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# Branchial nitrogen cycle symbionts can remove ammonia in fish gills

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## Summary

**Knowledge of the mechanisms by which fish excrete their metabolic nitrogenous waste and insights into nitrogen cycling in aquaculture systems is of utmost importance to improve the sustainable commercial production of fish. In fish, most nitrogenous waste is excreted via the gills as ammonia, a potentially toxic nitrogenous compound. In this study; activity assays, physiological experiments, molecular analysis and microscopy were used to show that the gills of fish harbor a unique combination of hitherto overlooked nitrogen-cycle microorganisms that can theoretically detoxify excreted ammonia by converting it into inert dinitrogen gas. By doing so, these microorganisms may benefit from the ammonia supply by the host and prevent the build-up of this compound to toxic concentrations. This novel relationship between vertebrates and microorganisms may shed new light on nitrogen handling by ammonotelic fish species.**

## Introduction

Fish in aquaculture are usually fed protein-rich diets to maximize growth performance (Ip *et al.*, 2004; Chakraborty *et al.*, 1992), but this also leads to high levels of metabolic nitrogenous waste. Most fish are ammonotelic; nitrogenous waste is directly excreted as ammonia via specific ammonia transporters in the fish gill (Chakraborty *et al.*, 1992; Evans *et al.*, 2005; Nakada *et al.*, 2007). Ammonia is toxic to fish (Schram *et al.*, 2010) and concentrations in the gills should be kept low to prevent tissue damage.

The gills of fish harbour an own microbial community; the composition of the microbiota is substantially different from the microbial community of the water (Trust, 1975; Wang *et al.*, 2010). It was thought that these microorganisms only belong to a few taxa (Steinum *et al.*, 2009) but since the application of new sequencing techniques it appears that the gill microbiota is more complex than thought before (Lowrey *et al.*, 2015). Because of the constant supply of nitrogen, gills would be an ideal niche for N-cycle microorganisms. However, so far the presence of N-cycle microorganisms in the gills of fish has not been shown. A consortium of ammonia-oxidizing and nitrite-reducing microorganisms can theoretically detoxify excreted ammonia into harmless dinitrogen gas within the fish' gill. Symbiosis like this are only demonstrated for invertebrates, as reviewed by Dubilier *et al.* (2008).

We investigated the presence of a N-cycle bacteria consortium in two related fish species; common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*). It was shown that fish indeed produce dinitrogen gas. Ammonia oxidizing and denitrifying bacteria could be detected in gill tissue using molecular techniques (PCR and FISH) and microscopy (FISH and TEM). Altogether, the data in this study show that the gills of fish harbor an active consortium of N-cycle bacteria and teleostean fish which may shed new light on nitrogen handling by these animals.

## Results and discussion

### *Ammonia excretion by fish*

We compared the amounts of ammonia excreted by carp which were fed continuously using an automated

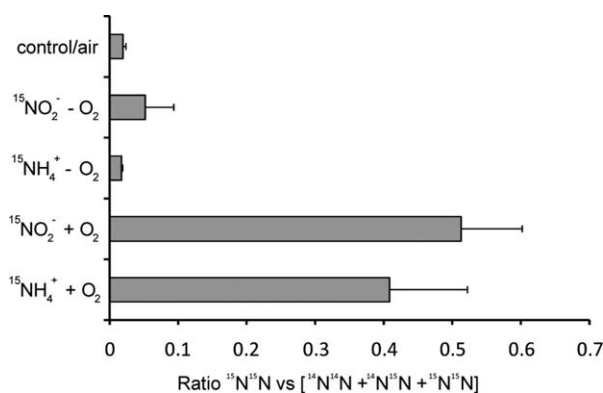
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feeding system which supplied small portions of food over the whole day (demand-fed; Klaren *et al.*, 2013) or fed once a day (hand-fed). Ammonium concentrations in tanks with demand-fed fish were relatively stable during the day (Supporting Information Fig. S1), whereas ammonium concentrations in tanks with hand-fed fish peaked 1–5 h post feeding. These data were used to calculate the ammonium excretion by these groups of fish as a percentage of total nitrogen input (based on the amount of eaten food). When corrected for the total consumption of food and nitrogen used for growth, 18% and 31% of the nitrogen intake was detected as ammonium in the water for demand-fed and hand-fed fish, respectively. This indicates that less nitrogenous waste could be traced back in the tank water for fish which were demand-fed. Production of dinitrogen gas by a microbial consortium may explain the discrepancy in ammonia excretion. As shown in Supporting Information Fig. S1, demand-fed fish are excreting ammonium over the whole day and microorganisms in the gills therefore face a constant supply of ammonium. This may result in an increased ammonia turnover by the microflora of the fish gills, explaining the difference in the amount of nitrogen traced back in the water.

#### Nitrogen gas production by microorganisms present in fish gills

In order to test if fish indeed produce nitrogen gas, fish were transferred to airtight tanks with a small air space filled with an argon/O<sub>2</sub> gas mixture (80/20%). Nitrogen gas accumulation in this headspace was followed by GC measurements. Dinitrogen gas was indeed produced by carp ( $303 \pm 113 \mu\text{mol}$  in 150 min,  $n \geq 5$ ) and zebrafish ( $1.2 \pm 0.9 \mu\text{mol}$  in 65 min,  $n = 3$ ). Unfed fish produced significantly ( $P < 0.05$ ) less nitrogen gas compared to fed fish ( $26 \pm 4 \mu\text{mol}$  in 150 min and  $0.4 \pm 0.04 \mu\text{mol}$  in 65 min for unfed carp and zebrafish, respectively), suggesting that nitrogen gas production depends at least partly on the excretion of ammonia arising from dietary protein catabolism. Exposure of zebrafish to <sup>15</sup>N-labelled ammonium via the ambient water did not lead to production of <sup>29</sup>N<sub>2</sub> or <sup>30</sup>N<sub>2</sub> showing that water-borne ammonia does not significantly contribute to nitrogen gas production.

The gills are the main excretory organs for ammonia, it was therefore hypothesized that the conversion from ammonia into dinitrogen gas occurs in the gills. To test whether dinitrogen gas was indeed produced by microorganisms associated with gills, dissected carp gills were incubated with <sup>15</sup>N-labelled ammonium or <sup>15</sup>N-labelled nitrite in the presence or absence of oxygen (Fig. 1). In the presence of oxygen, gills produced <sup>30</sup>N<sub>2</sub> from both <sup>15</sup>N-labelled ammonium and nitrite. Addition

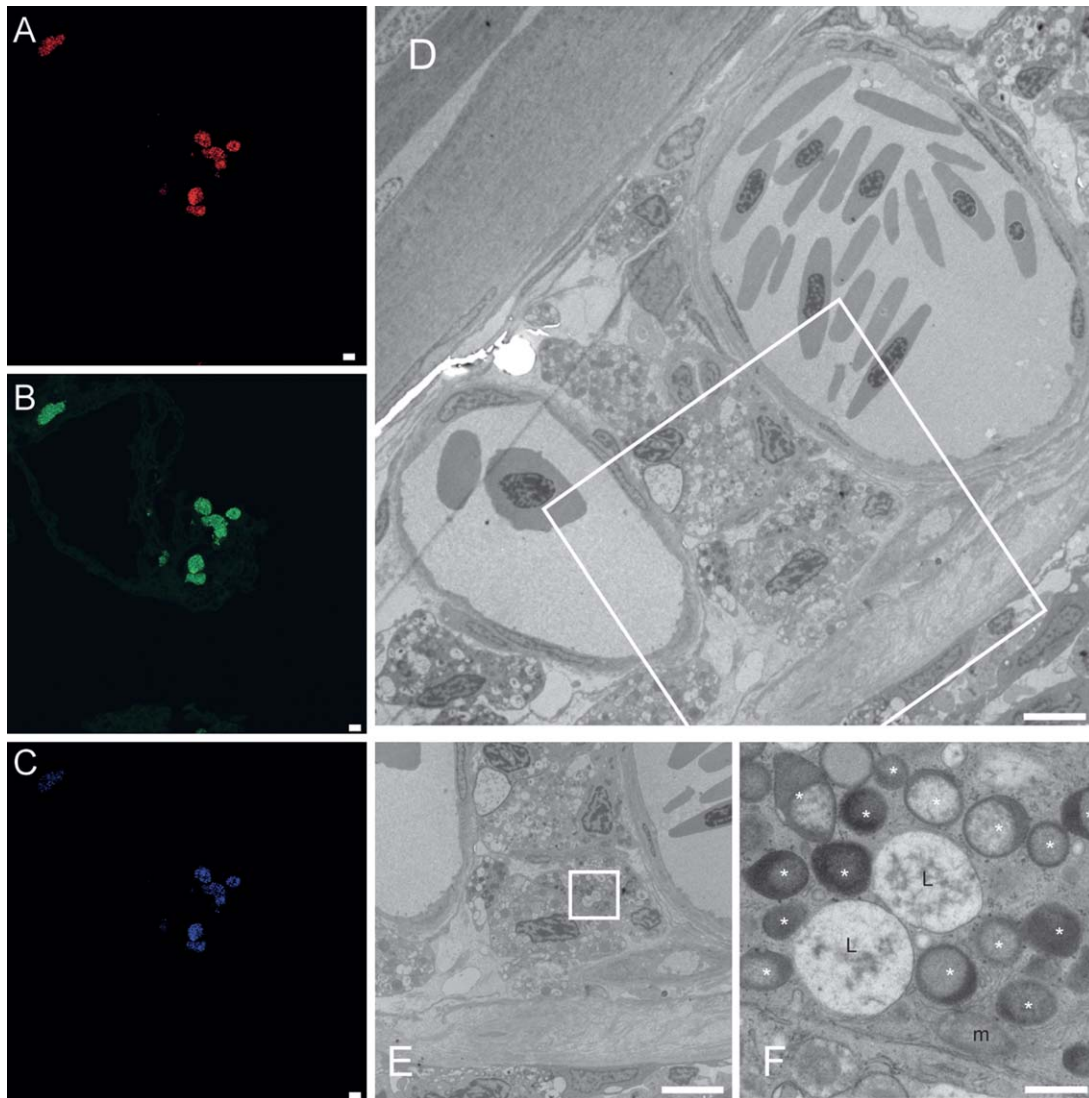


**Fig. 1.** Nitrogen gas production by dissected gills of common carp. Dissected gills were incubated for 90 min with <sup>15</sup>N-labelled ammonium or nitrite in the presence or absence of oxygen. Production of labelled nitrogen gas is shown by an increase in the ratio of <sup>30</sup>N to total nitrogen gas. Values are the mean  $\pm$  SD ( $n = 6$ ).

of <sup>15</sup>N-labelled ammonium in the absence of oxygen did not lead to labelled dinitrogen gas formation. These results suggest that dinitrogen gas is produced by the simultaneous activity of ammonia-oxidizing and denitrifying microorganisms. The first group requires oxygen to form nitrite, whereas denitrifying bacteria do not require oxygen for the reduction of nitrite to dinitrogen gas. Generally it is assumed that denitrification is inhibited by oxygen but activity of denitrifying microorganisms in multicellular hosts has been described before; also in the presence of oxygen (Schl ppy *et al.*, 2010). The execution of the experiments from this and other studies using non-vertebrate hosts were comparable to the experiments performed in this study. In our experiments, no denitrification activity could be observed within 90 min of incubation with nitrite under anoxic conditions. However, it has been shown before that aerobic denitrification sometimes has a long lag phase before conversion of nitrate or nitrite into dinitrogen gas is observed (Su *et al.*, 2015). Indeed, prolonged incubation (4 h) of the gills under anoxic conditions with nitrite eventually resulted in <sup>30</sup>N<sub>2</sub> production but since the gill structure was completely disintegrated after this incubation time, it is difficult to draw conclusions from these observations.

#### Molecular analysis of fish gills

DNA isolated from gills (van Kessel *et al.*, 2011) was used for PCR analysis with primers targeting marker genes from both ammonia-oxidizing (ammonia monooxygenase subunit A, *amoA*) and denitrifying (nitrite reductase, *nir*) microorganisms (Supporting Information Table S1). The *amoA* gene of archaea was not detected, while both *amoA* and 16S rRNA genes of *Nitrosomonas*-like ammonia-oxidizing bacteria (AOB) were



**Fig. 2.** Microscopic observations of gill tissue. Fluorescence *in situ* hybridization (FISH) on the gills of common carp (A., B., C.). A. FISH using eubmix (Eub I, II, III, labelled with Cy3) targeting all bacteria. B. FISH targeting beta-proteobacteria (bet42a + competitor, labelled with FLUOS). C. FISH targeting beta-proteobacterial ammonia-oxidizing bacteria (NSO1225, labelled with Cy5). The bacterial clusters are targeted by the probes. Many bacteria within the clusters are ammonia-oxidizing bacteria. Scale bar represents 10  $\mu\text{m}$ . Transmission electron microscopy on gills of common carp (D., E., F.). D. Clusters of bacteria are found close to blood vessels within the carp gill. E. Bacteria reside in host cells. F. Magnification of the bacterial cells in the carp gills. Scale bars represent 5  $\mu\text{m}$ , 5  $\mu\text{m}$  and 500 nm for panels D, E, and F, respectively.

retrieved (Supporting Information Fig. S2). The same analysis was performed using water samples from the aquaculture units in which the fish were kept. The prevailing *Nitrosomonas* species in the water was phylogenetically different from the species associated with the gills. Nitrite reductases (*nirS* and *nirK*) were used as marker genes for denitrifying bacteria. Only PCRs targeting the *nirS* gene yielded a product (Supporting Information Fig. S3). Organisms containing these *nirS* sequences have been shown before in gills of different fish species (Wang *et al.*, 2010; Steinum *et al.*, 2009). However, it has not been shown so far that these bacteria are indeed able to denitrify in the fish gills.

#### Microscopy on fish gills

Fluorescence *in situ* hybridization (FISH, Supporting Information Table S2) and transmission electron microscopy (TEM) were applied to carp gills to study the microbial consortium in more detail. Carp gills harbour clusters of bacteria in close proximity to blood vessels (Supporting Information Fig. S4). These bacterial clusters hybridized with specific probes against *Nitrosomonas* species (Fig. 2). Not all bacteria targeted by the betaproteobacterial probe were targeted by the *Nitrosomonas*-specific probe. The presence of other betaproteobacterial species in the gills is in line with the results

obtained with the molecular analysis targeting the *nirS*; sequences obtained were also belonging to betaproteobacterial species. The bacterial clusters were localized intracellularly (Fig. 2), which is similar to mussel symbionts (Duperron *et al.*, 2005). Intracellular bacteria in fish gills are known, but these bacteria are surrounded by double membranes (Toenshoff *et al.*, 2012). The bacteria in our study were surrounded by a single membrane. Furthermore, fish having intracellular bacteria suffer from tissue reactions (Toenshoff *et al.*, 2012). Our carp were healthy and no signs of tissue damage or disturbances were observed, indicating that the intracellular bacteria that were observed in our study are most likely not pathogenic.

## Conclusions

In conclusion, these results show that gills of carp and zebrafish harbour several N-cycle bacteria (ammonia oxidizers and denitrifiers) in their gills. According to the activity assays performed in this study, ammonia excreted by fish is directly converted into dinitrogen gas by this microbial consortium. This may be beneficial to many ammonotelic fish because it can make the gills less vulnerable to high ammonia concentrations, but this has to be investigated in more detail. By performing more animal experiments using fish fed nitrogen-poor food, the influence of the amount of ammonia that is available to the bacteria can be investigated. Furthermore, the microbial population in the gills of ammonotelic and ureotelic fish can be compared. Ureotelic fish excrete most of their nitrogenous waste as urea instead of ammonia and are therefore less vulnerable to toxic concentrations of ammonia in their gills. This is the first study which indicates that the microflora of fish gills harbours bacteria which might be beneficial for the fish and this can be of great importance for our knowledge about nitrogenous waste excretion in fish.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

#### Methods.

**Table S1.** Primer specifications.

**Table S2.** Probe specifications.

**Fig. S1.** Ambient ammonium concentrations under three different feeding regimes of carp. Two groups of fish are hand fed once a day (10:00 AM and 10:00 PM, closed squares and triangles respectively), resulting in a peak in ammonium excretion starting 1 hour after feeding. Ammonium concentration in the water is back at base line level 10 hours after feeding in both groups. Carp from the demand fed group eat continuously between 5:00 AM and the end of the experiment (registered meal requests in the upper part of the graph) resulting in a stable ammonium concentration between 9:00 AM and the end of the experiment. Water exchange, recirculation and biofilter activity was exactly same in all three tanks; therefore differences in total ammonium excretion are therefore only related to the feeding regime. Based on ammonium concentration in the water, food consumption and growth it could be calculated

that demand fed fish excrete less nitrogenous waste in the form of ammonium compared to fish which were hand fed only once a day. Under these same conditions 18% of the nitrogen input in demand fed fish was traced back in the water as ammonium whereas this was  $31 \pm 2\%$  for fish that were fed once a day.

**Fig. S2.** Phylogenetic relationship of the AmoA protein sequences obtained from the gills (zebrafish and carp) and from aquaculture water. The tree was calculated using the Neighbour-joining algorithm with Kimura 2-parameter correction. Bootstrap values of 1000 replicates are shown at the nodes. The scale bar represents 0.1 amino acid changes per position. The closely related methane monooxygenase subunit PmoA from *Methylocystis* sp. m261 is used as an outgroup.

**Fig. S3.** Phylogenetic relationship of the *nirS* gene sequences obtained from carp gills. The tree was calculated using the Neighbour-joining algorithm with Kimura 2-parameter correction. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. There were a total of 373 positions in the final dataset. Bootstrap values of 500 replicates are shown at the nodes. The scale bar represents 0.05 nucleotide changes per position.

**Fig. S4.** Fluorescence in situ hybridization (FISH) on gills of common carp. **A.** Phase contrast image with visible bacterial clusters (arrows) surrounding a blood vessel (\*). **B.** FISH using eubmix (Eub I, II, III, labelled with Cy3) targeting all bacteria. Scale bar represents 10  $\mu\text{m}$ .