Cold-Resistant Heterotrophic Ammonium and Nitrite-Removing Bacteria Improve Aquaculture Conditions of Rainbow Trout (*Oncorhynchus mykiss*)

Alireza Neissi¹ · Gholamreza Rafiee¹ · Hamid Farahmand¹ · Shadi Rahimi² · Ivan Mijakovic²,³

Received: 1 December 2019 / Accepted: 19 February 2020 / Published online: 11 March 2020
© The Author(s) 2020

Abstract
The aim of this study was isolation and characterization of heterotrophic bacteria capable of ammonium and nitrite removal at 15 °C (optimal temperature for growing rainbow trout *Oncorhynchus mykiss*). Environmental isolates were grown in liquid media containing ammonium or nitrite, and best strains in terms of growth and ammonium or nitrite removal were identified via 16S rRNA sequencing. Dyadobacter sp. (no. 68) and Janthinobacterium sp. (no. 100) were selected for optimal adaptation to growth at 15 °C and best ammonium and nitrite removal (*P* < 0.05), respectively. A heterotrophic ammonium and nitrite removal (HAN) microbial complex, containing selected strains, was prepared and applied in a trout culture system. After 10 days, the effect of microbial HAN complex was investigated in terms of ammonium and nitrite removal, as well as stress and immune indices present in the plasma of cultivated trout. Compared to a standard cultivation setup, addition of the HAN complex had a clear beneficial effect on keeping the un-ionized ammonia and nitrite level below prescribed standards (*P* < 0.05). This resulted in reduction of stress and immune reactions of cultivated fish (*P* < 0.05), leading to an augmentation of final weight and survival. Application of the selected microbial complex resulted in a significant improvement of the aquaculture ecosystem.

Keywords Rainbow trout (*Oncorhynchus mykiss*) · Heterotrophic bacteria · Ammonium and nitrite removal · Cold adaptation · Stress · Innate immune response

Introduction
Aquaculture associated to inland water sources is one of the most intensely growing industries, and its growth potential is limited by availability of freshwater sources worldwide [1, 2]. Reusing water is one of the solutions proposed to reduce water consumption in aquaculture. However, the water contains high levels of ammonium, which represents an obstacle for fish breeding [3–5]. Ammonia and nitrite are recognized as the major environmental stressors for fish [6, 7]. Exposure to such stressors results in physiological responses leading weakness in their immune system, which makes them more prone to infections [6, 8] and reduces overall growth and production yield [9].

Attempts have been made to operate freshwater fish farming using some level of water recycling, such as minimal water exchange or recirculating aquaculture systems (RAS) [10, 11]. Nitrification is a microbial process that could be used to reduce or eliminate unwanted nitrogen in recycled water for aquaculture [12–14], and thus make water recycling feasible on a larger scale. Nitrification can be performed in two forms, with autotrophic and heterotrophic bacteria [15, 16], and these bacteria can sometimes work in association [16, 17]. It has
been reported that autotrophic ammonium and nitrite removal occurs more rapidly in the presence of heterotrophic strains [18–23].

Rainbow trout (Oncorhynchus mykiss) is one of the major cold water species growing worldwide [24]. Trout are generally on-grown in raceways or ponds supplied with flowing water, but some are produced in cages and recirculating aquaculture systems (RAS). In these systems, biofilters based on microorganisms convert harmful components such as ammonium to nitrite and nitrate [25]. The suitable growth temperature for most aerobic heterotrophic species used in biofilters is 28 °C [26], whereas the optimal temperature for trout culture is around 15 °C [17]. Therefore, there is a considerable challenge of finding microorganisms that can grow and efficiently remove ammonium at lower temperatures required for trout aquaculture [27].

The purpose of this study was isolation and characterization of heterotrophic bacteria that remove ammonium and nitrite and can operate at lower temperatures. The aim was to apply such bacteria to rainbow trout culture systems operated at 15 °C, in order to improve environmental conditions in a rainbow trout recirculating aquaculture system and obtain higher production yields.

Materials and Methods

Sampling

Three different water sources were collected from different locations in Gothenburg, Sweden, including an artificial lake (57° 41’ 02.6” N, 11° 56’ 50.0” E) (SDL), a river (57° 41’ 49.4” N, 11° 55’ 04.5” E) (GR), and a natural lake (57° 40’ 42.4” N, 12° 03’ 25.7” E, 57° 40’ 42.4” N, 12° 03’ 25.7” E) (DL) (Supplemental Fig. 1).

Isolation of Ammonium and Nitrite Removing Bacteria

Three milliliters of water samples collected from various sources was inoculated in 47 ml of liquid medium adapted for ammonia-oxidizing bacteria (AOB) (containing 279 mg l⁻¹ ammonium [28, 29]) and 47 ml of liquid medium adapted for nitrite-oxidizing bacteria (NOB) (containing 427 mg l⁻¹ nitrite [30, 31]) and grown in 100-ml sterile flasks at 30 °C. The media were modified by addition of 330 mg l⁻¹ glucose and 60 mg l⁻¹ peptone as the carbon sources. After 2 weeks, old media were replaced with fresh ones. After 30 days, the cultures were serially diluted up to 10⁻⁸ and grown on AOB and NOB plates (containing 13 g l⁻¹ agar powder, bacteriological). Colonies potentially related to ammonium and nitrite removal were isolated and cultured separately.

Bacterial Growth and Ammonium or Nitrite Removal Activities

Colonies were grown in liquid AOB and NOB media for 5 days and then subcultured in 96-well plates at 30 °C. Absorbance at 600 nm was monitored every 30 min for 10 days in growth profiler (EnzyScreen Growth Profiler 960). For the selected colonies, ammonium and nitrite concentrations were measured using Hach spectrophotometer DR 3900 (according to the manufacturer’s protocol: LCK340 kits for nitrate assay, LCK303 for ammonium assay and LCK339 for nitrite assay).

Identification of Bacterial Strains

Genomic DNA of selected bacteria was extracted using DNeasy UltraClean Microbial Kit (Qiagen). The 16S rRNA fragments were amplified using a thermocycler (c1000 touch thermal cycler, BioRad, USA) after preparation with primstar PCR kit, using the following primers: 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-GGT TAC CTT GTT AGC CAG ACT T-3'. The size and quality of 16S rRNA fragments (expected size 1.5 kb) were checked by agarose gel electrophoresis (Supplemental Fig. 2). After a nano-drop quality assay, PCR-amplified 16S rRNA samples were sequenced (Eurofins, Germany) using the same forward and reverse primers that were used for amplification. The forward and reverse sequencing results were assembled using snap gene software and bacterial strains were identified using the blast portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed by neighbor-joining method, and reliability of each node was established by bootstrap methods using MEGA4 software.

Adaptation to Cold Temperature

Following the screening steps, the selected strains were adapted to low temperature. The strains were first grown at 30 °C for 3 days, then at RT (room temperature, 22.3 ± 2.8 °C) for 2 weeks, and finally at 15 °C for 2 weeks.

Microscopy Slide Preparing of Isolated Bacteria

1.2% agarose was completely dissolved in Tris-HCl (50 mM) by heating. The microscope slides were floated in agarose solution and then 5 µl of bacterial suspension was transferred onto the slides. The slides were covered by cover glass and observed under an optical microscope (× 1000 combined magnification).
Rainbow Trout Cultivation

The selected and adapted heterotrophic ammonium and nitrite removing bacteria (HAN) were mixed at a ratio of 2.5:1 (ammonium removing bacteria: nitrite removing bacteria) [32] at a dilution of $8.1 \times 10^9$ CFU/ml. One liter of HAN mix was transferred to a 400-l tank containing 50 rainbow trout (50.65 ± 3.89 g), at 14.5 ± 1.2 °C ($n = 3$). Negative control group tanks was selected without adding bacteria to the tanks ($n = 3$). HAN (bacterial complex) and negative control tanks were filled with water before the trout were added to each tank. Ammonium, nitrite, and nitrate concentrations were monitored for 10 days. The fish were fed once per day (1% body weight) with fish feed (CP = 38.7 ± 2.8). During the feeding period, water was not replaced, and only water evaporation (1–2% per day) was compensated. Each tank was a recirculating system with a 30 W waterproof pump at the bottom of each tank. Water from the bottom of each tank was pumped through a filter (containing washed sand, glass wool, and 2 cm$^2$ sponge particles) at the bottom of the tank and after filtration, it was returned into the tank. For aeration, a central air compressor was connected to each tank via an aquarium water hose connected to ceramic air stones (40 mm × 15 mm).

Blood Sampling

At the end of the breeding period, after 24 h of starvation, three fish from each experimental unit were randomly sampled. For this purpose, the fish were first anesthetized using benzocaine [33, 34] and then completely dried. Blood sampling from their caudal vein was performed using a 5-ml heparin syringe. Then, a part of each blood sample was centrifuged at 6000 g for 8 min to obtain plasma. The collected sera were stored at −80 °C until the desired parameters were measured. The remaining blood samples were used for investigation of hematological parameters.

Immunological and Stress Indicators

Immunological and stress indicators were measured from the obtained sera according to previously described protocols. The assays included quantification of lysozyme as described by Demers and Bayne [35], total protein as described by Nonaka, Iwaki, Nakai, Nozaki, Kaidoh, Natsuume-Sakai, and Takahashi [36], respiratory burst activity as described by Secombes [37], bacterial activity as described by Buduño, Cal, Piazzon, and Lamas [38], complementary activity assay as described by Yano [39], total immunoglobulin as described by Siwicki and Anderson [40], glucose measurement according to Pottinger and Carrick [41], and cortisol assay as described by Pickering, Pottinger, and Sumpter [42].

Hematological Parameters

Hematocrit, hemoglobin, and number of red blood cells were measured in all experimental groups. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured using the following formulas [43, 44].

\[
\text{MCV} = \frac{1000}{RBC} \\
\text{MCH} = \frac{RBC}{Hb} \\
\text{MCHC} = \frac{Hb}{Ht}
\]

Statistical Analysis

The normality of data was assessed by the Kolmogorov-Smirnov test. One-way analysis of variance (one-way ANOVA) was used for comparing data means. The significance level between treatments was determined by the Tukey test at 5% level. Statistical analysis was performed by SPSS 17 software in the Windows environment.

Ethics

Ethical permission for the research obtained from the Ethics Committee of the University of Tehran.

Results

In order to isolate bacteria that can thrive at low temperature and effectively remove ammonium and nitrite, water samples were collected from three different water sources in Gothenburg, Sweden (Supplemental Fig. 1). Freshwater sources included a river (GR), and a natural (DL) and artificial lake (SDL). After water sampling from different locations, water quality indicators were measured in all collected samples (Table 1). The results

| Indicator            | Water sources |
|----------------------|---------------|
| GR                   | SDL           | DL           |
| pH                   | 7.3           | 7.6           | 7.4           |
| Temperature °C       | 4°            | 4°            | 4°            |
| NH$_4$-N (mg l$^{-1}$) | 0.135         | 0.051         | 0.006         |
| NO$_2$-N (mg l$^{-1}$) | 0.026         | 0.024         | 0.025         |
| NO$_3$-N (mg l$^{-1}$) | 0.079         | 0.352         | 0.053         |

Artificial lake (SDL), river (GR), and a lake (DL)
showed that different water sources had different concentration of ammonium, nitrite, and nitrate. Among the samples, the DL water contained the lowest ammonium concentration (0.006 mg l\(^{-1}\)), followed by SDL (0.051 mg l\(^{-1}\)) and GR (0.135 mg l\(^{-1}\)) water. Nitrite is the most ephemeral form of nitrogen which is rapidly converted to other products. Nitrite concentrations were similar in all samples.

Isolation and Characterization of Ammonium-Removing *Dyadobacter* sp. (no. 68) and Nitrite-Removing *Janthinobacterium* sp. (no. 100)

To find a solution for accumulation of unwanted ammonium and nitrite in an aquaculture system, bacterial strains with optimal growth at 15 °C and ammonium and nitrite removal ability were isolated and identified. Among the selected isolated in this study. 

![Fig. 1](image.png)

**Fig. 1**  
[a] Growth rate of isolated heterotrophic ammonium removing bacteria for 10 days.  
[b] Ammonium removal activity of selected bacteria after 10 days.  
[c] Phylogenetic tree of ammonium removing bacteria isolated in this study.  
[d] Microscopic observation of *Dyadobacter* sp.  
[e] Ammonium removal activity of *Dyadobacter* sp. at different temperatures.
environmental strains with ammonium removal capacity (17.12–24.75% NH4-N removal), samples no. 2 (DL), 6 (SDL), 62 (GR), 68 (DL), and 117 (GR) had superior growth characteristics, with strain no. 68 being clearly the best performer in terms of growth and ammonium removal activity (Fig. 1a, b). Interestingly, strain no. 68 was isolated from the water source collected from DL region with lowest ammonium concentration. Since the use of a new strain in aquaculture
The effect of heterotrophic ammonium and nitrite removal set (HAN) on concentration of un-ionized ammonia, nitrite, nitrate, dissolved oxygen, pH, and temperature in a rainbow trout system after 9 days. Recommended limits according to Timmons and Ebeling [48]

| Indicators             | Group          | Range limit |
|------------------------|----------------|-------------|
| NH3-N μg l⁻¹           | HAN*           | 12.5        |
|                        | Control        | 10.5 ± 4.9b |
| NO2-N μg l⁻¹           | HAN*           | 1000        |
|                        | Control        | 9.85 ± 1.6b |
| NO3-N mg l⁻¹           | HAN*           | <400        |
|                        | Control        | 15.61 ± 5.36 |
| Temperature °C         | HAN*           | <16         |
|                        | Control        | 14.15 ± 1.17 |
| pH                     | HAN*           | 6.5 ± 0.17a |
|                        | Control        | 6.85 ± 0.09b |
| Dissolved oxygen (mg l⁻¹) | HAN*        | >6          |
|                        | Control        | 8.65 ± 2.12 |

The table shows values of mean ± SD of three experimental repetitions. Values within the same row with different superscript differ significantly (p < 0.05)

*Heterotrophic ammonium and nitrite removal set (HAN)

system is restricted and one should avoid strains that may infect the fish, the selected strains need to be identified prior to application. Among the isolated heterotrophic ammonium removing bacteria, strains no. 2, 6, 62, 68, and 117 were identified using 16S rRNA sequencing (Supplemental Fig. 3). Figure 1c shows the phylogenetic tree of different heterotrophic ammonium removing species that were isolated in this study. The phylogenetic tree revealed the most closely related relatives of the selected ammonium removing strains. It has shown that the closest evolutionary strain with 94.62% similarity to the best performing strain no. 68 was *Dyadobacter hamtensis* (NR 042226.1) (marked by black arrow in Fig. 1c). Microscopic observation of this strain showed that the cells aggregate in chains which can be helpful for biofloc formation in biofilters and thereby suitable for aquaculture wastewater treatment (Fig. 1d). The isolated strain of *Dyadobacter* sp. (no. 68) is not pathogenic and based on the growth profile and ammonium removal activity, it was selected as the best candidate for further analysis.

All selected environmental strains exhibited some capacity to remove nitrite, with their activity ranging from 10.57 to 49.37% nitrite removed from the medium in 10 days (Fig. 2b). Among the selected environmental strains, no. 100 exhibited the best growth and highest nitrite removal activity (Fig. 2a). The group of best performers, among heterotrophic nitrite removing bacteria, strains no. 3 (GR), 9 (SDL), 16 (GR), 84 (SDL), 100 (GR), and 154 (DL) were identified using 16S rRNA sequencing (Supplemental Fig. 4). Figure 2c shows the phylogenetic tree of all heterotrophic nitrite removing species isolated in the current study. Phylogenetic tree showed that among the selected nitrite removing strains, 84, 100, and 154 were close to *Janthinobacterium* genus which can suggest a link between the performance of this genus and nitrite removal. Furthermore, it indicated that the closest strain with 95.33% similarity to strain no. 100 was non-pathogenic *Janthinobacterium svalbardensis* (KR 085903.1) (marked by black arrow in Fig. 2c, D). Therefore, this strain was selected for further analysis as the optimal nitrite remover. It was already reported that the genus Janthinobacterium exhibited an impressive heterotrophic nitrifying efficiency with significant nitrite removal activity [45, 46]. Therefore, it can reduce nitrite to nitric oxide by the nitrite reductase proteins [47].

**Adaptation of Dyadobacter sp. and Janthinobacterium sp. to 15 °C**

Different bacterial strains may not have the ability to adapt and perform certain metabolic processes at low temperatures. On the other hand, rainbow trout is a cold water fish; therefore, the selected strains need to operate at 15 °C in order to be successfully implemented in trout aquaculture. To examine the ammonium removal activity at lower temperatures, the isolated *Dyadobacter* sp. and *Janthinobacterium* sp. were grown at RT (room temperature, 22.3 ± 2.8 °C) and 15 °C. The results showed that *Dyadobacter* sp. retained sufficient ammonium removal activity at lower temperatures (Fig. 2e). The ammonium removal activity commenced around day 6 and continued progressively to day 15. Among the strains isolated in this study, *Dyadobacter* had the highest efficiency of ammonium removal and hence, it was selected for further experiments in trout culturing system. For *Janthinobacterium* sp., the results showed nitrite removal activity at 15 °C and RT starting from day 3. From day 3 until day 9, there was no significant difference in nitrite removal between RT and 15 °C. On days 12 and 15, the highest nitrite removal activity of 11.6 and 13.25 mg l⁻¹ day was observed in RT samples, respectively. This investigation indicated that *Janthinobacterium* sp. was able to perform effective nitrite removal at both RT and 15 °C (Fig. 2e) and represents a good candidate for implementation in trout culturing systems.

---

**Table 2** The effect of heterotrophic ammonium and nitrite removal set (HAN) on concentration of un-ionized ammonia, nitrite, nitrate, dissolved oxygen, pH, and temperature in a rainbow trout system after 9 days. Recommended limits according to Timmons and Ebeling [48]

| Indicators             | Group          | Range limit |
|------------------------|----------------|-------------|
| NH3-N μg l⁻¹           | HAN*           | 12.5        |
|                        | Control        | 10.5 ± 4.9b |
| NO2-N μg l⁻¹           | HAN*           | 1000        |
|                        | Control        | 9.85 ± 1.6b |
| NO3-N mg l⁻¹           | HAN*           | <400        |
|                        | Control        | 15.61 ± 5.36 |
| Temperature °C         | HAN*           | <16         |
|                        | Control        | 14.15 ± 1.17 |
| pH                     | HAN*           | 6.5 ± 0.17a |
|                        | Control        | 6.85 ± 0.09b |
| Dissolved oxygen (mg l⁻¹) | HAN*        | >6          |
|                        | Control        | 8.65 ± 2.12 |

The table shows values of mean ± SD of three experimental repetitions. Values within the same columns with different superscript differ significantly (p < 0.05)

*Heterotrophic ammonium and nitrite removal set (HAN)
Ammonium and Nitrite Levels in Rainbow Trout Culture Were Reduced in the Presence of *Dyadobacter* sp. and *Janthinobacterium* sp.

To determine the ammonium and nitrite removal activity of selected cold-adapted bacteria in trout culture, fresh colonies of *Dyadobacter* sp. (no. 68) and *Janthinobacterium* sp. (no. 100) were used to inoculate AOB and NOB liquid medium and grown for 2 weeks, respectively. The culture of *Dyadobacter* sp. (no. 68) and *Janthinobacterium* sp. (no. 100) were applied to the trout breeding system as a mixed culture (*Dyadobacter* sp. (no. 68) to *Janthinobacterium* sp. (no. 100) ratios, 2.5:1) [32]. After 9 days, un-ionized ammonia and nitrite removal activity of the mixed culture was investigated. As expected, the results showed that the un-ionized ammonia concentration in untreated negative control group was above the recommended limits for trout culture. The results also showed that the survival rate in the HAN-treated population was higher than in the control group, and the observed differences were statistically significant (Table 3).

Environmental disturbances in aquaculture can lead to increased growth of opportunistic pathogens and decreased appetite of the fish, that negatively affect growth and survival [52, 53]. The trout growth in HAN-treated group increased compared to the control group. The results also showed that the survival rate in the HAN-treated population was higher than in the control group, and the observed differences were statistically significant (Table 3).

Fish gills are responsible for oxygen uptake, and the oxygen is subsequently transported to different fish organs. Any abnormality in fish respiratory system can affect the hematological parameters. Thus, we have investigated these parameters in HAN-treated fish compared to the control group. The results of blood indices showed that hemoglobin, MCV, MCHC%, and hematocrit were higher in the control group compared with the HAN-treated group (Table 4). Red blood cell numbers showed no significant differences between the groups. Increased un-ionized ammonia level can result in fish respiratory disorders in a fish farming environment. Therefore, hemoglobin and hematocrit increase observed in the control group might be correlated with the need to increase of oxygen carrying capacity. However, further investigation of these strains for longer treatment periods and with higher fish density will be required to test this hypothesis.

Environmental disturbances can lead to increased growth of opportunistic pathogens and decreased appetite of the fish, that negatively affect growth and survival [52, 53]. The trout growth in HAN-treated group increased compared to the control group. The results also showed that the survival rate in the HAN-treated population was higher than in the control group, and the observed differences were statistically significant (Table 3).

Environmental disturbances in aquaculture can lead to increased growth of opportunistic pathogens and decreased appetite of the fish, that negatively affect growth and survival [52, 53]. The trout growth in HAN-treated group increased compared to the control group. The results also showed that the survival rate in the HAN-treated population was higher than in the control group, and the observed differences were statistically significant (Table 3).

**Table 4** Hematological indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC)) in different rainbow trout experimental groups (HAN and control) after 9 days.

| Group | Indicators | Hemoglobin (g/dl) | Hematocrit % | RBC (10⁶ µl⁻¹) | MCV (fl) | MCHC% |
|-------|------------|------------------|-------------|----------------|----------|-------|
| Control | 63.75 ± 2.21 | 39.5 ± 1.29 | 1.24 ± 0.03 | 318.68 ± 12.59 | 51.41 ± 1.45 |
| HAN* | 59.5 ± 1.5 | 38 ± 1.81 | 1.25 ± 0.014 | 304.04 ± 8.23 | 47.60 ± 1.15 |

The table shows values of mean ± SD of three experimental repetitions. Values within the same columns with different superscript differ significantly (p < 0.05)

*Heterotrophic ammonium and nitrite removal set (HAN)

**Trout Stress Was Alleviated, and Aquaculture Productivity Increased in the HAN-Treated Group**

Environmental disturbances in aquaculture can lead to increased growth of opportunistic pathogens and decreased appetite of the fish, that negatively affect growth and survival [52, 53]. Stressors, such as ammonium and nitrite, cause systemic shocks. In this study, the groups were under stress. Our results showed that the glucose and cortisol levels in HAN-treated group were lower than the control at the end of the experiment (Fig. 3), indicating that the fish experienced less environmental stress in HAN-treated group. Growth of opportunistic pathogens also increase in stress conditions [55]. This typically triggers immune system
response. Therefore, we have investigated the innate immune response of trout in HAN-treated group compared with the control group. The results showed that the total plasma protein, total globulin, complement activity, lysozyme, respiratory burst activity, and bacterial activity in control group were lower than HAN group at the end of the experiment (Fig. 3), also corroborating the notion that HAN-treated group experienced less stress.

![Comparison of biological parameters](image)

**Fig. 3** Comparison (mean ± SD) of total protein (a), total globulin (b), complement activity (c), lysozyme (d), respiratory burst activity (e), bacterial activity (f), cortisol (g), and glucose (h) in rainbow trout sera exposed to microbial complex after 9 days. Start (starting point), control (no microbial complex), and HAN (microbial complex of *Dyadobacter sp.* + *Janthinobacterium sp.*) \( (P < 0.05) \)
In a RAS system, biological filters are usually used to reduce ammonia. Ammonia is available in two forms, as un-ionized ammonia and ionized ammonium. In terms of toxicity, the un-ionized form is important for aquatic animals [56]. As both groups of heterotrophic and autotrophic bacteria have nitrification ability [56], there is a competition for nitrification between these two groups in a RAS system. Heterotrophic bacteria win this competition for nitrification when dissolved organic carbon (DOC) to nitrogen ratio increases [56, 57]. This ratio could be increased by the activity of heterotrophic bacteria, Accumulation of dead bacteria as well as ammonia assimilation into microbial biomass [57, 58]. It has been reported that by increasing this ratio heterotrophic nitrifiers could have five times higher growth and 2–3 times higher activity compared to autotrophic bacteria [59]. In the current study, heterotrophic *Dyadobacter* sp. and *Janthinobacterium* sp. from environmental isolates were

**Discussion**

In a RAS system, biological filters are usually used to reduce ammonia. Ammonia is available in two forms, as un-ionized ammonia and ionized ammonium. In terms of toxicity, the un-ionized form is important for aquatic animals [56]. As both groups of heterotrophic and autotrophic bacteria have nitrification ability [56], there is a competition for nitrification between these two groups in a RAS system. Heterotrophic bacteria win this competition for nitrification when dissolved organic carbon (DOC) to nitrogen ratio increases [56, 57]. This ratio could be increased by the activity of heterotrophic bacteria, Accumulation of dead bacteria as well as ammonia assimilation into microbial biomass [57, 58]. It has been reported that by increasing this ratio heterotrophic nitrifiers could have five times higher growth and 2–3 times higher activity compared to autotrophic bacteria [59]. In the current study, heterotrophic *Dyadobacter* sp. and *Janthinobacterium* sp. from environmental isolates were
applied for un-ionized ammonia and nitrite removal in trout culture system, respectively.

Prior studies showed that *D. hamtensis* is lipase, maltose, gelatinase and urease negative, glucose, arabinose, galactose and xylose positive, and possesses fermentation ability [60]. The studies have also reported that this species can grow at lower temperatures [60]. It has been previously demonstrated that *Dyadobacter* species can perform denitrification [61]. For example, it was shown that *D. fermentans* and *D. soli* MJ20 are capable of ammonium consumption [62]. In nature, distinct strategies are adopted by microorganisms to cope with nitrogen starvation. *Dyadobacter* can even grow on N-deficient Burk medium due to its ability of N₂ fixation [63]. However, in our study, ammonium was the only available nitrogen source in the medium for *Dyadobacter* and it was evidently able to grow on it and remove ammonium from the ammonium-enriched medium. Our results suggest that despite a modest decrease of activity at 15 ºC, this species can effectively lower ammonium concentration in aquaculture.

The genus *Janthinobacterium* has been isolated from many different water and soil sources so far [64–66]. It has optimal growth at 15 ºC and it can also grow at temperatures as low as 2 ºC, with a pH of 6.8 [67]. This genus is glucose and oxidase-positive, but indole and lactose negative [67]. Members of this genus are known to degrade nitrite at low temperatures [46]. Several studies report antifungal and antibacterial roles of the *Janthinobacterium* genus [68–71]. These roles are very important in aquaculture systems where opportunistic pathogens are a constant threat.

Some of the major environmental pollutants in trout culture are un-ionized ammonium (ammonia) and nitrite [72]. Ammonia is produced by protein catabolism in fish and excreted from the blood through the gills [73]. A decline in growth, tissue erosion (kidney, gill, and skin) and degeneration, immune suppression and high fish mortality is related to the accumulation of large amounts of ammonium in aquatic systems [74]. The results of the current study showed that selected bacterial complex decreased ammonia in trout culture system. In treated group, decreased mortality and increased growth was achieved. Furthermore, reduced stress and immune reactions in HAN-treated trout in comparison with the control group was observed. Stressors affect the activation of hypothalamus-pituitary-interrenal axis (HPI) in different organs, which is involved in immune and stress response in different species of teleost. Studies showed that although most fishes exhibit a general stress response, the pattern and magnitude of the response may be influenced by environmental factors such as ammonium, temperature, and salinity [75, 76]. Similar to the results of our control setup, cortisol levels were previously found to be increased, and immune responses were decreased in changing or stressful conditions for aquatic species [8, 54, 72].

In the present study, the HAN culture applied to the trout culture system effectively lowered the un-ionized ammonia concentration during trout cultivation. This resulted in improved physiological conditions of trout, with decrease in activation of immune system and enhanced growth (Fig. 4). Nitrite levels were kept in check by *Janthinobacterium* sp., despite the active ammonium to nitrite conversion by *Dyadobacter* sp., and they remained under acceptable limits at all times. Nitrogen immobilization may contribute to nitrogen removal in which microorganisms assimilate inorganic nitrogen such as ammonium and nitrite for the synthesis of proteins and other nitrogen-containing organic compounds [77, 78]. This process occurs frequently when nitrogen-poor organic matter is decomposed [77, 79]. This nitrogen utilization mechanism could be also suggested as a hypothetic mechanism of nitrogen removal using these bacteria. Thus, it can be concluded that the combined use of *Dyadobacter* sp. and *Janthinobacterium* sp. can be recommended for rainbow trout culture systems, as it leads to a considerable improvement of farming conditions. Continued research is recommended by applying longer study period and higher fish density as well as performing further analysis eg additional fish growth indices (SGR, FCR) and oxidative stress analysis. Additionally, further investigation is required to study different mechanism of ammonium and nitrite removal by described heterotrophic microorganisms.

**Funding Information** This study was supported by the University of Tehran, Department of Fisheries Sciences; Chalmers University of Technology, Division of Systems & Synthetic Biology, Department of Biology and Biological Engineering and Iran National Science Foundation. This work was also supported by a grant from the Carl Tryggers Foundation (CTS17:312).

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

**References**

1. Kalbassi MR, Abdollahzadeh E, Salari-Joo H (2013) A review on aquaculture development in Iran. Ecopersia 1:159–178
2. Hoseinifar SH, Sun Y-Z, Wang A, Zhou Z (2018) Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. Front Microbiol. 9:2429
3. De Leao SR, Zaniboni-Filho E, Baldisserotto B (2009) Effect of combined non-ionized ammonia and dissolved oxygen levels on the survival of juvenile dourado, *Salminus brasiliensis* (Cuvier). J World Aquacult Soc 40:695–701
4. Thurston RV, Phillips GR, Russo RC, Hinkins SM (1981) Increased toxicity of ammonia to rainbow trout (*Salmo gairdneri*) resulting from reduced concentrations of dissolved oxygen. Can J Fish Aquat Sci 38:983–988
5. Person-Le Ruyet J, Chartois H, Quemener L (1995) Comparative acute ammonia toxicity in marine fish and plasma ammonia response. Aquaculture 136:181–194
6. Ackerman PA, Wicks BJ, Iwama GK, Randall DJ (2006) Low levels of environmental ammonia increase susceptibility to disease in Chinook salmon smolts. Physiol Biochem Zool 79:695–707
7. Lewis Jr WM, Morris DP (1986) Toxicity of nitrite to fish: a review. Trans Am Fish Soc 115:183–195
8. Neissi A, Rafiee G, Nematollahi M, Razavi SH, Maniei F (2015) Influence of supplemented diet with Pediococcus acidilactici on non-specific immunity and stress indicators in green terror (*Aequidens rivulatus*) during hypoxia. Fish Shellfish Immunol 45:13–18
9. Russell N, Evans R, Ter Steeg P, Hellemons J, Verheul A, Abee T (2013) Biofilters treating dairy wastewater via nitrification and aerobic denitrification. Bioresour Technol 126:196–202
10. Decamp O, Conquest L, Forster I, Tacon A (2002) The nutrition of rainbow trout. *Aquaculture Society: Microbial approaches to aquatic nutrition*. Elsevier, pp. 55-88
11. Verstraete W, Focht D (1977) Biochemical ecology of nitrification and denitrification. *Advances in microbial ecology*. Springer, pp. 135–214
12. Foesel BU, Gieseke A, Schwermer C, Stief P, Koch L, Cytryn E, De Batist S (2013) The culturing of biological filters. *Aquaculture Society: Microbial approaches to aquatic nutrition*. Elsevier, pp. 109–130
13. Schreier HJ, Mirzoyan N, Saito K (2010) Microbial diversity of biological filters in recirculating aquaculture systems. *Curr Opin Biotechnol* 21:318–325
14. Stephey JR, McCaig AE, Smith Z, Prosser JI, Embley TM (1996) Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. *Appl Environ Microbiol* 62:4147–4154
15. Chen J, Han Y, Wang Y, Gong B, Zhou J, Qing X (2016) Start-up and microbial communities of a simultaneous nitrogen removal system for high salinity and high nitrogen organic wastewater via heterotrophic nitrification. *Bioresour Technol* 216:196–202
16. Chen Q, Ni J (2012) Ammonium removal by Agrobacterium sp. LAD9 capable of heterotrophic nitrification–aerobic denitrification. *J Biosci Bioeng* 113:619–623
17. Qu D, Wang C, Wang Y, Zhou R, Ren H (2015) Heterotrophic nitrification and aerobic denitrification by a novel groundwater origin cold-adapted bacterium at low temperatures. *RSC Adv.* 5:5149–5157
18. Clark C, Schmidt E (1966) Effect of mixed culture on *Nitrosomonas europaea* simulated by uptake and utilization of pyrurate. *J Bacteriol* 91:367–373
19. Jones RD, Hood MA (1980) Effects of temperature, pH, salinity, and inorganic nitrogen on the rate of ammonium oxidation by nitrifiers isolated from wetland environments. *Microb Ecol* 6:339–347
20. Su J-J, Yeh K-S, Tseng P-W (2006) A strain of *Pseudomonas* sp. isolated from piggery wastewater treatment systems with heterotrophic nitrification capability in Taiwan. *Curr Microbiol* 53:77–81
21. Robertson L, Cornelisse R, De Vos P, Hadioetomo R, Kuenen J (1989) Aerobic denitrification in various heterotrophic nitrifiers. *Antonie Van Leeuwenhoek* 56:289–299
22. Verstraete W, Focht D (1977) Biochemical ecology of nitrification and denitrification. *Advances in microbial ecology*. Springer, pp. 135–214
23. Sakai K, Ikehata Y, Benaga Y, Wakayama M, Moriguchi M (1996) Nitrite oxidation by heterotrophic bacteria under various nutritional and aerobic conditions. *J Fermont Bioeng* 82:613–617
24. FAO (2011) Cultured aquatic species information programme. *Oncorhynchus mykiss* (Walbaum, 1792): Fisheries and aquaculture department
25. Cohen Y (2001) Biofiltration—the treatment of fluids by microorganisms immobilized into the filter bedding material: a review. *Bioresour Technol* 77:257–274
26. Tao W, He Y, Wang Z, Smith R, Shaya Y, Pei Y (2012) Effects of pH and temperature on coupling nitrification and anammox in biofilters treating dairy wastewater. *Ecol Eng* 47:76–82
27. Taotao Z, Dong L, Huiping Z, Shubo X, Wenzin Q, Yingjiu L, Jie Z (2015) Nitrogen removal efficiency and microbial community analysis of ANAMMox biofilter at ambient temperature. *Water Sci Technol* 71:725–733
28. Bollmann A, French E, Laanbroek HJ (2011) Isolation, cultivation, and characterization of ammonia-oxidizing bacteria and archaea adapted to low ammonium concentrations. *Methods in enzymology*. Elsevier, pp. 55-88
29. Verhagen FJ, Laanbroek HJ (1991) Competition for ammonium between nitrifying and heterotrophic bacteria in dual energy-limited chemostats. *Appl Environ Microbiol* 57:3255–3263
30. Spieck E, Lipski A (2011) Cultivation, growth physiology, and chemotaxonomy of nitrite-oxidizing bacteria. *Methods in enzymology*. Elsevier, pp. 109–130
31. Hankinson T, Schmidt E (1988) An acidophilic and a neutrophilic Nitrobacter strain isolated from the numerically predominant nitrite-oxidizing population of an acid forest soil. *Appl Environ Microbiol* 54:1536–1540
32. Li B, Irvin S, Baker K (2007) The variation of nitrifying bacterial population sizes in a sequencing batch reactor (SBR) treating low, mid, high concentrated synthetic wastewater. *J Environ Eng Sci* 6:651–663
33. Gilderhus PA, Marking LL (1987) Comparative efficacy of 16 anesthetic chemicals on rainbow trout. *Nat Am Fish Manag* 7:288–292
34. Soivio A, Nyholm K, Huhti M (1977) Effects of anaesthesia with MS 222, neutralized MS 222 and benzocaine on the blood constituents of rainbow trout, *Salmo gairdneri*. *N Am J Fish Manag* 7:288–292
35. Demers NE, Bayne CJ (1997) The immediate effects of stress on plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture 175:351–359*
36. Nonaka M, Iwaki M, Nakai C, Nozaki M, Kaidoh T, Natsuume-Sakai S, Takahashi M (1984) Purification of a major serum protein component of mammalian complement. *J Biol Chem* 259:6327–6333
37. Sekon S, Ohara H, Ohtani H, Fujita T, Matsuura Y, Tsukita S, Ibusuki T, Hara T, Takahashi M, Hirata H (1995) Expression of the mRNAs encoding the b subunit of the complement component C4 in the liver of rainbow trout. *Comp Biochem Phys A* 145:108–113
38. Demers NE, Bayne CJ (1997) The immediate effects of stress on plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Dev Comp Immunol 21:363–373*
39. Pottinger T, Carrick T (1999) A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture 175:351–363*
Cold-Resistant Heterotrophic Ammonium and Nitrite-Removing Bacteria Improve Aquaculture Conditions of... 277

Pickering A, Pottinger T, Sumpter J (1987) On the use of dexamethasone to block the pituitary-interrenal axis in the brown trout, *Salmo trutta* L. Gen Comp Endocrin 65:346–353

Campbell T, Murr F (1990) An introduction to fish hematology. Compend Contin Educ Pract Vet 12:525–532

Grant KR (2015) Fish hematology and associated disorders. Vet Clin North Am Exot Anim Pract 18:83–103

Chen Y, Jin P, Cui Z, Xu T, Zhao R, Zheng Z (2019) Identification and characterization of Janthinobacterium svalbardensis F19, a novel low-C/N-tolerant denitrifying bacterium. Appl Sci 9:1937

Yang M, Lu D, Qin B, Liu Q, Zhao Y, Liu H, Ma J (2018) Highly efficient nitrogen removal of a coldness-resistant and low nutrient needed bacterium, *Janthinobacterium* sp. M-11. Bioreour Technol 256:366–373

Cho Y-J, Jung Y-J, Hong SG, Kim O-S (2017) Complete genome sequence of a psychrotolerant denitrifying bacterium, *Janthinobacterium svalbardensis* PAMC 27463. Genome Announc 5:e01178–e01117

Timmons MB, Ebeling JM (2010) Recirculating aquaculture. Cayuga Aqua Ventures Ithaca, USA

Svobodova Z, Machova J, Poleszczuk G, Hůča J, Hamáčková J, Kroupova H (2005) Nitrite poisoning of fish in aquaculture facilities with water-recirculating systems. Acta Vet Brno 74:129–137

Verstraete W, Alexander M (1972) Heterotrophic nitrification by *Arthrobacter* sp. J Bacteriol 110:955–961

Neilands J (1967) Hydroxamic acids in nature. Science 156:1443–1447

Conte F (2004) Stress and the welfare of cultured fish. App Anim Behav Sci 86:205–223

Yavuzcan Yildiz H, Robaina L, Pirhonen J, Mente E, Dominguez D, Parisi G (2017) Fish welfare in aquaponic systems: its relation to water quality with an emphasis on feed and faeces—a review. Water 9:13

Tort L (2011) Stress and immune modulation in fish. Dev Comp Immunol 35:1366–1375

Segner H, Sundh H, Buchmann K, Douxfils J, Sundell KS, Mathieu C, Ruaane N, Jutfelt F, Toftsen H, Vaughan L (2012) Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. Fish Physiol Biochem 38:85–105

Avnimelech Y (2009) Biofloc technology: a practical guide book. World Aquaculture Society

Martínez-Córdova LR, Emerenciano M, Miranda-Baeza A, Martínez-Porchas M (2015) Microbial-based systems for aquaculture of fish and shrimp: an updated review. Rev Aqua 7:131–148

Moss S (2002) Dietary importance of microbes and detritus in penaeid shrimp aquaculture. Microbial approaches to aquatic nutrition within environmentally sound aquaculture production systems: 1–18

Gray AC (1981) Biological wastewater treatment: theory and applications. JSTOR

Chaturvedi P, Reddy G, Shivaji S (2005) *Dyadobacter hamtensis* sp. nov., from Hamta glacier, located in the Himalayas, India. Int J Syst Evol Microbiol 55:2113–2117

Allen MA (2014) Analysis of a bacterial nitrification Community in Lake Superior Enrichment Cultures. Bowling Green State University

Phophilusitthiphong Y, Vatanyoopaisarn S (2019) *Dyadobacter* and *Sphingobacterium* isolated from herbivore manure in Thailand and their cellulolytic activity in various organic waste substrates. Agric Nat Resour 53:89–98

Suyal DC, Kumar S, Yadav A, Shouche Y, Goel R (2017) Cold stress and nitrogen deficiency affected protein expression of psychrotrophic *Dyadobacter psychrophilus* B2 and *Pseudomonas jessenii* MP1. Front Microbiol 8:430

Kim SJ, Shin SC, Hong SG, Lee YM, Lee H, Lee J, Choi I-G, Park H (2012) Genome sequence of *Janthinobacterium* sp. strain PAMC 25724, isolated from alpine glacier cryoconite. Am Soc Microbiol

Shoemaker WR, Muscarella ME, Lennon JT (2015) Genome sequence of the soil bacterium *Janthinobacterium* sp. KBS0711. Genome Announce 3:e00689–e00615

Smith HJ, Foreman CM, Akiyama T, Franklin MJ, Devitt NP, Ramarat J (2016) Genome sequence of *Janthinobacterium* sp. Cg23_2, a Violacein-producing isolate from an Antarctic supraglacial stream. Genome Announce 4:e01415–e01415

Shivaji S, Ray M, Kumar GS, Reddy G, Saisree L, Wynn-Williams D (1991) Identification of *Janthinobacterium lividum* from the soils of the islands of scotia ridge and from Antarctic peninsula. Polar Biol 11:267–271

Asencio G, Lavin P, Alegría K, Domínguez M, Bello H, González-Rocha G, González-Arnava M (2014) Antibacterial activity of the Antarctic bacterium *Janthinobacterium* sp. SMN 33.6 against multi-resistant Gram-negative bacteria. Electronic J Biotech 17(1):1–5

Brucker RM, Harris RN, Schwantes CR, Gallagher TN, Flaherty DC, Lan BA, Minbiole KPC (2008) Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander plethodon cinereus. J Chem Ecol 34(11):1422–1429

Rebollar EA, Simonetti SJ, Shoemaker WR, Harris RN, Cullen D (2016) Direct and Indirect Horizontal Transmission of the Antifungal probiotic bacterium *Janthinobacterium lividum* on green frog (*Lithobates clamitans*) tadpoles. App Env Microbiol 82 (8):2457–2466

Pidot SJ, Coyne S, Kloss F, Hertweck C (2014) Antibiotics from neglected bacterial sources. Int J Med Microbiol 304(1):14–22

Liang Z, Liu R, Zhao D, Wang L, Sun M, Wang M, Song L (2016) Ammonia exposure induces oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas of pacific white shrimp (*Litopenaeus vannamei*). Fish Shellfish Immunal 54:523–528

Randall DJ, Wright PA (1987) Ammonia distribution and excretion in fish. Fish Physiol Biochem 3(3):107–120

Ding Z, Kong Y, Zhang Y, Li J, Cao F, Zhou J, Ye J (2017) Effect of feeding frequency on growth, body composition, antioxidant status and ammonia-N stress in juvenile oriental river prawn, *Macrobrachium nipponense* (L). Fish Shellfish Immunal 68:428–434

Kansarsi AR, Balasch JC, Vallesjos-Vidal E, Parra D, Reyes-López FE, Tort L (2018) Comparative immune- and stress-related transcript response induced by air exposure and *Vibrio anguillarum* bacterin in rainbow trout (*Oncorhyncus mykiss*) and gilthead seabream (*Sparus aurata*) mucosal surfaces. Front Immunol

Kansarsi AR, Parra D, Reyes-López FE, Tort L (2017) Modulatory in vitro effect of stress hormones on the cytokine response of rainbow trout and gilthead sea bream head kidney stimulated with *Vibrio anguillarum* bacterin. Fish Shellfish Immunal 70:736–749

Janssen B (1996) Nitrogen mineralization in relation to C: N ratio and decomposability of organic materials progress in Nitrogen cycling studies. Springer, pp.69–75

Mengel K (1996) Turnover of organic nitrogen in soils and its availability to crops. Plant Soil 181(1):93–93

Robertson GP, Grollman P (2007) Nitrogen transformations soil microbiology, ecology and biochemistry. Elsevier, pp.341–364