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Article type : Original Article

Application of the consortia of nitrifying archaea and bacteria for fish transportation may be beneficial for fish trading and aquaculture

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Running title: Nitrifying consortia for fish transportation

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ARE.14678

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ABSTRACT

The growing popularity of the aquarium trade is greatly increasing the demand for many ornamental fishes. While shipping technology has made the world-wide transportation of ornamental fish possible, a significant portion of the fish caught for the aquarium trade perish in transport before being sold to hobbyists. One of the major causes of fish death in transport is ammonia building up to toxic levels in the shipping bags. In order to solve this problem, we investigated the effectiveness of using nitrifying consortia in reducing the ammonia buildup in marine fish bags during transport. A pre-activated nitrifying consortium was effective in safely maintaining low ammonia levels during a three-day experiment. We found that both ammonium chloride and urea can activate nitrifying consortia. Activation of nitrifiers by urea is not only novel but beneficial due to being less harmful to fish in comparison to ammonia. We also discovered that unexpectedly one nitrifying consortium examined mainly contained ammonia-oxidizing archaea. The confirmation of the concept of the use of activated nitrifying consortia and the usefulness of nitrifying archaea for fish transportation may be beneficial for the fish trading and aquaculture.

KEYWORDS

fish packaging, ornamental fish, aquaculture, ammonia, nitrification, nitrifying consortia, ammonia-oxidizing archaea

1. INTRODUCTION

Currently, fish are the most popular target of international wildlife trade (Smith et al., 2009). The retail value of the world aquarium industry in 1995 was roughly estimated at between 4 and 15 billion US dollars with aquatic life sales alone estimated at $900 million (Ellis, 1999). The quickly growing popularity of the marine aquarium trade is increasing the demand for many reef fishes (Rhyne et al., 2012; Palmtag, 2017). Most of these reef fishes (90-95%) must be captured as they cannot be reared in captivity (Lecchini et al., 2006 and references therein; Palmtag, 2017). While the development of shipment technology has made the transportation of reef fish possible (Watson, Kilgore & Martinez, 2010), a significant portion of the fish caught for the aquarium trade perish during capture, shipment and handling before being sold to hobbyists (Wabnitz, Taylor, Green & Razak, 2003). Currently, the Philippines and Indonesia supply the majority of marine aquarium life, most are exported to the USA, Europe, and Japan (Palmtag, 2017). These fish losses during transportation are going to become more critical with the increasing demand from hobbyists in large developing nations such as China and India (Rhyne et al., 2012).

While fish packaging methods have been improved, currently, one of the major causes of fish death in transport is from ammonia concentrations building up to toxic levels in shipping bags (Lim, Dhert & Sorgeloos, 2003; Watson et al., 2010). Ammonia is a metabolic waste released primarily through the fish gills (Wright & Anderson, 2001). At high concentrations, ammonia could cause direct gill damage and stress for fish. High metabolic wastes are built after shipment because of extremely high fish loading densities (Lim et al., 2003). One solution to keep the ammonia level low is by increasing the volume of water per fish. However, the weight of the water makes fish shipment more expensive. Therefore, fish distributors must balance the cost of increased water volume and the risk of fish death due to uncontrolled ammonia production. One possible approach is the use of chemical additives such as sedatives and salt, which are widely used to aid in alleviating stress and trauma to fish in fish transportation without increasing the volume of water needed per fish. However, their negative impacts on water quality parameters, such as pH, have been reported (Watson et al., 2010). Thus, safer and robust alternative approaches are required (Lim et al., 2003).

In the present study, as a novel approach, we investigate the effectiveness of using nitrifying microbial consortia in reducing the ammonia buildup in marine fish bags during shipment. The same idea was once tested for a freshwater system (Dhanasiri, Kiron, Fernandes, Bergh & Powell, 2011). Nevertheless, no subsequent studies have been conducted, and nothing is known about marine fish shipping, which is more critical in terms of fish loss. Thus, we investigate the effectiveness of using

nitrifying consortia in reducing the ammonia buildup in marine fish bags during transport. Our data demonstrated the application of activated nitrifying consortia for fish transportation might be beneficial for fish trading and aquaculture.

2. MATERIALS AND METHODS

2.1 Fish transport bag experiments

Fish packaging experiments were conducted three times with three different marine ornamental fish species each time, which is common in the aquarium trade (**Table 1**). According to Rhyne et al. (2012), Yellowtail blue damselfish (*Chrysiptera parasema*) is ranked the sixth, Blue-green chromis (*Chromis viridis*) is listed in the first and Banggai cardinal fish (*Pterapogon kauderni*) is ranked in the tenth in marine aquarium fish imported into the USA. Banggai cardinal fish (*Pterapogon kauderni*) used were aquacultured. All fish were purchased from local retailers. Fish were maintained in 40 to 80-liter tanks with internal filters and sand. Salinity was maintained within the range of 30-37 ppt. Water quality was routinely monitored. Experiments were conducted with three replicates and monitored for three days. In each experiment, water quality parameters were monitored using a YSI Professional Plus Multi-Parameter Instrument for the measurements of temperature, dissolved oxygen, and pH. The sensor probe was inserted into the fish bag, and water was gently agitated. Fish density was adjusted to one fish per 100 ml of artificial seawater (Instant Ocean, Blacksburg, VA, USA). The fish transport bags (20 cm x 40 cm) sealed with rubber bands were stored in a polystyrene box under the dark condition, which is similar to a real small ornamental fish transportation process (Watson et al., 2010). Subsamples of water (2 to 5 ml) were taken daily from the bags for nutrient analysis. Total ammonia nitrogen (TAN) concentration was determined using the salicylate method (Hach, Loveland, CO, USA) at a wavelength of 655 nm. Nitrite concentration was determined using the Griess method at a wavelength of 545 nm (Martens-Habbena, Berube, Urakawa, de la Torre & Stahl, 2009). Nitrate concentration was determined using the cadmium method (Hach, Loveland, CO, USA) at a wavelength of 400 nm. All measurements were carried out in 10 mm path-length plastic cuvettes with 1.0 ml volume by using a Hach DR 2400 spectrophotometer. This research was conducted by following FGCU Institutional Animal Care and Use Committee protocol #1314-02.

2.2 Commercially available microbial consortia

Three commercially available nitrifying microbial products, nitrifying consortium A (One and Only, Dr.Tim's Aquatics, Moorpark, CA), consortium B (Microbe-Lift Nite Out II, Ecological Laboratories, Cape

Coral, FL, USA), and consortium C (API QuickStart, Mars Fishcare North America, Chalfont, PA, USA) were used in fish transport bag experiment 1. To test the nitrification activity of each microbial consortium, we incubated 4 ml of each product in 36 ml of artificial seawater medium containing 5 mg-N/L ammonia and 0.456 mg-P/L phosphate. The cultures were incubated in the dark at 20°C without shaking. Changes in TAN, nitrite, and nitrate concentrations were colorimetrically monitored using the spectrophotometer, as described above.

2.3 Pre-activation of the nitrifying consortium A

In experiments 2 and 3, the nitrifying consortium A was activated with urea (28 mg-N/L as final concentration) for two weeks before use. Changes in TAN, nitrite, and nitrate concentrations were colorimetrically monitored using the spectrophotometer, as described above.

2.4 Molecular characterization of microbial consortia

A portion of each product (10 ml) was filtered through 0.2 µm cellulose nitrate membrane filters (47mm diameter, ThermoScientific Nalgene Analytical Test Filter Funnels) to collect microbial biomass. Each consortium was tested as a duplicate from two individual bottles. Quarter size of the filter was cut out and inserted into a FastPrep Lysing Matrix E tubes (MP Biomedicals, Solon, OH, USA), and we carried out DNA extraction using a modified phenol-chloroform extraction method as described previously (Urakawa, Martens-Habbena & Stahl, 2010). Additionally, we extracted two more DNA samples from two aquarium biofilters from two fish tanks; biofilter sample 1 was from a Blue-green chromis tank, and biofilter sample 2 was from a Banggai cardinal fish tank. In total, eight DNA samples were sequenced using the Illumina MiSeq platform (RTL Genomics, Lubbock, Texas, USA). We used 16S rRNA primers (515F GTG CCA GCM GCC GCG GTA A and 806R GGA CTA CHV GGG TWT CTA AT), which cover the hypervariable region (V4) and can amplify both Archaea and Bacteria. Data analysis and annotation were performed as described previously (Sanchez, Vivian-Rogers & Urakawa, 2019). The genetic distances of operational taxonomic unit (OTU) centroids and reference 16S rRNA gene sequences were calculated using the Kimura's two-parameter model and visualized as neighbor-joining trees with bootstrap value supports using MEGA 7 (Kumar, Stecher & Tamura, 2016). General statistics of sequence data and clustering analysis were implemented using the PAST ver. 3.14 (Hammer, Harper & Ryan, 2001).

2.5 Chemical ammonia remover

Prior to the second fish transport bag experiment, we tested a chemical ammonia remover (Prime, Seachem Laboratories, Madison, GA, USA) to examine the ammonia removal efficiency. The chemical ammonia remover was adjusted to be six different concentrations (0%, 0.0025%, 0.0125%, 0.1%, 0.25% and 0.5% vol/vol) in 100 ml of artificial seawater, which was amended with ammonium chloride (5 mg-N/L as the final concentration) in 100 ml glass beakers (n = 3). Ammonia concentration was measured after 10 and 60 min using the spectrophotometer as described above. We used the chemical ammonia remover in the second fish transport bag experiment (0.5% as a final concentration) and the third fish transport bag experiment (0.25% as a final concentration).

2.6 Hydrophilic acrylic polymer sponge

Commercially available hydrophilic acrylic polymer sponge material (Poly-Filter, Poly-Bio-Marine, Reading, PA, USA) was used to test the potential efficiency of ammonia removal from the fish transportation bags. The dry weight of each sponge was measured before the experiment. We tested the polymer sponge in ammonia amended (4.5 mg-N/L) artificial seawater (Instant Ocean, Blacksburg, VA, USA) and compared with non-filter control. The sterilized 100 ml bottles were shaken with 25 rpm. Ammonia concentration was measured after 72 h.

2.7 Statistical analysis

General descriptive statistics were calculated for biotic and abiotic data sets using the Data Analysis Tools in Microsoft Excel. The majority of data were presented as mean ± one standard deviation unless denoted. Regression analyses were performed between two variables of interest. Additional statistical analyses (i.e., Student's *t*-test, and multiple comparison tests) and the visualization of data were implemented using Microsoft Excel and SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used for the assessment of multiple sample comparisons. The Shapiro-Wilk test was used as a normality test, and the Bonferroni test was used for a post hoc test in the one-way ANOVA unless denoted.

3. RESULTS

3.1 Fish transport bag experiment 1

Three commercially available microbial consortia were used in the first fish transport bag experiment using Yellowtail blue damselfish (*Chrysiptera parasema*) (**Table 1 and Fig. 1**). Constant production of ammonia by fish was observed, and the TAN concentration reached to 5.4 ± 0.4 mg-N/L (n = 12) after 72

h in all fish bags (**Fig. 1a**). We detected a weak but significant nitrification activity from the nitrifying consortium A (p = 0.003, n = 6, paired t-test between day 0 and 1, and day 2 and 3 pairs), but not observed from two other consortia (B and C) and the control sample (**Fig. 1b**). No significant increase or decrease of nitrate was observed in all fish bags (p = 0.641, n = 8, Wilcoxon Signed Rank Test) (**Fig. 1c**). During the experiment, we observed negligible changes in the temperature (21.1 ± 0.2°C) and salinity (31.2 ± 0.2 ppt) in all the bags (**Table 1**). Dissolved oxygen was saturated in all the bags throughout the experimental period (128.9 ± 40.7%), and no significant difference was found in the treatments (p = 0.918) (**Table 1**). The mean pH decreased from 8.1 to 7.3 on the first day for all the bags, and no significant difference was found between the treatments (p = 0.108) (**Fig. 1d**). The daily TAN removal rate calculated from the nitrite production of nitrifying consortium A was 35 µg-N/L day, which was much smaller than the ammonia produced by fish (2 mg-N/day). Therefore, we found that no nitrifying consortia efficiently worked within three days of the experimental period.

3.2 No fish culture experiment of nitrifying consortia

Since the observed nitrification activity of three microbial consortia used in the first fish transport bag experiment was not sufficient to reduce the accumulation of ammonia, we hypothesized that 72 hours were too short for the nitrifying consortia to exert their nitrification ability. To examine this hypothesis, we inoculated three nitrifying consortia into an artificial seawater medium supplemented with ammonium chloride (2 mg-N/L as final concentration) and monitored for ten days (**Fig. 2**). A removal of TAN was observed in the nitrifying consortium A, while the consortium B and C were less effective (**Fig. 2a**). This result was in accordance with our first fish bag experiment. In the consortium A, we found a clear nitrite peak at Day 3 (**Fig. 2b**), and the nitrate accumulation was observed during Day 5 to Day 7, showing that nearly all ammonia (2 mg-N) was converted into nitrate at the end of the experiment. The nitrifying consortium B and C did not show any nitrification activity in our experimental setting (**Fig. 2b and c**). Our data supported that the commercially available nitrifying consortia might require the pre-activation (i.e., pre-incubation) before use if the nitrification activity is necessary to be effective within three days.

3.3 Activation of a nitrifying consortium

We decided to activate the nitrifying consortium A prior to the fish bag experiment for 18 days (**Fig. 2d**). We tested ammonium chloride (140 mg-N/L), and 10 mM urea (280 mg-N/L) to activate ammonia-oxidizing microorganisms in the consortium A. Nitrite production patterns between urea and ammonium chloride were 2:1, which followed predicted stoichiometric patterns of urea and ammonia oxidation,

191 respectively (**Fig. 2d**). In this culture condition, the maximum nitrite production efficiency was identical
192 in these two substrates: 21.6 mg-N/L day and 22.0 mg-N/L day in ammonia and urea incubations,
193 respectively.

194 **3.4 Chemical ammonia remover**

195 Prior to the second fish transport bag experiment, we explored the potential usefulness of the chemical
196 ammonia remover to examine the ammonia removal efficiency. The chemical reaction of ammonia
197 removal instantly occurred within 10 min, and no noticeable concentration change was observed after
198 prolonged incubation (1 h). With increasing the concentration of the chemical ammonia remover, the
199 ammonia removal performance was enhanced (**Fig. 3a**). The highest concentration of water conditioner
200 (0.5%) removed almost 90% of ammonia within 10 min, suggesting the potential effectiveness of the
201 chemical ammonia remover for fish shipping (**Fig. 3a**). We used this concentration (0.5%) to remove
202 ammonia from the fish bags in the second fish bag experiment.

203 **3.5 Fish transport bag experiment 2**

204 In this second fish bag experiment, we compared the effectiveness of the pre-activated nitrifying
205 consortium A and the chemical ammonia remover using Banggai cardinalfish (**Fig. 4**). The water
206 temperature and salinity of fish bags were at 24.6 ± 1.3 °C and 33.4 ± 0.4 ppt throughout the experiment
207 (**Table 1**). Oxygen varied 83.5 to 105.5% in this experiment except for the bags containing the chemical
208 ammonia remover, in which fish mortality was observed at day 1, and the mean value of oxygen decreased
209 to 76.7 ± 16.4%. No saturated oxygen levels found in this second fish bag experiment were attributed to
210 the difference of the type of bag between this experiment (Ziploc) and the other two fish bag experiments
211 (fish transportation bag) (**Table 1**). The mean pH decreased from 8.0 to 7.1 on the first day for all the
212 bags, and no major difference was found in the treatments.

213 We observed improved strong nitrite and nitrate production patterns in the bag that used the pre-
214 activated nitrifying consortium A (**Fig. 4**). In the control bags, ammonia reached up to 8 mg-N/L (**Fig. 4a**).
215 We observed fifty percent of ammonia removal in the bag of pre-activated nitrifying consortium A.
216 Although no ammonia accumulation was detected in the bag amended with the chemical ammonia
217 remover, one fish was deceased, and another fish would have perished without intervention.
218 Subsequently, we stopped the experiment of the water conditioner after 24 h. The daily ammonia
219 removal rate was 2.5 mg-N/L day, which was a substantial improvement and more than 60 times higher
220 compared to the original consortium A used in the fish transfer bag experiment 1. Particularly after 24 h,

nearly all produced TAN was immediately converted to nitrate, suggesting the effectiveness of the pre-activation strategy of the nitrifying consortium and potential usefulness of the nitrifying consortia to fish transportation.

3.6 Ion-exchange sponge filter

Before the third fish transport bag experiment, we tested ion-exchange sponge filters in seawater (**Fig. 3b**) and freshwater (**Fig. 3c**) as an alternative approach to removing ammonia. The reduction of ammonia was detected on the first day in the seawater experiment and the first two days in the freshwater experiment, while we observed no apparent decrease of ammonia in both control experiments (**Fig. 3b & c**). Unexpectedly, a part of removed ammonia was released into the water on Day 2 and 3 in the seawater and Day 3 in the freshwater conditions.

3.7 Fish transport bag experiment 3

In this experiment, we reduced the amount of the chemical ammonia remover to minimize the chemical toxicity on fish and added an ion-exchange sponge filter as an additional approach (**Fig. 5**). No apparent changes were observed in temperature (20.6 ± 0.2 °C) and salinity (23.4 ± 0.3 ppt) in all the bags (**Table 1**). Oxygen was saturated in all the bags (190.4 ± 50.9%) throughout the experiment, and we found no difference in the treatments (**Table 1**). The average pH decreased from 8.1 to 7.4 on the first day for all the bags except for the bags of ion-exchange sponge filters in which the mean pH level was significantly higher than the control bag (p <0.001) (**Fig. 5d**).

Results showed the effectiveness of the chemical ammonia remover and nitrifying consortia, however, the effectiveness of the ion-exchange sponge filter on ammonia removal was not observed (**Fig. 5a**). The daily ammonia removal rate was 0.52 mg-N/L day, which was lower than that of fish experiment 2 (**Fig. 5a**). We attributed it as the difference in water temperature between these two experiments (24.6°C and 20.6°C in the fish experiments 2 and 3, respectively) (**Table 1**). We found a stoichiometric interaction between TAN removal and nitrite production (**Fig. 5a & b**). In spite of approximately 2 mg-N/L of ammonia was oxidized into nitrite during this experiment, the nitrate accumulation was not fit in ammonia oxidation and nitrite oxidation (**Fig. 5c**). A very similar result was obtained from the second fish bag experiment in which TAN removal and nitrite production coordinated, but surplus nitrate was produced (**Fig. 4c**). We attributed unmatched stoichiometry between ammonia oxidation and nitrate production as a carryover of trace amount of urea from the pre-incubation of the nitrifying consortium (**Fig. 2d**). As evidence, it did not occur in the first fish bag experiment in which the nitrifying consortia

were not pre-incubated with urea. In this first experiment, the stoichiometry of TAN, nitrite, and nitrate matched each other.

3.8 Molecular characterization of microbial consortia

Traditionally, nitrifying consortia are prepared from ammonia-oxidizing bacteria (AOB) species, such as *Nitrosomonas*. However, to our surprise, high-throughput sequencing of 16S rRNA gene amplicons revealed that the microbial community of consortium A and biofilters resembled each other at the phylum level and Thaumarchaeota were major ammonia-oxidizing archaea (AOA) (**Fig. 6**). AOA consisted of 27.7 ± 6.7% and 15.0 ± 8.8% of total microbial communities of the consortium A and biofilters, respectively (**Table 2**). We obtained similar microbial community profiles in a different batch of the product analyzed as duplicated samples (**Fig. 6**). All nitrifying consortia (consortium A, B, and C) contained AOB belonging to a variety of lineages in the genera *Nitrosomonas* and *Nitrosospira* (**Fig. 7**). Gammaproteobacterial AOB, such as *Nitrosococcus*, was not found in any samples. AOB consisted of 0.3 to 4.6% of total microbial communities of consortia and biofilters (**Table 2**). AOA species belonging to *Nitrosopumilus* dominated nitrifier communities of two different biofilters while *Nitrosocosmicus* dominated nitrifier communities in consortium A (**Fig. 8**). Various nitrite-oxidizing bacteria (NOB) containing *Nitrospira* (groups I, II, IV, and VI), *Nitrospina*, and *Nitrobacter* were found in the nitrifying consortia and saltwater biofilters, however, "*Candidatus* Nitrotoga" was not found (**Fig. 9**). The relative abundance of NOB population ranged between 0.1 to 1.5% of total microbial communities of nitrifying consortia and biofilters (**Table 2**).

4. DISCUSSION

In healthy fish rearing conditions, we can manage ammonia and other nitrogenous wastes with biological filtration units. However, in a sealed bag, nitrification is not functional. The resulting surge of ammonia level is a significant problem of fish transportation (Watson et al., 2010). Nitrifying bacteria and archaea are chemolithotrophs and play a vital role in the maintenance of water quality in aquarium and aquaculture settings by means of ammonia removal (Schreier, Mirzoyan & Saito, 2010). Ammonia-oxidizing bacteria and archaea oxidize ammonia and convert it into nitrite, nitrite-oxidizing bacteria convert nitrite into nitrate. These nitrifiers fix carbon dioxide as a carbon source and ammonia and nitrite as energy sources. These canonical nitrifiers require oxygen for the oxidation of ammonia and nitrite. Thus, the carbon dioxide produced by fish in the bag will be efficiently removed by nitrifiers. Because oxygen is used for the oxidation of ammonia and nitrite, the bacteria only consume molecular oxygen when ammonia or nitrite presents (Martens-Habbena et al., 2009). Thus, the nitrification activity in fish

transportation bags is regulated by the metabolism of fish. This type of reaction differs from chemical approaches. It could unlock other benefits such as modifying nitrifying consortia by mixing probiotic bacteria to antagonize the growth of fish pathogens in fish transportation bags.

Overall, we were able to demonstrate the effective use of nitrifying consortia in live-fish transport. We found that the ammonia removal efficiency differs with each nitrifying consortium. No products achieved sufficient removal of ammonia within the tested period (72 h) despite using dosages that far exceeded the manufacturers' recommendations. In our study, a pre-activated nitrifying consortium demonstrated a prominent effect to safely maintain a low TAN level within three days of experiments, although more strict microbial control techniques should be developed in the future to manage a much lower level of ammonia and nitrite. The aquarium industry uses sedatives (e.g., metomidate, benzocaine), which slow down respiration and metabolism of fish, thus decreasing the rate at which water quality deteriorates (Neiffer and Stamper, 2009; Watson et al., 2010). The proposed approach using nitrifying consortia in fish transportation could potentially replace the use of chemical tranquilizers in the future.

We found that both ammonium chloride and urea could activate nitrifying consortia. Urea is used as an alternative energy source of ammonia for a wide range of ammonia-oxidizing microorganisms (Prosser, Head & Stein, 2014; Qin et al., 2014). Because urea is less toxic for fish in comparison with ammonia (Knud-Hansen & Pautong, 1993), and many AOB and AOA species can use urea as an alternative energy source, the application of urea may be the best tactic to activate nitrifying microorganisms in aquaria and aquaculture facilities in the future.

Commercially available ion-exchange sponge filter was not likely handling the level of ammonia-based on the ammonia removal rate in our study. On the other hand, the use of the chemical ammonia remover for fish transportation seems an excellent approach due to its convenience and high efficiency. The effectiveness of sodium hydroxymethanesulfonate product for reducing TAN in a small-scale rotifer batch cultures have been reported previously (Riche, Pfeiffer & Garcia, 2006). Thus, we anticipate that commercially available chemical ammonia removers containing sodium hydroxymethanesulfonate as the main ingredient could be possibly used in a variety of aquaculture settings (Bentley, Carroll & Watanabe, 2008). Although the high concentration of the chemical ammonia remover used in this study (20 x of recommended use) may be harmful to some sensitive fish (e.g., Banggai cardinal fish), the moderate concentration of the chemical ammonia remover can sufficiently keep the ammonia level low in the fish transportation bags without causing mortality (**Fig. 5a**).

Surprisingly diverse species of nitrifying microorganisms were retrieved from nitrifying consortia and aquarium biofilters. This result is important because the difference of the AOB community was

attributed to the primary reason for the variation of TAN concentrations in the previous freshwater study (Dhanasiri et al., 2012). The composition of nitrifying microorganisms in two different aquarium biofilters was quite similar. *Nitrosopumilus* spp. were major AOA, and this observation was consistent with a previous report (Urakawa, Tajima, Numata & Tsuneda, 2008). Among AOB communities, cluster 1 *Nitrosospira*, cluster 6b *Nitrosomonas*, and *Nitrosomonas* sp. Nm143/NS20 lineages were three major AOB in accordance with previous reports (Foesel et al., 2008; Urakawa et al., 2008; Keuter, Beth, Quantz, Schulz & Spieck, 2017). It should be noted that all lineages reported here were mainly documented from marine environments (Purkhold, Wagner, Timmermann, Pommerening-Röser & Koops, 2003; Urakawa et al., 2006, 2008). Each nitrifying consortium had a unique combination of nitrifying microorganisms. In the products' instruction for use, these two nitrifying consortia (B and C) direct to use more doses for seawater than freshwater aquaria, indicating that the main nitrifiers included in these products likely prefer freshwater conditions to grow. The nitrifying consortium B mainly contained the members of cluster 7 *Nitrosomonas* as main AOB and cluster 6b *Nitrosomonas* related to *Nitrosomonas marina* (**Fig. 8**). The nitrifying consortium C contains mostly the members of cluster 7 *Nitrosomonas* and cluster 1 *Nitrosospira*. In general, cluster 7 *Nitrosomonas* are salt-tolerant terrestrial/brackish water group (Prosser et al., 2014). Especially, *Nitrosomonas mobilis* has been isolated from brackish water as well as sewage disposal plants (Prosser et al., 2014). Cluster 1 *Nitrosospira* species have only been found from marine environments, and no culture representatives are available (Prosser et al., 2014). *Nitrosomonas marina* is ubiquitous and considered as the most useful AOB in recirculating aquaculture systems (Burrell, Phalen & Hovanec, 2001; Foesel et al., 2008). The active nitrifying consortia tested in the previous freshwater study also documented that *N. marina*-like freshwater AOB was prominent in the community (Dhanasiri et al., 2011). Thus, these two products can be used to inaugurate the nitrogen cycle in a new aquarium in both marine and freshwater conditions.

Unexpectedly, major ammonia oxidizers found in consortium A were Archaea identified as the members of *Nitrosocosmicus*, which have been found in aquaculture biofilters (Bartelme, McLellan & Newton, 2017). It was the first observation in which AOA were seen as a central component of the commercially available nitrifying consortia. This consortium also contained cluster 8 *Nitrosomonas* (*Nitrosomonas nitrosa* as closest) and cluster 6a *Nitrosomonas* (*Nitrosomonas ureae* as closest). We found reasonable interaction between the function of nitrifying consortia and nitrifying microorganisms contained in the commercial products. The most robust nitrification activity was found in the nitrifying consortium comprising the highest relative abundance of nitrifiers (30.0 ± 6.1%).

Nitrospira is a diverse group of nitrite-oxidizing bacteria and among the most environmentally prevalent nitrifiers (Daims et al., 2015; Keuter et al., 2017). *Nitrospira* spp. were contained in all tested nitrifying consortia and regarded as main NOB (**Fig. 9**). Although some *Nitrospira*, which have a capability of complete oxidation of ammonia (comammox) to nitrate, were documented from a freshwater recirculating aquaculture system, comammox bacteria were not found in this study (van Kessel et al., 2015). It has been reported that comammox bacteria are more prominent in freshwater environments and can be a plausible explanation of why our study did not detect this group of nitrifying bacteria (Daims et al., 2015).

5. CONCLUSION

In conclusion, we examined the effectiveness of the utilization of commercially available nitrifying microbial consortia in reducing the ammonia buildup in marine fish bags during transportation. Pre-activated nitrifying consortia show a remarkable ability to maintain a low ammonia level for three days. We also demonstrated that nitrifying archaea could be the main component of available nitrifying consortia and was effective in removing ammonia from fish transport bags. Since oxygen is consumed for the oxidation of ammonia and nitrite, the bacteria only consume molecular oxygen when ammonia or nitrite presents. Thus, the nitrification activity in fish transportation bags is regulated by fish metabolism. This nature of reaction differs from chemical approaches. It could unlock other benefits such as modifying nitrifying consortia by mixing probiotic bacteria to antagonize the growth of fish pathogens in fish transportation bags. In the present study, we used 16S rRNA gene amplicon sequencing to characterize microbial consortia. Although the method is widely implemented, DNA-based analysis cannot discriminate active and inactive populations. Including the RNA-based sequencing approach helps identify functionally active members in the consortia. The concept of the use of activated nitrifying microbial consortia and the usefulness of AOA as the members of nitrifying consortia for fish transportation may be beneficial for fish trading and aquaculture.

Acknowledgments

We thank Mr. Joe Veradino at Joefish Aquatics for his valuable suggestions, encouragement, and aquarium donation. We also thank Dr.Tim's aquatics and Ecological Laboratories for providing their product free of charge. We appreciate Dr. Timothy Hovanec at Hovanec Consulting, and Dr. Matthew Palmtag and Dr. Gregory Tolley at Florida Gulf Coast University (FGCU) for their support and suggestions. We thank Derek Borgeson for his assistance. Finally, we gratefully appreciate the financial support from the Office of Research and Graduate Studies and the Office of Undergraduate Scholarship at FGCU, and

the Florida Sea Grant College Program with support from the National Oceanic and Atmospheric Administration, Office of Sea Grant, U.S. Department of Commerce (grant No. PD-15-2). A part of the research was also supported by the NSF Division of Environmental Biology Ecosystem Science Cluster grant DEB-1664052.

Conflict of interest

Our experimental data do not guarantee or reflect the quality of commercially available microbial consortium products used in this study under the normal usage conditions. We performed our research with the overdosage of the manufacturer's recommendation. We received nitrifying consortia as free of charge from Dr.Tim's aquatics and Ecological Laboratories.

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Data availability

The high-throughput 16S rRNA gene sequence data were deposited in the GenBank under BioProject number PRJNA598062.

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Table 1. Summary of fish transportation experiments

	Experiment 1	Experiment 2	Experiment 3
Fish	Yellowtail blue damselfish *Chrysiptera parasema*	Banggai cardinal *Pterapogon kauderni*	Green chromis *Chromis viridis*
No. of fish in a bag	4	2	3
Volume of water (ml)	400	200	300
Type of bag	Fish transportation bag	Ziploc bag	Fish transportation bag
Temperature (°C)	21.1 ± 0.2	24.6 ± 1.3	20.6 ± 0.2
Salinity (ppt)	31.2 ± 0.2	33.4 ± 0.4	23.4 ± 0.3
Dissolved oxygen (%)	128.9 ± 40.7	86.9 ± 36.9	194.0 ± 50.9
Treatment	Three different nitrifying consortia were compared.	Chemical water conditioner and the most effective activated nitrifying consortium were compared.	Chemical ammonia remover (reduced concentration), ion-exchange filter, and the most effective activated nitrifying consortium were compared.

493 Table 2. Relative abundance of nitrifying microorganisms in consortium and aquarium biofilter samples

Sample	Nitrifying microorganisms (%)	AOA (%)	AOB (%)	NOB (%)
Consortium A	30.0 ± 6.1	27.7 ± 6.7	0.8 ± 0.1	1.5 ± 0.7
Consortium B	0.4 ± 0.1	nd	0.3 ± 0.2	0.1 ± 0.1
Consortium C	4.6 ± 4.3	nd	4.6 ± 4.3	nd
Biofilters	16.7 ± 8.6	15.0 ± 8.8	0.7 ± 0.2	0.9 ± 0.1

494 Data are shown as mean and range (n = 2). nd indicates not detected.

Figure legends

Fig. 1. Fish transport bag experiment 1 showing the nitrification activity of three consortia. Data are shown as mean ± standard error (*n* = 3) of (a) TAN, (b) nitrite, (c) nitrate and (d) pH.

Fig. 2. Evaluation of a pre-incubation process in the succession of nitrogen species. The nitrification activity of three consortia was tested in an artificial seawater medium with ammonium chloride. The experiment was conducted without fish. The succession of nitrogen species is shown as (a) TAN, (b) nitrite, and (c) nitrate when ammonium chloride was supplied as a sole ammonium source. (d) collateral experimentation of nitrifying activity of consortium A when urea was supplied as a nitrogen source. All data are shown as mean ± standard error (*n* = 3).

Fig. 3. Effectiveness of the chemical ammonia remover (a) and the ion-exchange filters on TAN concentrations in seawater (b) and freshwater (c). The experiment was conducted without fish, and 4.5 mg-N/L of ammonia was added and incubated with the ion-exchange filters. Data are shown as mean ± standard error (*n* = 3).

Fig. 4. Fish transport bag experiment 2 showing the effectiveness of the consortium A and the chemical ammonia remover. Data are shown as mean ± standard error (*n* = 3) of (a) TAN, (b) nitrite, (c) nitrate and (d) pH. The chemical ammonia remover completely stopped the accumulation of ammonia but caused the mortality of fish. As a consequence, the experiment was stopped after day 1.

Fig. 5. Fish transport bag experiment 3 showing the effectiveness of the consortium A, chemical ammonia remover, and ion-exchange filter. The succession of nitrogen species and pH when ammonium chloride was supplied as a sole ammonium source. Data are shown as mean ± standard error (*n* = 3) of (a) TAN, (b) nitrite, (c) nitrate and (d) pH.

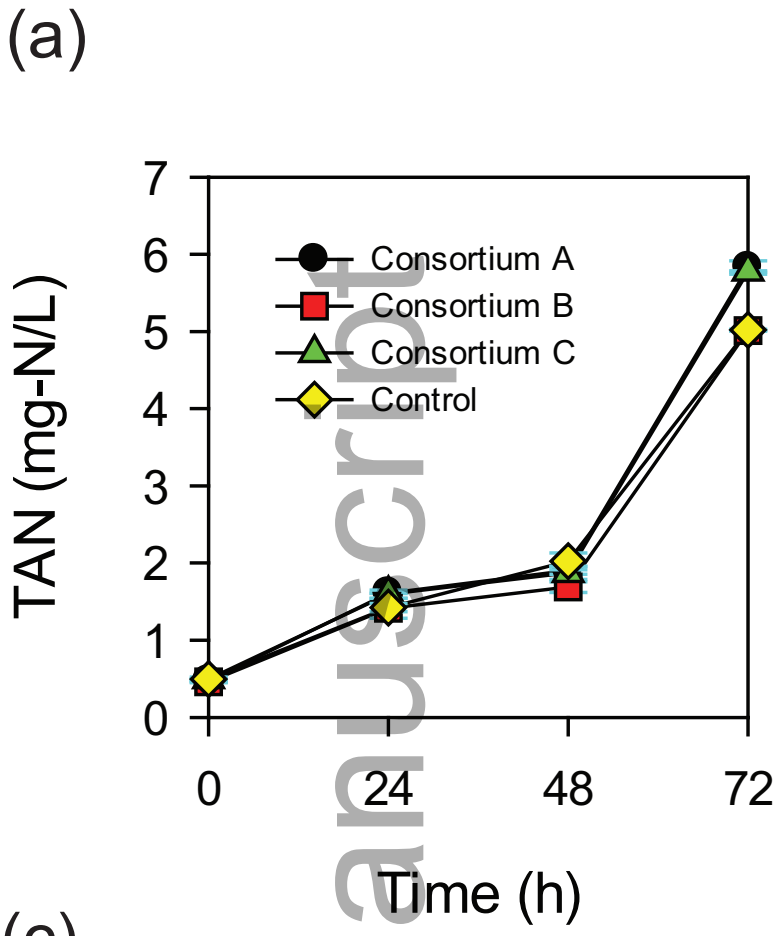
Fig. 6. Heat map of the relative abundance of sequencing reads showing microbial composition at the phylum level. Proteobacteria are shown at the class level. Intense blue colors indicate high standardized relative abundance values (row Z-scores), while green colors indicate low standardized relative abundance values. Samples and taxa were clustered using the Euclidean distance method and hierarchical clustering with the average linkage method.

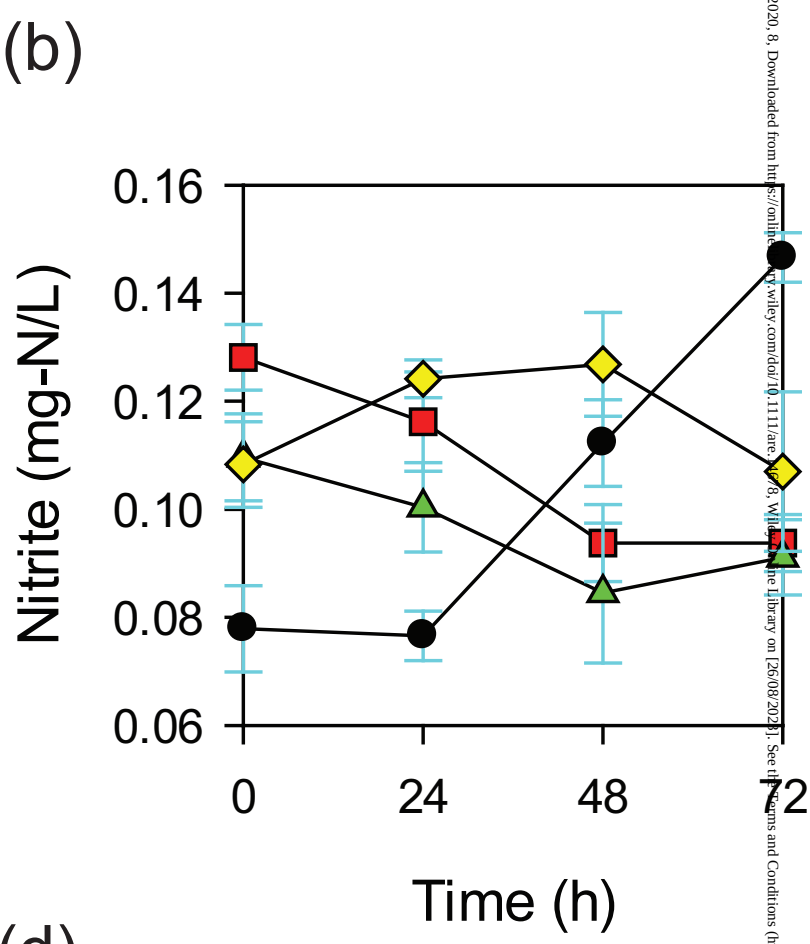
Fig. 7. Neighbor-joining tree of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. Bootstrap values (numbers next to the branches) were calculated from 1,000 iterations; values less than 50% are omitted. The scale indicates the number of substitutions per site. There were a total of 253 nucleotide

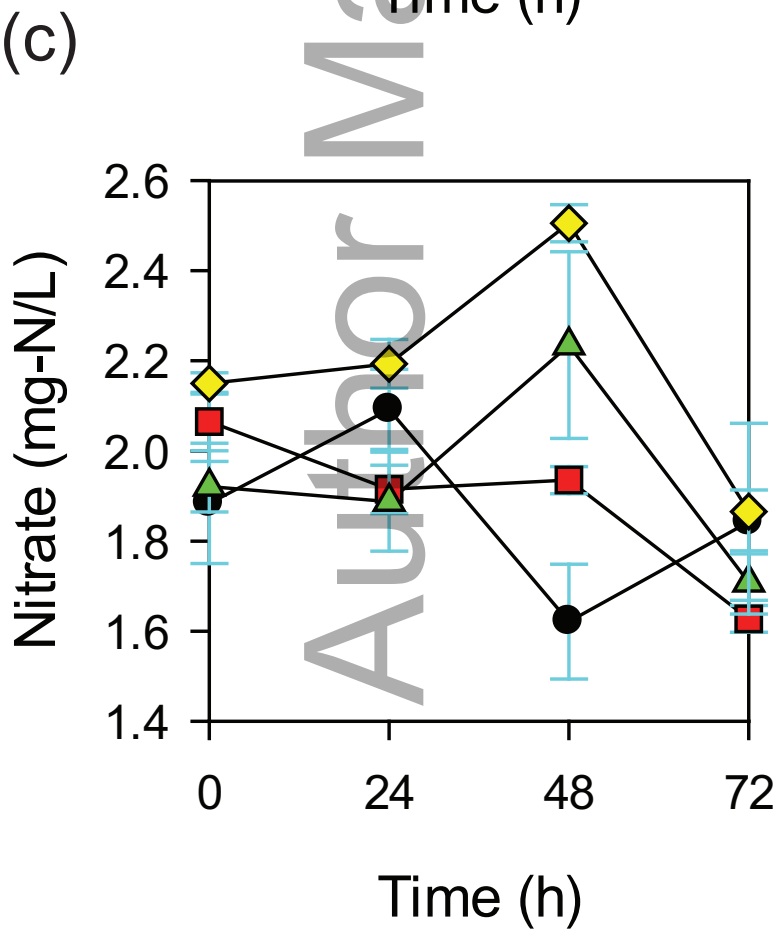
positions in the final dataset. All positions with less than 90% site coverage were eliminated. Parentheses following each OTU indicate the percentage of sequences recovered from each sample. Data are shown as the mean of duplicated samples. The OTU sources are shown as A, B, C (each consortium), and F (biofilter).

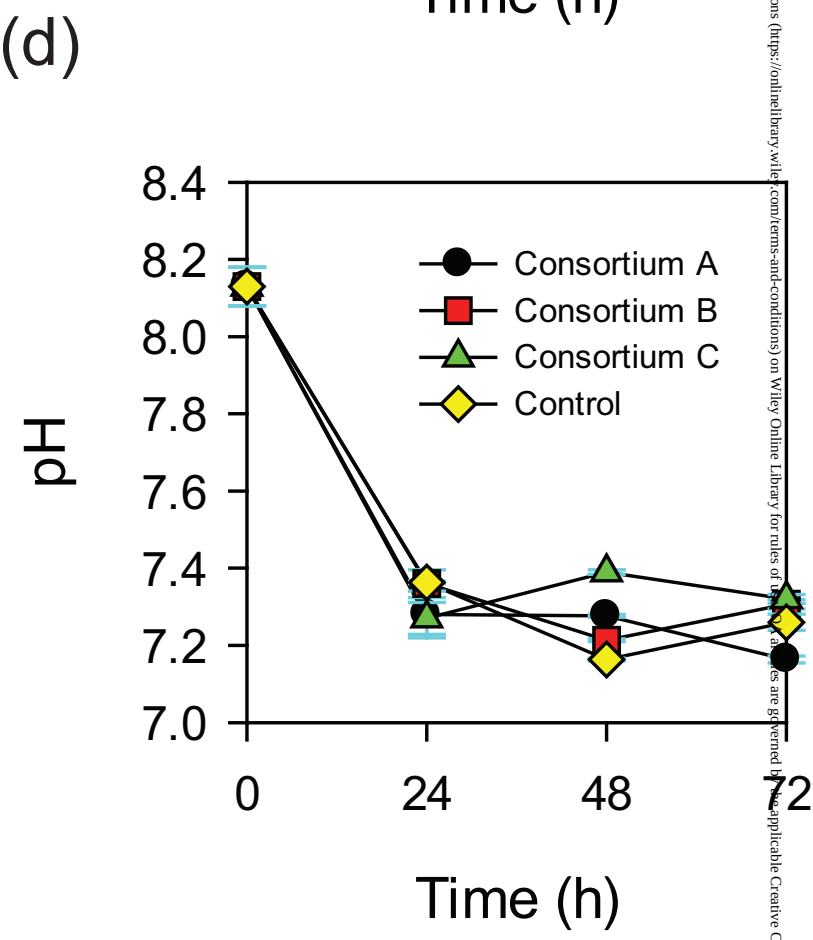
Fig. 8. Neighbor-joining tree of ammonia-oxidizing archaea based on 16S rRNA gene sequences. Bootstrap values (numbers next to the branches) were calculated from 1,000 iterations; values less than 50% are omitted. The scale indicates the number of substitutions per site. There were a total of 252 nucleotide positions in the final dataset. All positions with less than 90% site coverage were eliminated. Description of the candidatus status of some microorganisms is omitted. Parentheses following each OTU indicate the percentage of sequences recovered from each sample. Data are shown as the mean of duplicated samples. The OTUs fell into the *Nitrosocosmicus,* and *Nitrosopumilus* clades were found in the consortium A and the biofilter (F), respectively.

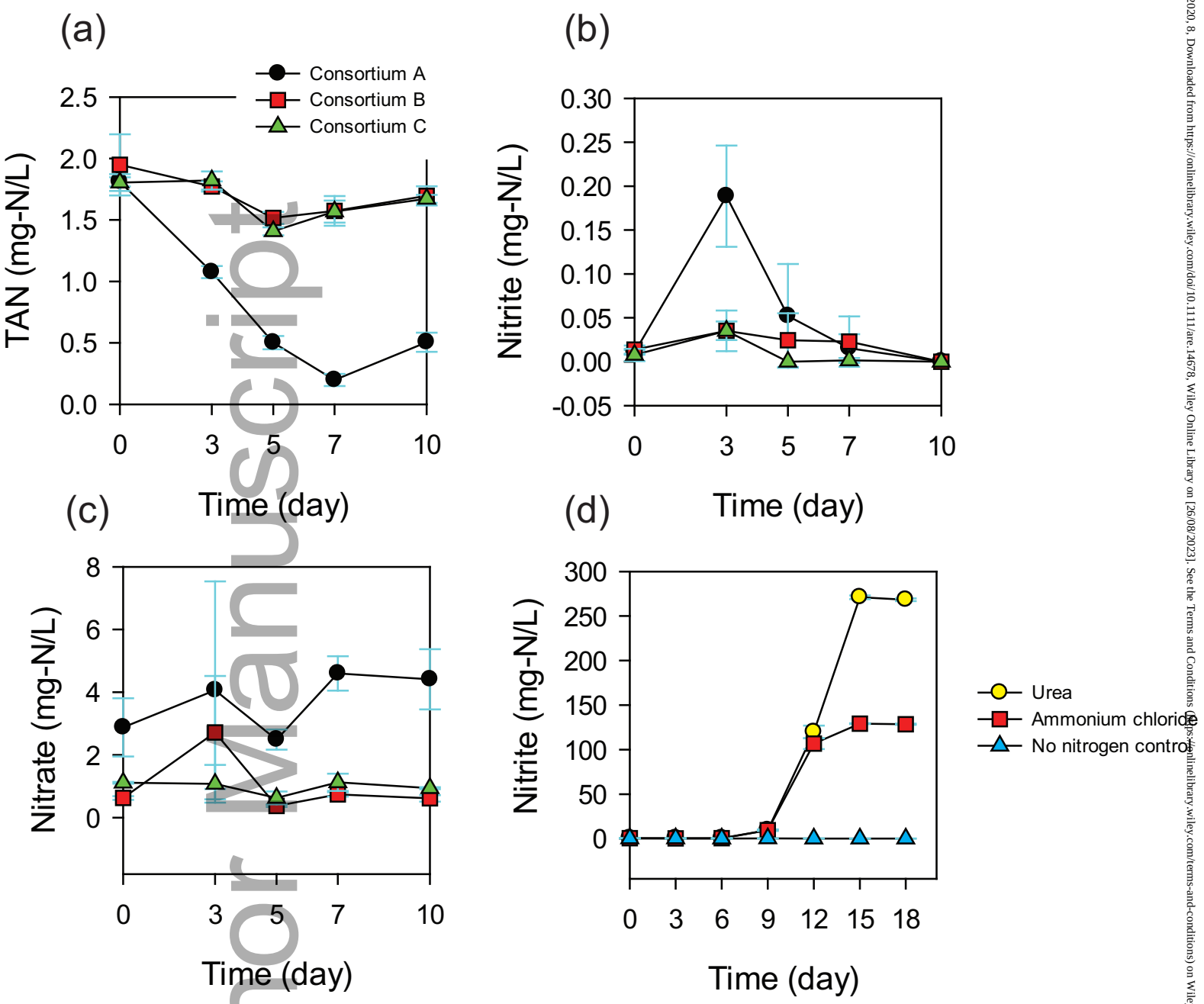
Fig. 9. Neighbor-joining tree of nitrite-oxidizing and comammox bacteria based on 16S rRNA gene sequences. Bootstrap values (numbers next to the branches) were calculated from 1,000 iterations; values less than 50% are omitted. The scale indicates the number of substitutions per site. There were a total of 253 nucleotide positions in the final dataset. All positions with less than 90% site coverage were eliminated. Description of the candidatus status of some microorganisms is omitted. Parentheses following each OTU indicate the percentage of sequences recovered from each sample. Data are shown as the mean of duplicated samples. The OTU sources are shown as A, B, C (each consortium), and F (biofilter).











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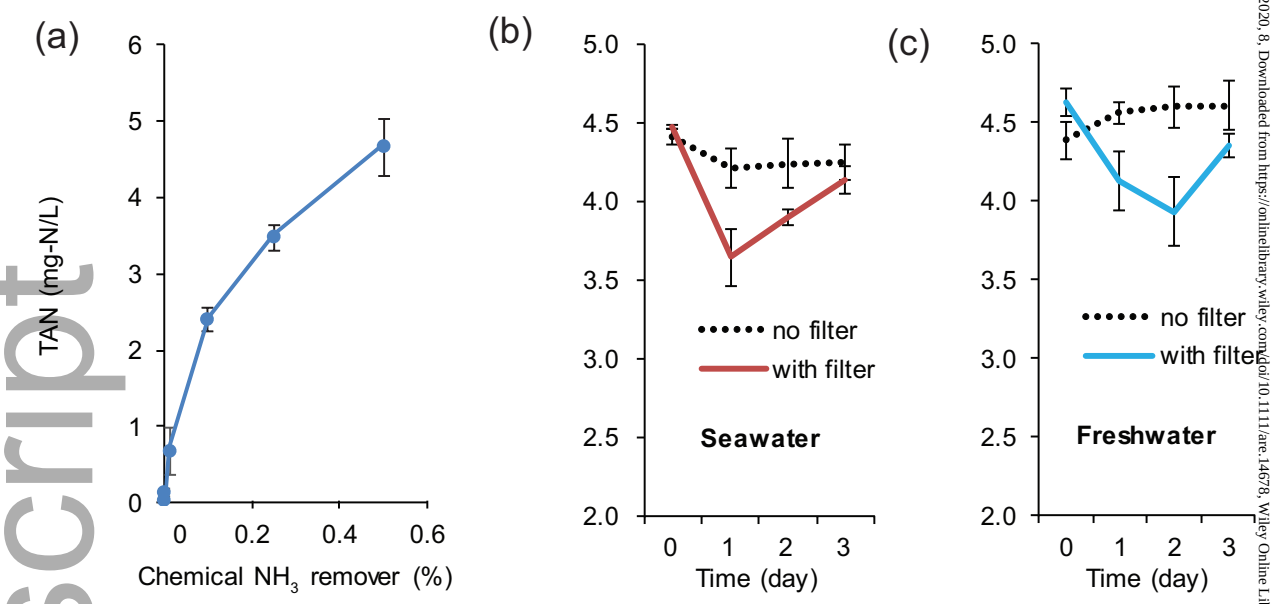

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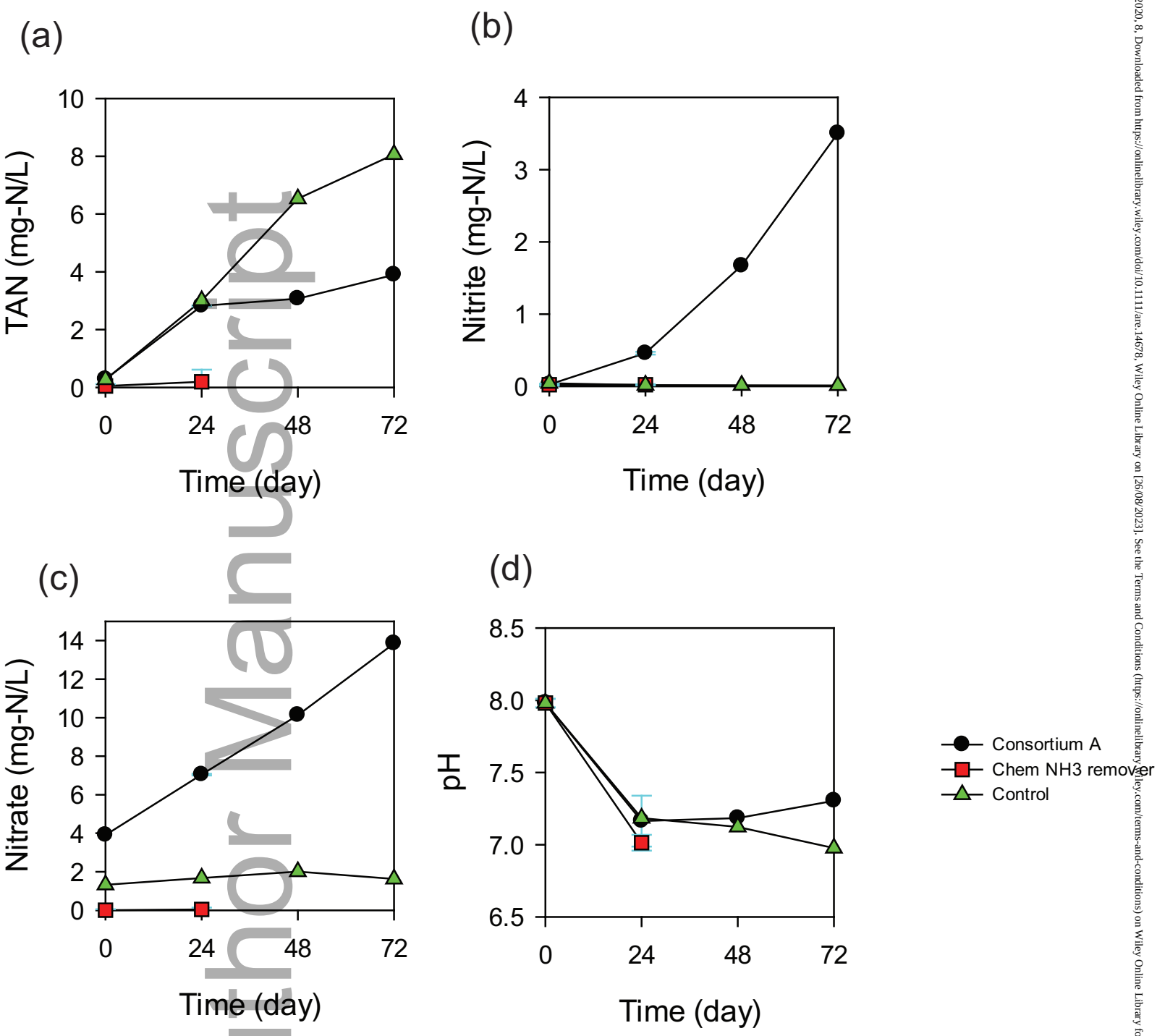

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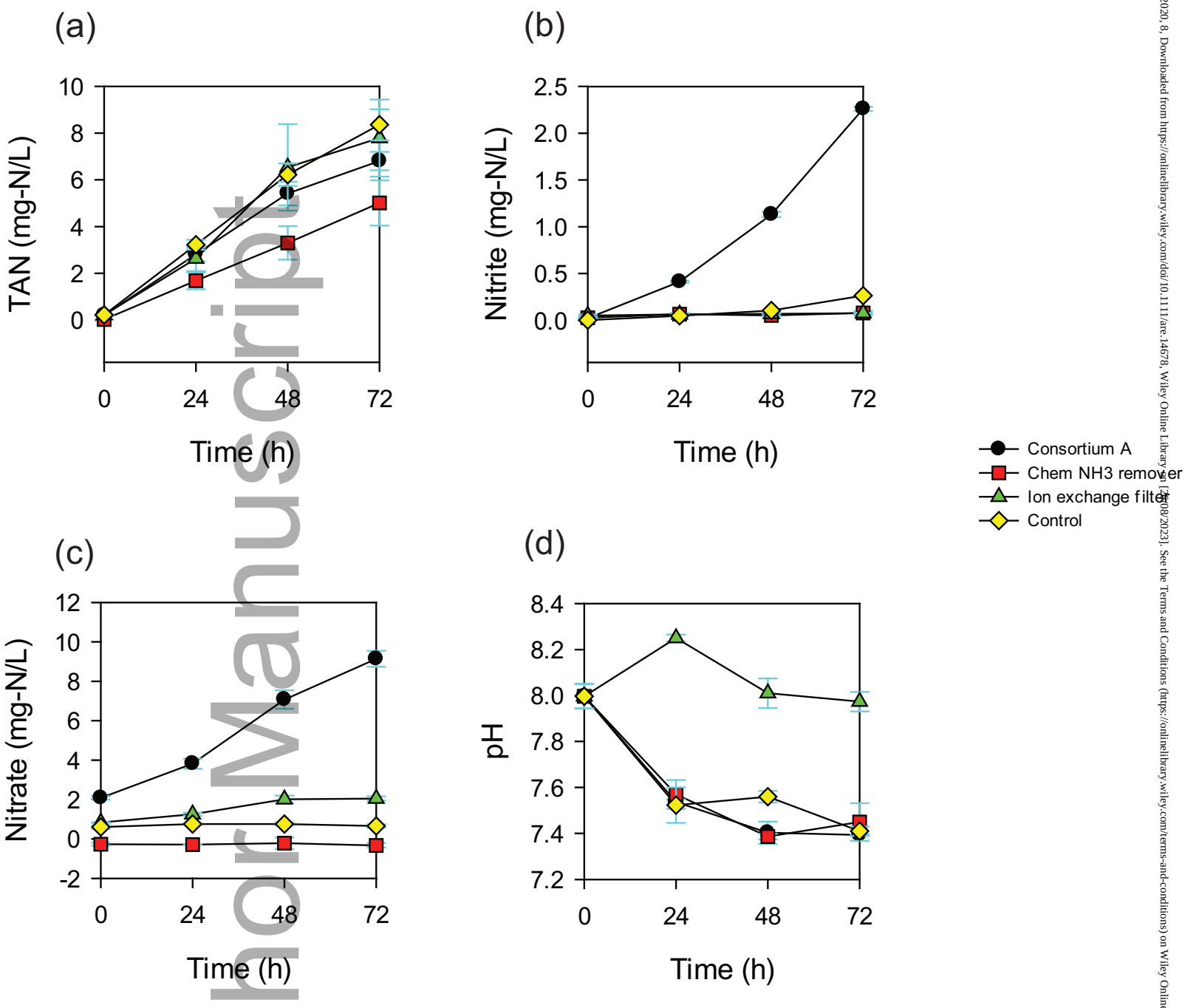

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