for the multiple dose nitrate treatment. Mean values and standard deviations of the biological replicates are reported.

Treatments	pH	Formate (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Total organic acids (mM)
N-0	7.45±0.05	0.02±0.02	2.48±0.41	0.85±0.14	0.07±0.03	3.42±0.6
N-12.5	7.69±0.01	0.08±0.05	1.29±0.22	0.16±0.03	0.05±0.01	1.58±0.31
N-25	7.90±0.01	0	0.06±0.08	0	0.04	0.1±0.08
N-50	8.78±0.03	0	0.06±0.04	0	0	0.06±0.04

Table 4. The pH and concentrations of the organic acids after incubation time for the microbially assisted nitrate treatment. Mean values and standard deviations of the biological replicates are reported.

Treatments	pH	Formate (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Total organic acids (mM)
Control	7.26±0.03	0	2.07±0.1	1.03±0.20	0.08±0.01	3.18±0.31
N	8.09±0.03	0	0	0	0.01±0.02	0.01±0.02
B	7.22±0.02	0.04±0.03	2.74±0.16	1.27±0.08	0.08	4.09±0.27
N+B	8.08±0.03	0	0	0	0.02±0.02	0.02±0.02

Figure captions

Figure 1. Concentration of H₂S (A) and CO₂ (B) in the headspaces of the vials and concentration of nitrate (NO₃-N) (C), and sulphate (SO₄) (D) in the liquid phase for the control (N0) and treatments at 50 (N-50), 100 (N-100) and 200 (N-200) mg/L NO₃⁻. Nitrate was added on day 0 and day 14. Values represent mean of biological replicates, and error bars represent standard deviation of the biological replicates. Timing of nitrate addition is indicated by * symbols.

Figure 2. Concentration of H₂S (A) and CO₂ (B) in the headspace of the vials and nitrate (NO₃-N) (C), and sulphate (SO₄) (D) in the liquid phase for the control (N0) and treatments at 12.5 (N-12.5), 25 (N-25) and 50(N-50) mg/L NO₃⁻. Nitrate was added every 4 days during 56 days of incubation. Values represent mean of biological replicates, and error bars represent standard deviation of the biological replicates. Timing of nitrate addition is indicated by * symbols.

Figure 3. Concentration of H₂S (A) and CO₂ (B) in the headspace of the vials and nitrate (NO₃-N) (C), and sulphate (SO₄) (D) in the liquid phase for the control (Control), 50 mg/L NO₃⁻ treatment (N), 10⁷ CFU/mL *Bacillus licheniformis* treatment(B) and their mixture thereof (N+B). Nitrate was added every 4 days during 44 days of incubation (indicated by *). Values represent mean of biological replicates and error bars represent standard deviation of the biological replicates.