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Comparison of the gill and gut microbiomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) and their RAS environment

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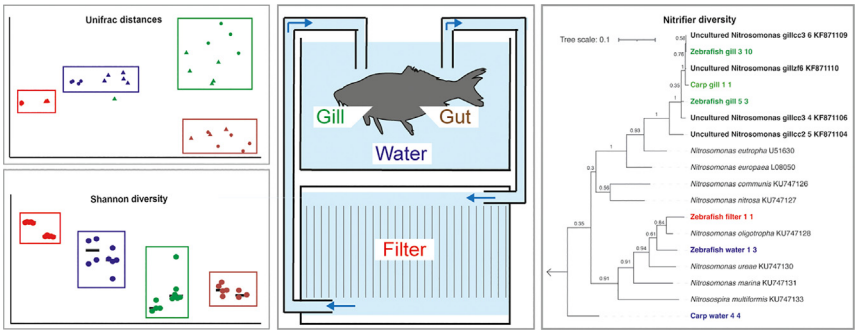
HIGHLIGHTS

- RAS compartment and organ-specific characteristics shape the structure of fish mucosal microbiomes.
- Fish-associated microbiomes are distinct from communities in the surrounding RAS compartments.
- A small core of highly abundant species shared between species constitutes the gill microbiome.
- Gills of zebrafish and carp contain *Nitrosomonas* spp. distinct from RAS water and biofilter.

GRAPHICAL ABSTRACT



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ABSTRACT

Recirculating aquaculture systems (RAS) are increasingly being used to grow fish, as intensive water reuse reduces water consumption and environmental impact. RAS use biofilters containing nitrogen-cycling microorganisms that remove ammonia from the aquaculture water. Knowledge of how RAS microbial communities relate to the fish-associated microbiome is limited, as is knowledge of fish-associated microbiota in general. Recently, nitrogen-cycling bacteria have been discovered in zebrafish and carp gills and shown to detoxify ammonia in a manner similar to the RAS biofilter. Here, we compared RAS water and biofilter microbiomes with fish-associated gut and gill microbial communities in laboratory RAS housing either zebrafish (*Danio rerio*) or common carp (*Cyprinus carpio*) using 16S rRNA gene amplicon sequencing. The phylogeny of ammonia-oxidizing bacteria in the gills and the RAS environment was investigated in more detail by phylogenetic analysis of the ammonia monooxygenase subunit A (*amoA*). The location from which the microbiome was sampled (RAS compartments and gills or gut) had a stronger effect on community composition than the fish species, but species-specific differences were also observed. We found that carp- and zebrafish-associated microbiomes were highly distinct from their respective RAS microbiomes, characterized by lower overall diversity and a small core microbiome consisting of taxa specifically adapted to the respective organ. The gill microbiome was also defined by a high proportion of unique taxa. Finally, we found that *amoA* sequences from the gills were distinct from those from the RAS biofilter and water. Our results showed that the gut and gill microbiomes of carp and zebrafish share a common and species-specific core microbiome that is distinct from the microbially-rich RAS environment.

1. Introduction

Aquaculture is a rapidly growing industry that reached a record output of 87.5 million tons of aquatic animals for human consumption last year,

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corresponding to a production of aquatic animals in 2020 30 % higher than the average in the 2000s, and >60 % above the average in the 1990s (FAO, 2022). The use of recirculating aquaculture systems (RAS) increases the sustainability of fish production as it reduces land and water use compared to other forms of aquaculture. RAS also reduces environmental impacts of aquaculture and decreases the chance of disease outbreaks (Bregnballe, 2010).

To maintain water quality, biofilters are applied to remove waste compounds excreted by the fish. Most fish are ammonotelic and excrete their nitrogenous waste mainly as ammonia through the gills (Randall and Wright, 1987; Evans et al., 2005). Thus, biofilters in RAS contain a diverse microbial community including nitrifying microorganisms in the oxic parts and anaerobic microorganisms such as denitrifying and anammox bacteria in the anoxic zones of the biofilter. Together, these microorganisms are able to remove ammonia *via* nitrite and nitrate to dinitrogen gas (van Kessel et al., 2010). Furthermore, RAS also contain microorganisms in the rearing water or microorganisms that live in association with the animals, and each RAS compartment has its specific microbial community composition (Rurangwa and Verdegem, 2015). A question specific to RAS is how the microbial communities in the RAS compartments affect (body site-specific) microbiomes of the fish kept in the system and how the type of fish affects the microbial community in different RAS compartments. Although water coming from the biofilter compartment is usually treated with UV light and/or ozone to prevent growth of potentially pathogenic bacteria, microorganisms (including biofilter-associated nitrifying bacteria) are still present throughout the system (Rurangwa and Verdegem, 2015; Minich et al., 2020).

External mucosal surfaces of fish, like the skin and gills, are in close contact with the surrounding water, but also the gut mucosal microbiome is affected by the microbial community present in the water (Bugten et al., 2021). Despite these effects, different fish organs (such as gills, skin, gut) are known to possess specific microbiomes that are distinct from the water and from one another (Minich et al., 2020; Minich et al., 2021). This is likely the result of differences in organ physiology and distinct micro-environments compared to the rearing water (*e.g.*, oxygen and nutrient availability), as well as the presence of specific mucosal immune factors at different body sites (Salinas, 2015). Fish mucus contains antimicrobial compounds and antibodies that can selectively inhibit growth of pathogens, while tolerating or even selecting for commensal or beneficial microorganisms (Gomez et al., 2013; Kelly and Salinas, 2017). The combination of these factors contributes to a unique composition of each mucosal microbiome in fish.

While research into the microbiomes of different fish body sites is growing, a lot remains to be explored. The body site-specific microbiomes of fish have been described in several species, although variation in composition can be significant even within species and under similar rearing conditions (Legrand et al., 2019). The few comparative studies between different fish species have revealed interesting patterns in how mucosal microbiomes are shaped by the environment and by interspecies differences. When gut and gill microbiomes of wild tropical reef fish species were compared, it was found that taxonomic relationships between fish affect their respective microbiome composition, but that there are some microorganisms that were enriched in specific body sites regardless of fish species' taxonomy (Pratte et al., 2018). In a similar experiment, Minich et al. (2022) found that body site was the strongest determinant of microbiome composition and suggested that anatomical and/or physiological aspects of fish organs select for certain microbial communities. Both studies were performed in wild fish, so it remains to be investigated to what extent the RAS environment influences the fish's microbiome of each body site. In a comparison between two salmon rearing systems (flow-through and RAS), it was found that the tank biofilm contributed more to the host microbiome than the surrounding water and that the microbial composition of different body sites was affected by the rearing system (Minich et al., 2020). In zebrafish (*Danio rerio*, Hamilton), a recent study demonstrated that a switch in RAS rearing system significantly affected the gut microbiome as well (Breen et al., 2019).

Most of the research into the microbiomes of fish has focused on the gut microbiome, since it plays a key role in digestion and hence growth, which is of key importance for aquaculture (reviewed in (Llewellyn et al., 2014; Ghanbari et al., 2015; Tarnecki et al., 2017)). These studies show that the gut microbiome of different fish species is quite similar in overall composition. Dominant bacterial groups include Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Verrucomicrobia, and there is evidence for a core gut microbiome in several species including zebrafish and common carp (*Cyprinus carpio*, L.) (Roeselers et al., 2011; van Kessel et al., 2011; Ghanbari et al., 2015; Tarnecki et al., 2017). In particular, *Cetobacterium somerae* (a Fusobacterial species) is common in freshwater fish gut microbiomes and can produce and probably provide the host with vitamin B12 (Tsuchiya et al., 2008; Roeselers et al., 2011; van Kessel et al., 2011). It is likely that there are mutualistic relationships in the gut of fish, but there is currently little knowledge about the functional roles of the gut microbiome in fish (Legrand et al., 2019; Perry et al., 2020).

Other mucosal microbiomes of fish have received comparatively little attention so far, even though these mucosal surfaces are equally important as a barrier against pathogens, and are distinct in their functions and ecology from the gut microbiome (Salinas, 2015; Kelly and Salinas, 2017; Legrand et al., 2019). The microbiomes of these organs likely are important to fish health (for example through the competitive exclusion of pathogenic species) and since the more external microbiomes of the skin and gills are in close contact with the surrounding water, it has been shown in some species that they are relatively more influenced by the RAS environment than the gut microbiome (Kelly and Salinas, 2017; Minich et al., 2021; Sehnal et al., 2021). In particular, the gills are an interesting mucosal surface to study, as the organ combines multiple vital functions such as gas exchange, osmoregulation, and nitrogen excretion (Evans et al., 2005; Sehnal et al., 2021; Lai et al., 2022). In addition, it is apparent that the gills are also an immunologically active tissue (Salinas, 2015). Data on the composition of teleost gill microbiomes have shown that Proteobacteria are the dominant phylum in most fish species (Lowrey et al., 2015; Legrand et al., 2018; Pratte et al., 2018). In addition, it was found that the gill microbiome was less diverse than the water microbial community (Pratte et al., 2018; Minich et al., 2021; Sehnal et al., 2021). Several factors were shown to influence gill microbiome composition, including health status, diet, and rearing system (Legrand et al., 2018; Pratte et al., 2018; Minich et al., 2020). However, research on the gill microbiome is rare and systematic comparisons of different fish species and between the gill and surrounding environment are limited (Sehnal et al., 2021).

In line with their key role in nitrogen excretion, fish gills present a suitable habitat for nitrogen-cycle microorganisms, in particular ammonia-oxidizing bacteria (AOB). The presence of these bacteria has been reported in several fish species, including zebrafish, common carp and Atlantic salmon from RAS systems, as well as sea cage cultured yellowtail kingfish and wild red snapper (*Lutjanus campechanus*, Poey) (Tarnecki et al., 2016; van Kessel et al., 2016; Legrand et al., 2018; Minich et al., 2020; Lorgen-Ritchie et al., 2022). In zebrafish and common carp, ammonia-oxidizing and denitrifying bacteria were found to act in concert and convert ammonia *via* nitrite or nitrate into dinitrogen gas (van Kessel et al., 2016; Mes et al., 2023). Interestingly, the phylogeny of these gill-associated AOB was found to be distinct from those present in the surrounding water. However, it remains unclear how abundant these nitrogen cycle bacteria are in zebrafish and common carp and how widespread this symbiotic relationship between nitrogen-cycling bacteria and fish is.

Overall, there is a lack of detailed knowledge on the extent to which RAS biofilter and water microbiomes affect the body site specific microbiomes of fish. In this study, we used 16S rRNA gene amplicon sequencing to compare the gill and gut microbiomes of zebrafish and common carp reared in a laboratory RAS with the microbiomes of their respective rearing water and RAS biofilter. We have chosen these two related fish species because carp are relevant for aquaculture whereas zebrafish are often used as model organisms in scientific research and both species harbor ammonia oxidizing bacteria in their gills (van Kessel et al., 2016; Mes et al., 2023). In addition, we examined the presence of

the marker gene for ammonia-oxidizing bacteria (*amoA*) in the gills and guts of both fish species, as well as in the rearing water and RAS biofilter. We hypothesized that the overall diversity of the microbiome in the rearing water and RAS biofilter would be higher than that of the fish-associated microbiome, and that body site and RAS location (rearing water or biofilter) would have a greater effect on microbiome composition than host species. We also hypothesized that the gill and gut microbiomes of both fish species would contain microorganisms adapted to the specific conditions of their respective organs, and thus that distinct ammonia-oxidizing *Nitrosomonas*-like bacteria would be present in the gills and RAS.

2. Materials and methods

2.1. Animals and recirculating systems

Common carp (obtained from Wageningen University) and zebrafish (cultured at Radboud University Nijmegen) were kept in recirculating systems at the Radboud University under standard husbandry conditions. No mortality was observed for at least 6 (carp) and 3 months (zebrafish). Carp were kept in groups of 20 individuals (12–15 cm, ~ 15 g) in a 200-L tank with tap water (pH 7.5–8.0) at 16 °C with 14 h light and 10 h dark cycles. The water was recirculated through a moving bed filter (120 L, Kaldness rings, surface area 800 m^2/m^3) fixed bed biofilter (60 × 65 × 85 cm, surface area 190 m^2/m^3), with subsequent UV treatment. Carp were fed twice per day with pelleted feed (Stella 2p, Skretting Nutreco, Amersfoort, The Netherlands; 47 % protein, 16 % fat, 2.6 % fibre, 6.1 % ash, 9.0 g/kg phosphorus) at ration sizes of 4 % of their body mass.

Zebrafish were kept in groups of 30 individuals (3.5 cm, ~ 250 mg) in 4 L tanks with tap water (pH 7.7–8.2; 27 °C; 14 h light, 10 h dark). The water was recirculated through a separate fixed-bed biofilter (106 × 43 × 4 cm, surface area 190 m^2/m^3) and UV-treated after biofiltration. Zebrafish were fed Gemma Micro 300 Zf (5 % of their body mass daily; Nutreco N.V., Amersfoort, The Netherlands; 59 % protein, 14 % fat, 0.2 % fibre, 14 % ash, 1.3 g/kg phosphorus), with addition of in-house reared live *Artemia* once a day.

2.2. Sample collection

Biomass for DNA extraction (5 samples each) was collected from the RAS biofilter, rearing water, gut content, and gill tissue of both fish species. Filter biomass was collected at the top, middle and bottom of the filter by taking 50 mL filter suspension with a syringe and left to settle to the bottom of the syringe (30 min, RT). Settled biomass was transferred to 2-mL Eppendorf tubes and centrifuged for 5 min at maximum velocity. Supernatant was removed and the pelleted biomass was frozen at −20 °C until DNA extraction. For each water sample, 1 L system water was collected from the inflow of the fish tank and filtered through a 0.22 μm mixed cellulose ester filter (Merck Millipore, Burlington, MA, USA) using a vacuum pump. Fish were euthanized by terminal anesthesia (overdose of buffered MS-222, pH 7.6) followed by spinal transection. The gut of each fish was removed, and the content was squeezed out using tweezers and collected in sterile 2-mL Eppendorf or 15-mL Greiner tubes. Gill arches of the same fish were removed aseptically and treated with a de-enrichment protocol to remove eukaryotic DNA directly after sampling as described elsewhere (Bruggeling et al., 2021). All samples were stored at −20 °C until further use.

2.3. DNA extraction

DNA was extracted from all samples using a cetyltrimethylammonium bromide (CTAB) method (Zhou et al., 1996). Briefly, samples were incubated in extraction buffer containing 10 mg/ mL proteinase K at 37 °C for 30 min, followed by 10 % SDS addition and incubation for 2 h at 65 °C. DNA was isolated by chloroform:isoamyl alcohol (24:1 *v*/v) extraction and isopropanol precipitation, followed by resuspension in MilliQ water. RNA was removed by addition of 0.5 μL RNase A (10 mg/mL) and incubating at 37 °C for 30 min. Then, DNA was re-extracted using phenol/chloroform extraction and precipitation in 70 % (*v*/v) ethanol. For the filtered water samples, the first incubation in extraction buffer was carried out by placing the filter in a 5-cm Petri dish. After this step, the buffer was collected in a 2-mL Eppendorf tube and the rest of the protocol was followed as described above. DNA integrity and quality were checked on a 1 % agarose gel and by Nanodrop analysis. Samples with sufficient DNA concentrations (>10 ng μL^{-1}) were used for 16S rRNA gene amplicon sequencing and *amoA* PCRs.

2.4. 16S rRNA gene amplicon sequencing and analysis

Sequencing libraries were constructed with the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Macrogen, Seoul, South Korea). Amplicon sequencing of the V3–V4 region of the bacterial 16S rRNA gene amplified with primers Bac341F (CCTACGGGNGGCWGCAG) (Herlemann et al., 2011) and Bac806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) was performed by Macrogen (Seoul, South Korea) using the Illumina platform (Illumina MiSeq, 2 × 300 bp). The number of samples per species, sample types successfully sequenced, and the respective average number of reads obtained are summarized in Supplementary Table S1. All biofilter samples and 9 out of 10 water samples contained sufficient DNA for amplification and sequencing. For the fish-associated microbiomes, 1 carp and 4 zebrafish gill, and 2 zebrafish gut samples did not pass the quality control step because the number of reads was too low. The remaining samples yielded an average of ≥55,000 reads after sequencing and were used for sequence analysis.

Sequence analysis was performed in R version 4.4.1. Raw reads were processed using the DADA2 pipeline (Callahan et al., 2016). Forward and reverse reads were quality-trimmed and primers were removed using DADA2 when also performing quality trimming, using the TrimLeft function, with length adjusted for 341F and 806R primers, after which the DADA2 algorithm was used to infer amplicon sequencing variants (ASVs) from the processed forward and reverse reads separately using the pooled samples. The forward and reverse ASVs were then merged (minimum overlap of 12 bases) to obtain the full-length amplicon ASVs. Chimeric ASVs were removed using the consensus-based chimera removal tool of DADA2, after which the taxonomic assignment of valid ASVs was determined using the naïve Bayesian classifier method with the SILVA SSU rRNA database (version 138) as training dataset (Quast et al., 2012). In total, 6662 ASVs were inferred after processing with DADA2. The inferred ASVs were aligned using the Multiple Alignment using Fast Fourier Transform (MAFFT) program and a neighbor-joining tree was calculated in MEGA X version 10.1.7 (Kumar et al., 2018; Madeira et al., 2019).

2.5. Ammonia monooxygenase subunit A PCRs

A functional gene-targeted PCR and sequencing approach was used to test for the presence of ammonia-oxidizing bacteria. We performed PCRs with primers targeting the ammonia monooxygenase subunit A (*amoA*) gene (Rotthauwe et al., 1997) to identify the presence of betaproteobacterial ammonia oxidizers. Obtained PCR products were purified using the GeneJET PCR cleanup kit (Thermo Fisher Scientific, Wilmington, USA) and ligated into the pGEM-T Easy vector (Invitrogen, Carlsbad, USA). Chemical competent *Escherichia coli* were transformed with the plasmids, which were subsequently isolated using the GeneJET Plasmid miniprep kit (Thermo Fisher Scientific, Wilmington, USA) and sequenced (Sanger sequencing, Baseclear N.V., Leiden, the Netherlands). Sequences were aligned with reference sequences using MUSCLE and a maximum-likelihood tree was calculated in MEGA X (version 10.1.7) (Edgar, 2004; Kumar et al., 2018).

2.6. Statistics

Statistical analyses were performed using the 'phyloseq' and 'microbiome' R packages (McMurdie and Holmes, 2013; Lahti et al.,

2017). ASVs that were present in less than three samples in total or could not be unambiguously assigned to any phylum were removed. Alpha diversity measures (Shannon diversity and Chao1 richness) were calculated from the untransformed ASV counts per sample. The effect of sample type and fish species on alpha diversity of the microbiomes was tested with a two-way analysis of variance (ANOVA; Graphpad prism 9.1, La Jolla, USA).

The differences in microbiome composition (beta diversity) between the samples were tested as well. Weighted Unifrac distances were calculated using log-transformed abundance data and a principal coordinate analysis was used to plot the calculated beta diversities. The R package 'vegan' was used to determine if the microbiomes were statistically different between sample type and species. A PERMANOVA analysis was performed with the *adonis* function with sample type and species as variables and 999 permutations (Oksanen et al., 2013).

The 'ALDEx2' package was used to identify differentially abundant microorganisms between groups (Fernandes et al., 2014). The analysis was based on a general linearized model with sample type (biofilter, water, gill, or gut) as factor, with ASVs clustered on the genus level as input. Data from carp and zebrafish systems were analyzed separately since a significant effect of species on beta diversity was found using the *adonis* function. Differentially abundant genera (FDR-corrected p value <0.05) with an effect size (>1) were distinguished in gill and gut samples.

ASVs forming the core microbiome of the gill and gut samples of both fish species were identified using the filtered ASV table, in which the cross-species core microbiome was defined as ASVs that were present in all gill or gut samples and the species-specific core microbiomes were defined as ASVs that were present in all gill or gut samples of either carp or zebrafish. Species-specific unique ASVs in each sample type were identified using the *psvenn* function of the 'MicEco' package, with a prevalence fraction of 0.5 (present in at least half of the samples of that type) (Russel, 2021). ASVs in the Venn diagram that were unique to each sample type with a relative abundance of >0.1 % were used for defining the unique microbiome of each sample type.

3. Results and discussion

In order to characterize and compare RAS- and animal-associated microbiomes in two fish species, we performed 16S rRNA gene amplicon sequencing on samples obtained from carp and zebrafish gills and guts, as well as from each species' RAS biofilter and water. We found that the gill and gut harbor microbiomes that are distinct from the RAS-associated microbial communities. Biofilter and water microbiomes were highly diverse and rich in bacterial taxa, while gill and gut microbiomes were characterized by a smaller number of bacterial taxa and small but relatively abundant cross-species core microbiomes as well as additional species-specific core microbiota. *AmoA* sequences obtained from the gills of carp and zebrafish were closely related to another, but distinct from those present in the surrounding water and biofilter.

3.1. RAS-associated microbiomes are more diverse than fish-associated microbiomes

We calculated the Chao1 and Shannon diversity measures of each sample type to estimate the overall community richness and diversity based on the inferred ASVs (Fig. 1). The RAS-associated microbiomes in both carp and zebrafish systems had a significantly higher ASV richness (Chao1 index; $F(3, 29) = 23.84$, $p < 0.001$), while the Shannon diversity index was also significantly affected by sample type ($F(3, 29) = 112.0$, $p < 0.001$), with the biofilter samples having the highest Shannon diversity, followed by the water samples. Fish species did not affect Shannon diversity, although a significant interaction effect was seen between species and sample type ($F(3, 29) = 5.683$, $p = 0.0035$). The gill and gut of each species had the lowest alpha diversity and there was no significant difference between the zebrafish and carp-associated microbiota. As hypothesized, fish-associated microbiomes had a lower number of ASVs than the surrounding water and biofilter. This difference has been observed in other fish species as well, for both gills and gut (Pratte et al., 2018; Minich et al., 2020; Bugten et al., 2021; Sehnal et al., 2021). More generally, host-association was found to be one of the most important factors influencing microbial richness (Earth microbiome project dataset (Thompson et al., 2017)). Our data confirm this finding for laboratory RAS ecosystems as well. A potential explanation for this reduced diversity is the effect of mucosal immunity on the microbiome composition in these fish-associated samples, which selects for commensal or symbiotic microorganisms (Salinas, 2015; Kelly and Salinas, 2017).

3.2. Microbial composition differs between RAS- and fish-associated microbiomes in both species

Besides the differences in diversity between RAS- and fish-associated microbiomes, their composition was also markedly different. Based on the Weighted UniFrac distances between samples, each sample type from the RAS system formed separate clusters (Fig. 2). In line with our hypothesis that sample type has a stronger effect on microbiome composition than host species, we found that a significant effect of the sample type that explained the majority (58.5 %) of the observed variance in microbial composition ($F = 28.9$, $p < 0.001$, $r^2 = 0.58$; Table S2). Host species explained a smaller proportion of variance ($F = 11.57$, $p < 0.001$, $r^2 = 0.078$; Table S2),

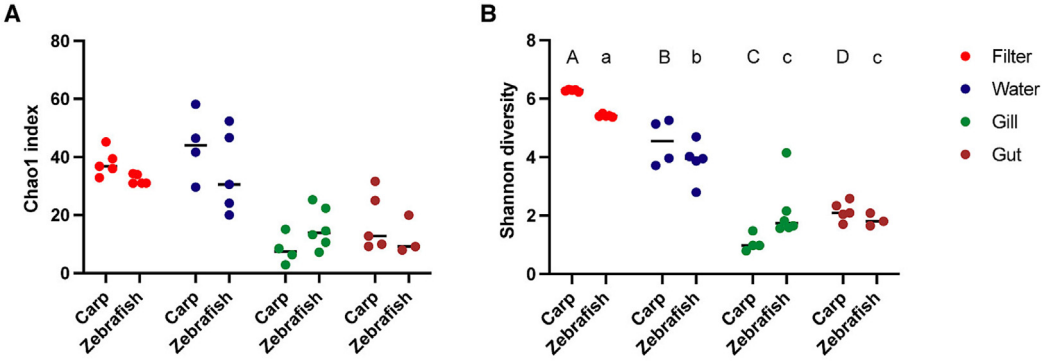

Fig. 1. Alpha diversity of zebrafish and carp RAS- and fish-associated microbiota. A) Chao1 index (ASV richness) of alpha diversity per sample type. Each dot corresponds to one sequenced sample, with bars indicating median values. Significance of species and sample type in the systems was tested using a two-way ANOVA. Sample type had a significant effect on Chao1 diversity index values ($F(3, 29) = 23.84$, $p < 0.001$). B) Shannon diversity index (ASV richness and evenness) of alpha diversity per sample type. Each dot corresponds to one sequenced sample, with bars indicating median values. Significance of the species and sample type was tested using a two-way ANOVA followed by multiple comparison testing. Sample type had a significant effect on the Shannon diversity index ($F(3, 29) = 5.683$, $p = 0.0035$), with an additional interaction effect observed between sample type and species ($F(3, 29) = 112.0$, $p < 0.001$). Carp and zebrafish samples were tested separately. Significant differences between sample types are indicated using different letters, with upper-case letters used for carp samples and lower-case letters for zebrafish samples.

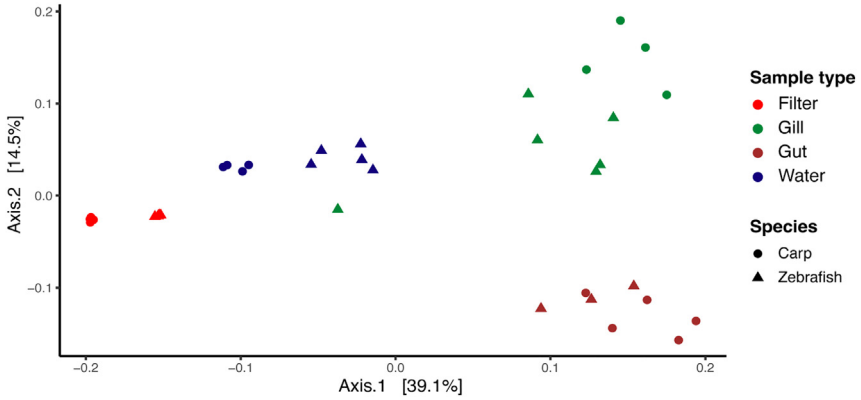

Fig. 2. Ordination plot of carp and zebrafish RAS- and fish-associated microbiomes. Weighted UniFrac distances were calculated from log-transformed abundance data to achieve homogeneity of variance between sample types. Colors indicate the sample types and species are distinguished by symbol. Significance of both factors was tested using a PERMANOVA test. Sample type and species significantly affected weighted UniFrac distances ($F = 28.9$, $r^2 = 0.58$, $p < 0.001$ and $F = 11.57$, $r^2 = 0.078$, $p < 0.001$, respectively).

which indicates that despite a large overall similarity between carp and zebrafish systems in the RAS- and fish-associated microbiomes, species-specific differences in microbiome composition remain. This strong effect of sample type is commonly seen in fish and aquaculture microbiome research. For the RAS environment, it was found that each compartment provides a specific biotope with a specific microbial community (reviewed by Rurangwa and Verdegem (2015)) and the same principle has been found to apply in several fish-associated microbiomes (Lowrey et al., 2015; Legrand et al., 2018; Minich et al., 2022).

RAS biofilter samples from carp and zebrafish systems had a similar composition (Fig. 2) and were characterized by a high abundance of Proteobacteria, Chloroflexi, and Nitrospirota, with zebrafish biofilters also containing Planctomycetes (Fig. 3). In accordance with their function in metabolizing nitrogenous compounds, Nitrosomonadaceae ASVs were abundant in both carp and zebrafish biofilter samples (9.6 % and 3.5 % relative abundance, respectively) and *Nitrospira* ASVs comprised an even larger share of the total community (36 % and 18 % relative abundance, respectively). No other known nitrifying microorganisms were found in the biofilter samples based on 16S rRNA gene sequencing, which indicates that Nitrosomonadaceae and *Nitrospira* are the key nitrifiers in our laboratory RAS. This is in line with other research on RAS biofilters, where these taxa are often the main nitrifiers present (Moschos et al., 2022). Many *Nitrospira* ASVs clustered most closely to known comammox species within *Nitrospira* lineage II (Fig. S1), which is an interesting finding that deserves further research, as it would be informative to know how abundant comammox *Nitrospira* are in RAS compared to canonical nitrite-oxidizing *Nitrospira* and other ammonia-oxidizing microorganisms. In a study focusing on RAS biofilter microbiomes, comammox *Nitrospira* were in fact the dominant nitrifier type (Bartelme et al., 2017) and they were previously isolated from a carp RAS biofilter (van Kessel et al., 2015).

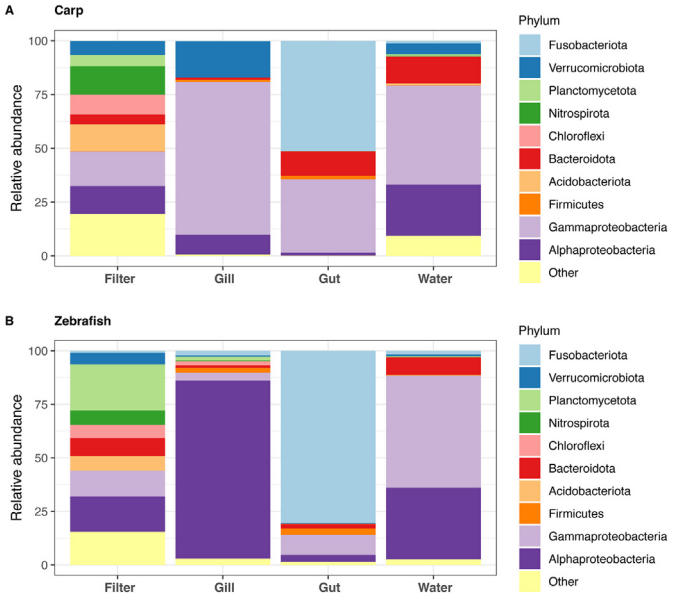

Fig. 3. Phylum-level composition of carp and zebrafish RAS- and fish-associated microbiomes. Major phylum level groupings are shown (>5 % relative abundance in any individual sample), with phyla below this value classified as 'Other' and with Proteobacteria split further to class level. A) Relative abundances of major phyla present in carp system compartments. B) Relative abundances of major phyla present in zebrafish system compartments. Abundances of phyla are shown as percentages of the total number of 16S rRNA gene copies recovered.

The water in the two systems contained different microbial communities, but on phylum level both were dominated by Alpha- and Gammaproteobacteria, together with Bacteroidota. The RAS water microbiome showed overlap with the biofilter microbiomes. A similar pattern was seen in salmon RAS, where the water samples were closest in composition to the biofilter (Schmidt et al., 2016). Since the water is in contact with the biofilter microbiome continuously, this is likely affecting its microbial composition, as observed in other RAS microbiome research (Moschos et al., 2022). The observed dissimilarity of the water and biofilter can be explained by the RAS layout: the biofilter is physically separated from the fish tanks and has microorganisms growing in biofilms on carrier material, and the water is treated with UV radiation before it enters the rearing tanks. The different conditions prevalent in the water and biofilter can lead to a divergence in community composition over time (Blancheton et al., 2013).

The gut microbiome composition of zebrafish and carp was similar on phylum level and was characterized by a dominance of Fusobacteriota and to a lesser extent Gammaproteobacteria and Bacteroidota (Fig. 3). This result is also in line with previous studies that have found a high degree of overlap between the gut microbiomes of different freshwater fish species (Sullam et al., 2012; Tarnecki et al., 2017). The similarity between the RAS-associated and gut microbiome was limited in our data. In zebrafish, it was found that a change in RAS rearing water caused changes in the gut microbiome (Breen et al., 2019), which can be contradictory to the limited overlap we observe in our laboratory RAS. However, in Atlantic salmon reared in RAS, an effect of the rearing water on the gut microbiome was found despite limited overlap between the water and gut microbiomes (Bugten et al., 2021). It would thus be worthwhile to investigate how the laboratory RAS environment induces changes in fish-associated microbiomes, even if the overall similarity is low.

The gill microbiome of both fish species was distinct from the RAS-associated and gut microbiomes and is dominated by Gammaproteobacteria in carp gills (up to 90 % of all sequences) and by Alphaproteobacteria in

zebrafish gills (Fig. 3). This is in line with research on the gill microbiome (Legrand et al., 2019; Sehnal et al., 2021). The microbial composition on a lower taxonomic level is close to other freshwater teleost species (Slinger et al., 2021; Wu et al., 2021), indicating that similar factors are shaping the gill microbiome of freshwater fish species. Tarnecki et al. (2017) showed that salinity is a key factor in shaping the gut microbiome of different fish species, and it would be interesting to determine if salinity also affects the gill microbiome of fish. It is possible that the differences in gill physiology between freshwater and marine fish in terms of osmoregulation and nitrogen excretion (Hwang et al., 2011; Wright and Wood, 2012) lead to a distinct gill microbiome and this warrants further study.

3.3. Fish-associated microbiomes have an abundant core that is partly shared between species

We investigated the gill and gut microbiomes of both fish species to determine genes-level bacterial taxa specifically enriched in these tissues, and the cross-species as well as species-specific core microbiomes. First, we determined differential abundance of bacterial genera for each sample type. In the carp and zebrafish RAS, 187 out of 455 and 103 out of 443 genera, respectively, were differentially abundant between sample types (Benjamini-Hochberg corrected $p < 0.05$). Most of these genera were enriched in the RAS-associated habitats, but a smaller number of genera had a significantly higher abundance in host-associated microbiomes (Table 1). For carp, 7 and 9 genera were specifically enriched in the gill and gut, respectively. For zebrafish, 11 genera were enriched in the gill compared to all other samples, but only 2 in the gut.

The gut microbiome of both fish species was characterized by a significant enrichment in *Cetobacterium* and *Aeromonas* genera. The cross-species core gut microbiome consists of 26 ASVs: 8 ASVs assigned to *Cetobacterium*, 4 *Aeromonas*, 3 Bacteroidales, 3 Rhizobiales, 2 *Crenobacter*, 2 *Plesiomonas* and 1 ASV of each of *Pseudomonas*, *Shewanella*, *Sphingomonas* and *Vibrio*. *Cetobacterium* and *Aeromonas* have been reported before in common carp and zebrafish gut samples (Roeselers et al., 2011; van Kessel et al., 2011; Meng et al., 2021). *Cetobacterium somerae* is commonly observed in other fish species, was shown to produce vitamin B12, and is therefore considered a beneficial member of the gut microbiome (Tsuchiya et al., 2008; Roeselers et al., 2011). The carp-specific core gut microbiome comprised 32 ASVs, predominantly *Cetobacterium,* Firmicutes and the gammaproteobacterial genera *Aeromonas*, *Burkholderiales* and *Vibrio*. The zebrafish-specific core gut microbiome consisted of 49 ASVs, with similar taxa represented as in the carp-specific core microbiome. The core gut microbiomes represented 99 % and 98 % of all bacterial reads in carp and zebrafish, respectively, while approximately half of the total ASVs were observed in only one gut sample for both species.

In the gills of both fish species, *Bradyrhizobium*, *Sphingomonas, Phyllobacterium* and *Variovorax* were significantly higher in abundance compared to other sample types. Interestingly, these taxa also make up the majority of the cross-species core gill microbiome on ASV level, which further included one *Methylobacterium* ASV (Table 2). This shared core microbiome is small in number of ASVs, but the high congruence of enriched taxa in the gills is notable. In particular, the existence of a cross-species gill microbiome shared between two separate RAS suggests that some microorganisms are suited to colonize the fish gill habitat effectively regardless of the fish species. Since the carp and zebrafish RAS were physically separated, direct transmission between fish cannot explain this shared microbiome. It is likely that these microorganisms originate from the water, where these ASVs were also detected, albeit in low abundances. Systematic comparisons of the gill microbiomes of multiple species are rare and are often performed with wild fish or animals kept in co-culture (Pratte et al., 2018; Kuang et al., 2020), so it remains to be determined if this pattern is conserved in other RAS gill microbiomes. While the functions of the identified shared core taxa are currently unknown, they may be involved in protecting the mucosal surfaces against pathogens. For example, *Sphingomonas* abundance was found to be inversely correlated to potentially pathogenic *Vibrio* species in the skin of European seabass (*Dicentrarchus labrax*, L.) (Rosado et al., 2021). Since *Sphingomonas* is one of the few bacterial genera to produce sphingolipids, and since symbiont-derived sphingolipids were found to modulate gill mucosal immunity in rainbow trout (*Oncorhynchus mykiss*, Walbaum), it would be interesting to study the involvement of these molecules in the establishment of symbiotic relationships with other fish species (Sepahi et al., 2016). *Methylobacterium* species had a similar inverse correlation with the opportunistic pathogen *Flavobacterium psychrophilum* in brook charr (*Salvelinus fontinalis*) skin microbiomes and also co-occurred together with *Sphingomonas* in healthy but not in stressed fish (Boutin et al., 2013; Boutin et al., 2014).

To further characterize the gill-associated microbiomes of both species, we investigated the ASVs that were unique to the gill (Fig. 4, Table S3) and the species-specific core ASVs. Both carp and zebrafish gill microbiomes contained a high proportion of unique ASVs: over half of the ASVs found in gill samples are unique to this tissue, which was considerably higher than for gut samples. For carp gills, the unique ASVs were mostly Burkholderiales related to *Ca.* Branchiomonas and *Ca.* Ichtyocystis and constituted the majority of the species-specific core which further consisted of *Cetobacterium* and 1 Parachlamydiaceae ASV. The Parachlamydiaceae and *Ca.* Branchiomonas and *Ca.* Ichthyocystis ASVs are intracellular bacteria that are associated with gill diseases (Toenshoff et al., 2012; Stride et al., 2014; Seth-Smith et al., 2016; Gjessing et al., 2021). Our carp did not show signs of disease on the gills, so this finding is surprising. The high abundance of these potentially pathogenic species has been reported for other healthy fish before (Mitchell et al., 2013; Brown et al., 2019). The carp core gill microbiome (shared and carp-specific) accounted for a large majority (96 %) of gill 16S rRNA sequence reads, while the majority of ASVs (75 %) were found in single carp gill samples only and constituted merely ~2.5 % of all sequences. Unique zebrafish gill ASVs were from the genera *Paracoccus*, *Enhydrobacter* and *Sphingomonas* and the zebrafish-specific core gill microbiome consisted of 3 *Sphingomonas*, 2 Rhizobiales, 2 Actinobacteria, 1 *Paracoccus* and 1 *Enhydrobacter* ASVs. Together, the shared and zebrafish-specific gill core microbiome accounted for 79 % of all sequence reads, while the large majority of ASVs (80 %) was only

Table 1
Differentially abundant genera in carp and zebrafish gill and gut samples. Differentially abundant genera were identified with the ALDEx2 generalized linear model and Kruskal-Wallis tests. The family to which each genus belongs is also given. Cut-offs used for differential abundance were an FDR-corrected p value < 0.05 and an effect size >1.

Carp		Zebrafish	
Family	Genus	Family	Genus
Gill			
Burkholderiales_IS[a]	2013Ark19i[b]	Xanthobacteraceae	Bradyrhizobium
Burkholderiales_IS[a]	Ca_Branchiomonas[b]	Comamonadaceae	Variovorax
Xanthobacteraceae	Bradyrhizobium	Corynebacteriaceae	Corynebacterium
Comamonadaceae	Limnohabitans	Staphylococcaceae	Staphylococcus
Sphingomonadaceae	Sphingomonas	Rhizobiaceae	Phyllobacterium
Rhizobiaceae	Phyllobacterium	Rhodobacteraceae	Paracoccus
Comamonadaceae	Variovorax	Moraxellaceae	Enhydrobacter
		Sphingomonadaceae	Stakelama[b]
		Sphingomonadaceae	Sphingomonas
		Propionibacteriaceae	Cutibacterium
		Beijerinckiaceae	Methylobacterium-Methylorubrum
Gut			
Fusobacteriaceae	Cetobacterium	Fusobacteriaceae	Cetobacterium
Aeromonadaceae	Aeromonas	Aeromonadaceae	Aeromonas
Shewanellaceae	Shewanella		
Chromobacteriaceae	Crenobacter		
Erysipelotrichaceae	Dielma		
Vibrionaceae	Vibrio		
Erysipelotrichaceae	ZOR0006		
Pasteurellaceae	Rodentibacter[b]		
Bacteroidaceae	Bacteroides		

[a] IS: *Incertae sedis*.
[b] This genus is only found in the microbiome of this body site and species.

Table 2
Core ASVs of carp and zebrafish gill microbiomes. ASVs were defined as cross-species core ASVs when identified in all gill samples of both species and as species-specific core ASVs when found in all gill samples of one species.

Cross-species core
ASV_2 Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Sphingomonas
ASV_5 Alphaproteobacteria_Rhizobiales_Xanthobacteraceae_Bradyrhizobium
ASV_6 Alphaproteobacteria_Rhizobiales_Beijerinckiaceae_Methylobacterium
ASV_14 Alphaproteobacteria_Rhizobiales_Rhizobiaceae_Phyllobacterium
ASV_22 Alphaproteobacteria_Rhizobiales_Xanthobacteraceae_Bradyrhizobium
ASV_59 Gammaproteobacteria_Burkholderiales_Comamonadaceae_Variovorax
ASV_359 Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Sphingomonas
ASV_532 Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Sphingomonas

Carp-specific core ASVs	Zebrafish-specific core ASVs
ASV_1 Gammaproteobacteria_Burkholderiales_Burkholderiales_Incertae_Sedis_2013Ark19i	ASV_184 Alphaproteobacteria_Rhodobacterales_Rhodobacteraceae_Paracoccus
ASV_3 Fusobacteria_Fusobacteriales_Fusobacteriaceae_Cetobacterium	ASV_216 Gammaproteobacteria_Pseudomonadales_Moraxellaceae_Enhydrobacter
ASV_7 Chlamydiae_Chlamydiales_Parachlamydiaceae_NA	ASV_469 Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Sphingomonas
ASV_56 Gammaproteobacteria_Burkholderiales_Comamonadaceae_Limnohabitans	ASV_732 Actinobacteria_Propionibacteriales_Propionibacteriaceae_Cutibacterium
ASV_133 Gammaproteobacteria_Burkholderiales_Burkholderiales_Incertae_Sedis_Ca_Branchiomonas	ASV_920 Alphaproteobacteria_Rhizobiales_Xanthobacteraceae_Bradyrhizobium
ASV_445 Gammaproteobacteria_Burkholderiales_Burkholderiales_Incertae_Sedis_2013Ark19i	ASV_951 Alphaproteobacteria_Rhizobiales_Xanthobacteraceae_Bradyrhizobium
ASV_477 Gammaproteobacteria_Burkholderiales_Burkholderiales_Incertae_Sedis_2013Ark19i	ASV_1142 Actinobacteria_Corynebacteriales_Corynebacteriaceae_Corynebacterium
ASV_3097 Gammaproteobacteria_Burkholderiales_Burkholderiales_Incertae_Sedis_2013Ark19i	ASV_1645 Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Sphingomonas
ASV_4869 Gammaproteobacteria_Burkholderiales_Burkholderiales_Incertae_Sedis_2013Ark19i	ASV_3563 Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Stakelama

found in single gill samples and accounted for 10 % of all amplicons. Thus, the core microbiome constitutes the majority of bacteria present in both fish species' gills.

Our results indicate that despite the close proximity to microbiologically rich surroundings, the gill and gut microbiome of carp and zebrafish remain distinct from the RAS-associated microbiomes. In the case of the gut microbiome, this pattern has been observed in zebrafish and other species (Breen et al., 2019; Bugten et al., 2021). Contrastingly, studies that have focused on the effects of the RAS environment on external microbiota are scarce, with to our knowledge only Atlantic salmon and yellowtail kingfish RAS having been investigated (Minich et al., 2020; Minich et al., 2021). The effects of the RAS environment on the fish-associated microbiomes can potentially vary over time, differ between species, and depend on operating conditions of the system (Fossmark et al., 2020; Bugten et al., 2021; Lorgen-Ritchie et al., 2022). Since we only examined a single RAS setup for each species and a single point in time, the temporal dynamics of the RAS microbiome remain unknown. While there was little direct overlap in microbial composition between the RAS microbiome and fish-associated microbiomes in our study, a number of highly abundant core gill and gut microbiota members were be detected throughout the system. This raises the possibility that the RAS environment contains rare members that are adapted to colonize fish microbiomes, or alternatively that the fish microbiota are able to spread microorganisms into the RAS ecosystem and *vice versa*. Furthermore, the ASVs unique to the gill and gut microbiome of fish are interesting targets to study for alternative transmission mechanisms other than *via* the water.

3.4. Gills of carp and zebrafish possess a distinct population of ammonia-oxidizing bacteria

As both the RAS biofilter and fish-associated microbiomes are potential habitats for ammonia-oxidizing bacteria, we assessed the diversity of ammonia-oxidizing bacteria based on the marker gene *amoA*. From both carp and zebrafish gills, we obtained *amoA* sequences that were closely related to *Nitrosomonas eutropha*, whereas the filter and water samples contained distinct *amoA* sequences (Fig. 5). Thus, it can be concluded that gill-associated *Nitrosomonas* species form a distinct group from those found in either tank water or RAS biofilters. Interestingly, the *amoA* sequences obtained from carp and zebrafish gills form a monophyletic cluster together with previously published sequences derived from gills (van Kessel

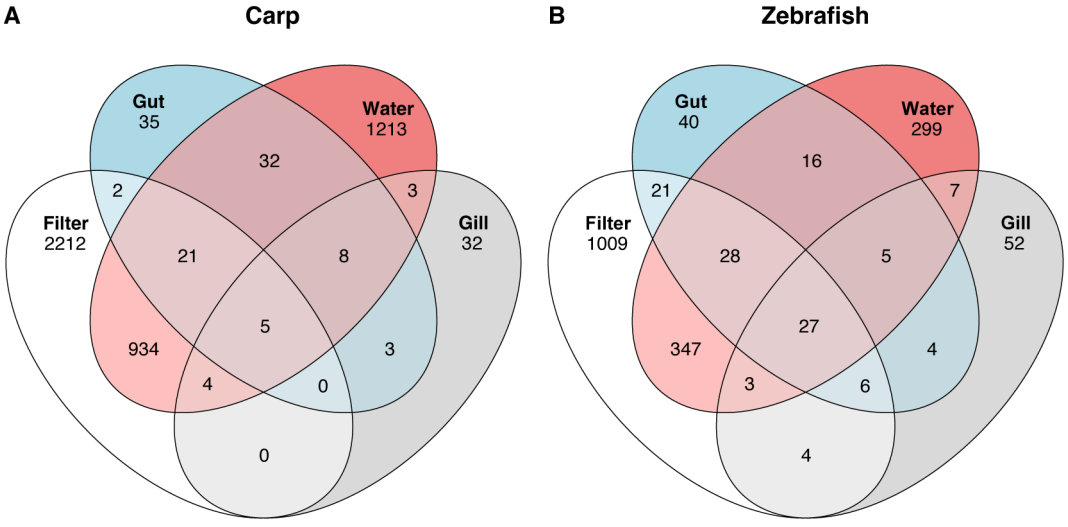

Fig. 4. Unique and shared ASVs in RAS- and fish-associated microbiomes of carp and zebrafish. Venn diagrams indicate the set of ASVs found in each respective microbiome for carp (A) and zebrafish (B) systems separately. Shared ASVs had to be present in at least half of the samples of the type.

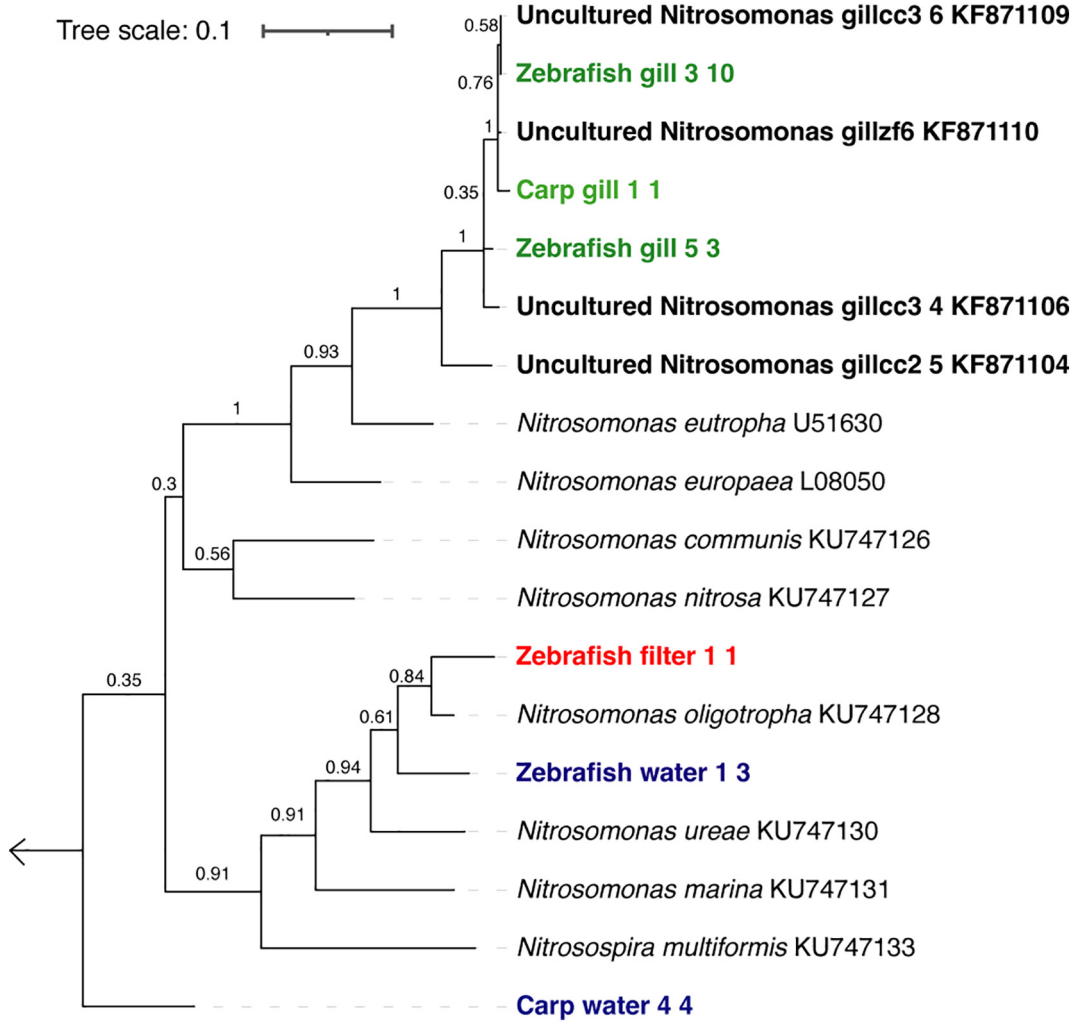

Fig. 5. Maximum likelihood tree of ammonia monooxygenase subunit A (*amoA*) gene sequences obtained from RAS, water and gill microbiomes. *amoA* sequences obtained in this study are colored according to the source of the sequence, with biofilter samples in red, water samples in blue and gill samples in green and are printed in bold. Previously published gill-associated *amoA* sequences are also printed in bold. Bootstrap values are given for all nodes in the tree. Methyloprofundus *pmoA* (LC616792) was used as outgroup for the tree.

et al., 2016). As this research was performed in the same facility but more than six years prior to our study, there seems to be a stable association of gill-associated *Nitrosomonas* and both fish species studied. As these specific *Nitrosomonas* species were not observed in the RAS environment, it is unlikely that the presence of these ammonia oxidizers in fish gills is dependent on their presence in the biofilter. Since the bacteria were found in multiple individual fish, it raises questions about the transmission of these gill-specific *Nitrosomonas* between fish if they are not originating from the RAS environment.

In contrast to *amoA*, no *Nitrosomonas* 16S rRNA gene sequences were obtained from the gills, which makes quantification and comparison of these bacteria to other fish gill microbiomes impossible. Samples from carp and zebrafish contained ASVs classified as Nitrosomonadaceae, but these were closely related to other Betaproteobacteria (*Usitatibacter rugosus*, 99.53 % similarity and *Hydrogenophilus*, 93 % similarity, respectively). Neither RAS had high abundances of *Nitrosomonas* ASVs in the water and biofilter samples, but ASVs within the closely related genus of *Nitrosospira* were more abundant. It is possible that the applied de-enrichment protocol did not remove host DNA sufficiently or removed bacterial DNA as well. Alternatively, it may have been the case that PCR-inhibitory compounds were still present in the sample, which lead to lower observed microbial diversity as reported in a recent study in fish gills (Clokie et al., 2022). In that study, the authors proposed to improve gill microbiome sampling by using filter swabs instead of whole tissue, which might allow quantification of the 16S rRNA sequence abundance of the gill-specific *Nitrosomonas* and would be an interesting option for follow-up research.

While *Nitrosomonas*-like 16S rRNA gene sequences have been found in the gills of multiple other fish species (Tarnecki et al., 2016; Legrand et al., 2018; Minich et al., 2020), further evidence for their nitrogen-cycling role as indicated by the presence of *amoA* sequences is currently lacking in the literature. Application of alternative methods to 16S rRNA amplicon sequencing, such as PCRs for nitrogen cycle marker genes or more advanced sequencing methods (shotgun metagenomic sequencing) will further our understanding of the symbiosis of nitrogen cycle bacteria with teleostean fish and lead to a more functional understanding of the gill microbiome.

4. Conclusion

In summary, we examined the gill and gut microbiomes of carp and zebrafish reared in RAS and compared the composition of these microbiomes with the microbial community of the circulating water and biofilters. We found that the fish-associated microbiomes were characterized by lower overall richness and a different composition than the RAS microbiomes. Community composition was largely determined by the environment, but a smaller effect of fish species was apparent for each

microbiome. Carp and zebrafish largely shared their core gill and gut microbiomes, which consisted of a small number of abundant ASVs. Finally, our investigation of ammonia-oxidizing bacteria revealed that gill-associated *Nitrosomonas*-like bacteria formed a distinct group from the ammonia-oxidizers present in the surrounding water and biofilter samples.

CRediT authorship contribution statement

WM, MG, SL, MK contributed to conception and design of the study. WM, MK performed the experiments. WM wrote the original draft, which was reviewed and edited by MJ, HS, SL, MG and MK. All authors contributed to manuscript revision, read, and approved the submitted version.

Data availability

All 16S rRNA gene sequence data generated in this project were deposited at NCBI under Bioproject number PRJNA940353. Ammonia monooxygenase A sequences were deposited at NCBI GeneBank under accession numbers OQ679757–OQ679762.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.165212.

References

Bartelme, R.P., McLellan, S.L., Newton, R.J., 2017. Freshwater recirculating aquaculture system operations drive biofilter bacterial community shifts around a stable nitrifying consortium of ammonia-oxidizing archaea and comammox Nitrospira. Front. Microbiol. 8, 101.

Blancheton, J., Attramadal, K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquac. Eng. 53, 30–39.

Boutin, S., Bernatchez, L., Audet, C., Derôme, N., 2013. Network analysis highlights complex interactions between pathogen, host and commensal microbiota. PLoS One 8 (12), e84772.

Boutin, S., Sauvage, C., Bernatchez, L., Audet, C., Derome, N., 2014. Inter individual variations of the fish skin microbiota: host genetics basis of mutualism? PLoS One 9 (7), e102649.

Breen, P., Winters, A.D., Nag, D., Ahmad, M.M., Theis, K.R., Withey, J.H., 2019. Internal versus external pressures: effect of housing systems on the zebrafish microbiome. Zebrafish 16 (4), 388–400.

Bregnballe, J., 2010. A guide to recirculation aquaculture: an introduction to the new environmentally friendly and highly productive closed fish farming systems.

Brown, R.M., Wiens, G.D., Salinas, I., 2019. Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol. 86, 497–506.

Bruggeling, C.E., Garza, D.R., Achouiti, S., Mes, W., Dutilh, B.E., Boleij, A., 2021. Optimized bacterial DNA isolation method for microbiome analysis of human tissues. MicrobiologyOpen 10 (3), e1191.

Bugten, A.V., Attramadal, K.J.K., Fossmark, R.O., Rosten, T., Vadstein, O., Bakke, I., 2021. Changes in rearing water microbiomes in RAS induced by membrane filtration alters the hindgut microbiomes of Atlantic salmon (Salmo salar) parr. Aquaculture 548 (2), 737661.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13 (7), 581.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., et al., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108 (supplement_1), 4516–4522.

Clokie, B.G.J., Elsheshtawy, A., Albalat, A., Nylund, A., Beveridge, A., Payne, C.J., et al., 2022. Optimization of low-biomass sample collection and quantitative PCR-based titration impact 16S rRNA microbiome resolution. Microbiol. Spectr. 10 (6), e02255-02222.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32 (5), 1792–1797.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85 (1), 97–177.

FAO, 2022. The State of World Fisheries And Aquaculture 2022. Towards Blue Transformation. FAO, Rome.

Fernandes, A.D., Reid, J.N., Macklaim, J.M., McMurrough, T.A., Edgell, D.R., Gloor, G.B., 2014. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome 2 (1), 1–13.

Fossmark, R.O., Vadstein, O., Rosten, T.W., Bakke, I., Košeto, D., Bugten, A.V., et al., 2020. Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (Salmo salar). Aquaculture 524, 735268.

Ghanbari, M., Kneifel, W., Domig, K.J., 2015. A new view of the fish gut microbiome: advances from next-generation sequencing. Aquaculture 448, 464–475.

Gjessing, M.C., Spilsberg, B., Steinum, T.M., Amundsen, M., Austbø, L., Hansen, H., et al., 2021. Multi-agent in situ hybridization confirms Ca. Branchiomonas cysticola as a major contributor in complex gill disease in Atlantic salmon. Fish Shellfish Immunol. Rep. 2, 100026.

Gomez, D., Sunyer, J.O., Salinas, I., 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunol. 35 (6), 1729–1739.

Herlemann, D.P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F., 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J. 5 (10), 1571–1579.

Hwang, P.-P., Lee, T.-H., Lin, L.-Y., 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am. J. Phys. Regul. Integr. Comp. Phys. 301 (1), R28–R47.

Kelly, C., Salinas, I., 2017. Under pressure: interactions between commensal microbiota and the teleost immune system. Front. Immunol. 8, 559.

Kuang, T., He, A., Lin, Y., Huang, X., Liu, L., Zhou, L., 2020. Comparative analysis of microbial communities associated with the gill, gut, and habitat of two filter-feeding fish. Aquac. Rep. 18, 100501.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1547.

Lahti, L., Shetty, S., Blake, T., Salojarvi, J., 2017. Tools for Microbiome Analysis in R. Version 1(10013). p. 504.

Lai, K.P., Zhu, P., Boncan, D.A.T., Yang, L., Leung, C.C.T., Ho, J.C.H., et al., 2022. Integrated omics approaches revealed the osmotic stress-responsive genes and microbiota in gill of marine medaka. mSystems 7 (2), e00047-00022.

Legrand, T.P., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., et al., 2018. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. Front. Microbiol. 8, 2664.

Legrand, T.P., Wynne, J.W., Weyrich, L.S., Oxley, A.P., 2019. A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. Rev. Aquac. 12 (2), 1101–1134.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 5, 207.

Lorgen-Ritchie, M., Clarkson, M., Chalmers, L., Taylor, J.F., Migaud, H., Martin, S.A., 2022. Temporal changes in skin and gill microbiomes of Atlantic salmon in a recirculating aquaculture system—why do they matter? Aquaculture 738352.

Lowrey, L., Woodhams, D.C., Tacchi, L., Salinas, I., 2015. Topographical mapping of the rainbow trout (Oncorhynchus mykiss) microbiome reveals a diverse bacterial community with antifungal properties in the skin. Appl. Environ. Microbiol. 81 (19), 6915–6925.

Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., et al., 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 47 (W1), W636–W641.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8 (4), e61217.

Meng, K.-F., Ding, L.-G., Wu, S., Wu, Z.-B., Cheng, G.-F., Zhai, X., et al., 2021. Interactions between commensal microbiota and mucosal immunity in teleost fish during viral infection with SVCV. Front. Immunol. 1074.

Mes, W., Kersten, P., Maas, R.M., Eding, E.H., Jetten, M.S.M., Siepel, H., et al., 2023. Effects of demand-feeding and dietary protein level on nitrogen metabolism and symbiont dinitrogen gas production of common carp (Cyprinus carpio, L.). Front. Physiol. 14.

Minich, J.J., Poore, G.D., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., et al., 2020. Microbial ecology of Atlantic salmon (Salmo salar) hatcheries: impacts of the built environment on fish mucosal microbiota. Appl. Environ. Microbiol. 86 (12), e00411–e00420.

Minich, J.J., Nowak, B., Elizur, A., Knight, R., Fielder, S., Allen, E.E., 2021. Impacts of the marine hatchery built environment, water and feed on mucosal microbiome colonization across ontogeny in Yellowtail Kingfish, *Seriola lalandi*. Front. Mar. Sci. 8, 516.

Minich, J.J., Härer, A., Vechinski, J., Frable, B.W., Skelton, Z.R., Kunselman, E., et al., 2022. Host biology, ecology and the environment influence microbial biomass and diversity in 101 marine fish species. Nat. Commun. 13 (1), 6978.

Mitchell, S.O., Steinum, T.M., Toenshoff, E.R., Kvellestad, A., Falk, K., Horn, M., et al., 2013. 'Candidatus Branchiomonas cysticola' is a common agent of epitheliocysts in seawater-farmed Atlantic salmon Salmo salar in Norway and Ireland. Dis. Aquat. Org. 103 (1), 35–43.

Moschos, S., Kormas, K.A., Karayanni, H., 2022. Prokaryotic diversity in marine and freshwater recirculating aquaculture systems. Rev. Aquac. 14, 1861–1886.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R., et al., 2013. Package 'vegan'. Community Ecology Package, Version 2(9). pp. 1–295.
Perry, W.B., Lindsay, E., Payne, C.J., Brodie, C., Kazlauskaite, R., 2020. The role of the gut microbiome in sustainable teleost aquaculture. Proc. R. Soc. B 287 (1926), 20200184.
Pratte, Z.A., Besson, M., Hollman, R.D., Stewart, F.J., 2018. The gills of reef fish support a distinct microbiome influenced by host-specific factors. Appl. Environ. Microbiol. 84 (9), e00063-00018.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (D1), D590–D596.
Randall, D.J., Wright, P.A., 1987. Ammonia distribution and excretion in fish. Fish Physiol. Biochem. 3 (3), 107–120.
Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., et al., 2011. Evidence for a core gut microbiota in the zebrafish. ISME J. 5 (10), 1595–1608.
Rosado, D., Xavier, R., Cable, J., Severino, R., Tarroso, P., Pérez-Losada, M., 2021. Longitudinal sampling of external mucosae in farmed European seabass reveals the impact of water temperature on bacterial dynamics. ISME Commun. 1 (1), 1–11.
Rotthauwe, J.-H., Witzel, K.-P., Liesack, W., 1997. The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63 (12), 4704–4712.
Rurangwa, E., Verdegem, M.C., 2015. Microorganisms in recirculating aquaculture systems and their management. Rev. Aquac. 7 (2), 117–130.
Russel, J., 2021. "MicEco". v0.9.15 ed.
Salinas, I., 2015. The mucosal immune system of teleost fish. Biology 4 (3), 525–539.
Schmidt, V., Amaral-Zettler, L., Davidson, J., Summerfelt, S., Good, C., 2016. Influence of fishmeal-free diets on microbial communities in Atlantic salmon (Salmo salar) recirculation aquaculture systems. Appl. Environ. Microbiol. 82 (15), 4470–4481.
Sehnal, L., Brammer-Robbins, E., Wormington, A.M., Blaha, L., Bisesi, J., Larkin, I., et al., 2021. Microbiome composition and function in aquatic vertebrates: small organisms making big impacts on aquatic animal health. Front. Microbiol. 12, 358.
Sepahi, A., Cordero, H., Goldfine, H., Esteban, M.Á., Salinas, I., 2016. Symbiont-derived sphingolipids modulate mucosal homeostasis and B cells in teleost fish. Sci. Rep. 6 (1), 1–13.
Seth-Smith, H., Dourala, N., Fehr, A., Qi, W., Katharios, P., Ruetten, M., et al., 2016. Emerging pathogens of gilthead seabream: characterisation and genomic analysis of novel intracellular β-proteobacteria. ISME J. 10 (7), 1791–1803.
Slinger, J., Adams, M.B., Stratford, C.N., Rigby, M., Wynne, J.W., 2021. The effect of antimicrobial treatment upon the gill bacteriome of Atlantic salmon (Salmo salar L.) and progression of amoebic gill disease (AGD) in vivo. Microorganisms 9 (5), 987.
Stride, M., Polkinghorne, A., Nowak, B., 2014. Chlamydial infections of fish: diverse pathogens and emerging causes of disease in aquaculture species. Vet. Microbiol. 170 (1–2), 19–27.
Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R., et al., 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol. Ecol. 21 (13), 3363–3378.
Tarnecki, A.M., Patterson, W.F., Arias, C.R., 2016. Microbiota of wild-caught Red Snapper Lutjanus campechanus. BMC Microbiol. 16 (1), 1–10.
Tarnecki, A.M., Burgos, F.A., Ray, C.L., Arias, C.R., 2017. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. J. Appl. Microbiol. 123 (1), 2–17.
Thompson, L.R., Sanders, J.G., McDonald, D., Amir, A., Ladau, J., Locey, K.J., et al., 2017. A communal catalogue reveals Earth's multiscale microbial diversity. Nature 551 (7681), 457–463.
Toenshoff, E.R., Kvellestad, A., Mitchell, S.O., Steinum, T., Falk, K., Colquhoun, D.J., et al., 2012. A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic salmon (Salmo salar). PLoS One 7 (3), e32696.
Tsuchiya, C., Sakata, T., Sugita, H., 2008. Novel ecological niche of Cetobacterium somerae, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett. Appl. Microbiol. 46 (1), 43–48.
van Kessel, M.A.H.J., Harhangi, H.R., van de Pas-Schoonen, K., van de Vossenberg, J., Flik, G., Jetten, M.S.M., et al., 2010. Biodiversity of N-cycle bacteria in nitrogen removing moving bed biofilters for freshwater recirculating aquaculture systems. Aquaculture 306 (1–4), 177–184.
van Kessel, M.A.H.J., Dutilh, B.E., Neveling, K., Kwint, M.P., Veltman, J.A., Flik, G., et al., 2011. Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (Cyprinus carpio L.). AMB Express 1 (1), 1–9.
van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B., et al., 2015. Complete nitrification by a single microorganism. Nature 528 (7583), 555–559.
van Kessel, M.A.H.J., Mesman, R.J., Arshad, A., Metz, J.R., Spanings, F.A.T., van Dalen, S.C.M., et al., 2016. Branchial nitrogen cycle symbionts can remove ammonia in fish gills. Environ. Microbiol. Rep. 8 (5), 590–594.
Wright, P.A., Wood, C.M., 2012. Seven things fish know about ammonia and we don't. Respir. Physiol. Neurobiol. 184 (3), 231–240.
Wu, Z., Zhang, Q., Lin, Y., Hao, J., Wang, S., Zhang, J., et al., 2021. Taxonomic and functional characteristics of the gill and gastrointestinal microbiota and its correlation with intestinal metabolites in NEW GIFT strain of farmed adult Nile tilapia (Oreochromis niloticus). Microorganisms 9 (3), 617.
Zhou, J., Bruns, M.A., Tiedje, J.M., 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62 (2), 316–322.