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HOST MICROBE INTERACTIONS

Impact of Sublethal Concentrations of Nitrite on Goldfish (*Carassius auratus*) Microbiomes

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Abstract

Elevated concentrations of nitrite are toxic to fish and can cause a myriad of well documented issues. However, the effects of sublethal concentrations of nitrite on fish health, and specifically, fish tissue microbiomes have not been studied. To test the effects of nitrite exposure, goldfish were exposed to sublethal concentrations of nitrite, 0.0 mM, 0.1 mM, and 1.0 mM, for 2 months. The bacteria in the nose, skin, gills, and water were then extracted and sequenced to identify changes to the microbial composition. The water microbiome was not significantly changed by the added nitrite; however, each of the tissue microbiomes was changed by at least one of the treatments. The skin and gill microbiomes were significantly different between the control and 1.0 mM treatment and the nose microbiome showed significant changes between the control and both the 0.1 mM and 1.0 mM treatments. Thus, sublethal concentrations of nitrite in the environment caused a shift in the fish tissue microbiomes independently of the water microbiome. These changes could lead to an increased chance of infection, disrupt organ systems, and raise the mortality rate of fish. In systems with high nitrite concentrations, like intensive aquaculture setups or polluted areas, the effects of nitrite on the microbiomes could negatively affect fish populations.

Keywords Nitrite · Sublethal · Microbiome · Aquaculture · Goldfish

Introduction

Nitrite is a common water pollutant which can be devastating to aquatic ecosystems. Elevated concentrations of nitrite can occur in natural waterways as a result of nutrient shifts in the water and pollution from industry, sewage, and agriculture [1]. Additionally, aquaculture setups are more likely to have elevated water nitrite concentrations due to fish nitrogen excretion, which tends to be high due to crowding, high-protein feed, nitrogenous fertilizer in the water source, and low oxygen environments [2]. Nitrite is a dangerous pollutant because, as it accumulates in vertebrates, it disrupts various physiological processes including decreasing oxygen uptake, increasing production of oxygen radicals, and methemoglobinemia [3–7]. As the aquatic environment becomes more anoxic, the relative abundance of nitrite in the water increases, leading to a higher risk of nitrite toxicity [8, 9]. As a result of its toxicity and pervasiveness in aquatic ecosystems, acute nitrite toxicity has been studied extensively in fish species [10–13]. These studies show that acute exposures to nitrite can disrupt communication, immune responses, and organ function. However, the effects of chronic sublethal concentrations, low concentration exposure over long periods of time, of nitrite on the tissue microbiomes have not been studied extensively. This study investigated 0.1 and 1.0 mM concentrations of nitrite as those concentrations can be found in natural waterways where nitrite had been recorded from 0.001 mM to higher than 1 mM [14, 15]. Additionally, in aquaculture systems with poor filtration and overcrowding, the nitrite concentration can exceed 3.0 mM [2, 13].

The microbiome, which is representative of all microbiota, including bacteria, fungi, and viruses, that reside on and within an organism, plays numerous roles in the health of an organism [16]. The microbiome varies between tissues as it assists with physiological functions ranging from digestion to stimulation of the immune system [17]. Each tissue requires a specific composition and abundance of bacteria to successfully carry out the tissue's function and

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prevent infection [18]. If the composition and abundance of these bacteria shifts, it can disrupt physiological functions or increase susceptibility to infection [19]. These changes can be caused by stress, disease, environmental changes, or pollution. However, the effects of nitrite pollution on the tissue microbiomes are unknown. Aquaculture setups with elevated nitrite concentrations have higher mortality rates due to infectious diseases. Thus, it is possible that elevated concentrations of nitrite in aquaculture set-ups disrupt the microbiome and leads to higher susceptibility to infectious diseases.

To best understand how nitrite impacts the fish microbiomes and their overall health, the nose, skin, and gill tissues were selected. Each of these tissues is one of the mucosal-associated lymphoid tissues (MALTs) which are a major part of the immune system [20–22]. MALTs depend on the microbiome to shape the immune response and serve as a barrier against pathogen colonization. Disruption of the tissue microbiomes has been shown to disrupt the immune response in these tissues and leave the host more susceptible to severe infections [23]. In order to understand how sublethal nitrite concentrations can impact fish health, its impact on the microbiomes and associated tissue functions must first be examined. Each tissue microbiome chosen is associated with the immune system, but also plays a unique and important physiological function in its specific tissue. If nitrite impacts the functionality of these microbiomes, it could impact the specific physiological roles of the associated organ along causing systemic issues.

Fish depend on olfaction as a primary sense due to its high sensitivity and effectiveness in aquatic environments. Olfaction is used to locate food sources, identify conspecifics, avoid predators, and navigate [24]. However, the role of the nasal microbiome in fish has not been well described despite olfaction mediating physiological and behavioral responses. The nasal microbiome has been found to regulate genes associated with maintaining healthy olfactory and vomeronasal receptors and regulates the pseudostratification of the olfactory epithelium [25]. Therefore, a disruption to the nasal microbiome could hinder the olfactory ability of fish exposed to nitrite which would impact feeding, mating, predator avoidance, and communication.

Similarly, little is known about the specific roles of the skin and gill microbiomes in fish outside of their role in the immune system. The skin microbiome is highly variable between regions of the skin, individuals, and across geographic region which makes it difficult to identify a core skin microbiome [26]. However, the mucus on the skin and the associated microbiome likely inhibits the growth of fungal pathogens and serves as a physical barrier to pathogens, similar to its role in terrestrial vertebrates [27, 28]. Alternatively, the gill microbiome is difficult to define because of the environment created within the gills due to the continuous water flow and acidic environment due to carbon dioxide excretion [29]. A stable microbiome may be limited to the lamellae and pharyngeal arches and likely overlaps in composition with the skin microbiome [21]. A disruption to the skin and gill microbiomes would likely negatively impact their ability to fulfill both their immune and tissue specific roles.

Each of these tissue microbiomes is important to the overall health of the host fish and disruption caused by nitrite pollution could have severe impacts on health and longevity. This study used goldfish, *Carassius auratus*, as a model fish species. Goldfish are used to study numerous physiological processes including the endocrine system, reproduction, and chemical communication and the results can be generally applied to other fish species. They are a freshwater fish related to other economically and scientifically important species, including zebrafish and carp. Goldfish are relatively tolerant of changes in pH, high turbidity, temperature fluctuations, variable salinity, and low levels of dissolved oxygen. This combined with their tolerance for handling and in-depth research into the species, makes it an ideal model fish species [30].

This study aims to determine how sublethal concentrations of nitrite impact the composition of these tissue microbiomes in comparison to both a control and the water microbiome. We hypothesize that sublethal concentrations of nitrite in the water shift the composition of the tissue microbiomes. If nitrite exposure shifts the composition of the tissue microbiomes, it may account for some of the problems associated with chronic nitrite exposure including stress, increased susceptibility to disease, and higher mortality rates. The deleterious effects of nitrite due to interference with the microbiome may happen at nitrite concentrations that are considered innocuous and acceptable in natural and aquacultural water systems. Under these circumstances, fish would not show any apparent physiological stress symptoms but would be more sensitive to disease. Findings from this study, focused on the sublethal concentrations of nitrite in the fish microbiome, may be used to refine conservation efforts, legislation, and management of aquaculture and natural aquatic systems.

Materials and Methods

Nitrite Exposure and Sample Collection

Goldfish were acquired from a local Texas Parks and Wildlife hatchery at approximately the same age (1 year old) and about 5 cm in length. They were allowed to acclimate in a recirculating living stream with aerated water at 25 °C for 1 week. Fish were fed TetraFin Goldfish Vitamin C Enriched flake food once a day for the duration of the experiment.

After acclimation, they were transferred and allowed to acclimate for 1 week in a continuous flow water aquarium system. The acclimation time chosen followed IACUC procedures, which requires 1 to 2 weeks of acclimation [31]. Additionally, the goldfish did not show any signs of stress, including loss of appetite, changes in swimming, or skin color after the 2 week-two step acclimation.

This system had a 200-L tote that served as a water reservoir for each of the three treatments and was feeding water by gravity to four aquariums. Each tote was refilled every 2 days, and at this time the appropriate nitrite concentration was mixed into the tote. The water continuously flowed from the tote to the aquariums and out of the aquariums to allow for a consistent concentration of nitrite while otherwise maintaining proper water quality by allowing a complete replacement of water in the aquarium twice a day. Each treatment had 4 replicate tanks with 10 fish per tank for a total of 40 fish per treatment.

After the acclimation period, the fish were exposed to one of three nitrite treatments (0.0 mM, 0.1 mM, or 1.0 mM) for 2 months. These sublethal concentrations must be equal to or less than 10% of the LC50, approximately 4.0 mM, to allow for a 2-month chronic exposure [6]. Nitrite solutions were made with sodium nitrite ($NaNO_2$). Each of the selected nitrite concentrations is sublethal and can be observed in polluted water ways and agricultural systems. During this time, water samples were collected, and nitrite, nitrate, ammonium, and pH levels were monitored every other day. Any goldfish that died were immediately recorded, removed, and properly discarded.

After 2 months, the fish were anesthetized with tricaine mesylate (MS-222) at a concentration of 0.2 g/L before being sacrificed. The nose was dissected out of the head and collected in its entirety. The tail was clipped to serve as a skin sample. The gills were also dissected out in one piece to preserve any bacteria that might be associated between the gill lamellae. A subsample of 2 L of water was collected from each tank to determine if the tissue microbiomes were unique from the water microbiome. All tools and surfaces were disinfected with 70% ethanol between each fish and tissue to minimize contamination. Each tissue was placed in a sterile microcentrifuge tube and stored at − 20 °C until processing. The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Texas State University (IACUC # 7074).

Analysis of Nitrite

The water that was collected during exposure was analyzed using the Invitrogen fluorometric Measure-iT High Sensitivity Nitrite Assay for the 0.0 mM treatment and the Sigma-Aldrich Nitrite/Nitrate colorimetric Assay Kit for the 0.1 mM and 1.0 mM nitrite treatments. To do the colorimetric assay, reference standards were made to create a standard curve. One hundred microliters of the standards and the samples were added to a 96-well plate and then 100 µL of Griess solution was added to each well. The plate was shaken and allowed to sit for 5 min before being read at 570 nm. Standards were also made to conduct the Measure-iT High Sensitivity Nitrite Assay, and the assay was conducted according to the assay protocols. The fluorescence was measured with an excitation/emission of 365/450 nm. The results for both assays were then analyzed using Prism 9 (GraphPad).

Microbiome Analysis of Tissue Samples

DNA was extracted from tissue samples from 2 fish from each of the 4 tanks from each treatment, giving a total of 8 different tissue samples per treatment. The DNA was extracted using the QIAamp BiOstic Bacteremia DNA Kit following manufacturer's instructions, which includes homogenization using a bead beater. All the tissues were processed in the same manner to allow comparison between the tissues. The water samples were filtered using Durapore 0.22 µm PVDF membranes and the DNA was extracted from the membrane using the Qiagen QIAamp BiOstic Bacteremia DNA Kit. All samples were stored at − 20 °C until they were ready to be amplified.

16 s rRNA Amplification and Sequencing

After extraction, the V4 region of the 16 s rRNA gene was then amplified using KAPA Taq and primers 515F (5′-GTGCCAGCMGCCGCGGTAA) and 806R (5′-GGACTACHVHHHTWTCTAAT). The PCR amplicons were checked using gel electrophoresis with a 1.5% agarose gel for 20 min at 90 V. Any samples that showed amplification underwent a second PCR to add barcode primers that would allow the identification of each sample after sequencing [32]. The second PCR products were purified using the Applied Biosystems ExoSap-IT PCR Product Cleanup kit and each sample was quantified using the Invitrogen Qubit dsDNA BR assay kit. A non-template negative control was created by following the above procedure but not introducing a tissue sample or DNA into the extraction kit. Any DNA detected in this sample could be used as a negative control to remove contamination. The samples were diluted to 10 ng/µL, combined to form a library, and stored at − 20 °C until sequencing.

The DNA was sequenced using Illumina MiSeq sequencing and was analyzed using R Studio. The samples were trimmed using a minimum quality score of 30 and filtered with a maximum of 5 ambiguous bases. The minimum overlap was determined for each tissue to maximize the number

of reads. All samples were then decontaminated using the 0.5 filter of R decontam.

The resulting ASVs were then analyzed using microbiomeanalyst.ca Marker Data Profiling. The minimum count of the low count filter was changed to 2 and the data transformation was changed to centered log ratio, while all other settings were left as the default.

The datasets presented in this study can be found in the National Center for Biotechnology Information (NCBI) BioProject online repository, https://www.ncbi.nlm.nih.gov/bioproject/ using accession number: PRJNA855906.

Statistical Analysis

The nitrite water concentration was analyzed using Prism 9 (GraphPad) to graph the nitrite concentrations with mean ± SEM to show the variation between the four treatment replicates. An ordinary one-way ANOVA, $P < 0.05$, was used to determine if the treatments were significantly different from each other.

The resulting amplicon sequence variants (ASVs) were then analyzed using microbiomeanalyst.ca Marker Data Profiling (McGill University) [33]. The minimum count of the low count filter was changed to 2 and the data transformation was changed to centered log ratio, while all other settings were left as the default. The relative abundance of each of the microbiomes was visualized using the stacked bar/area plot option with the desired taxonomy level. The stacked bar plot used percentage abundance showing the top 10 taxa based on the total number of taxa. Significance was determined using a DESeq2 (Bioconductor) differential abundance analysis method with an adjusted *P* value cutoff of 0.05.

The significant difference between overall microbial communities was determined using Past3 (PAlentological STatistics). This was determined using a one-way PERMANOVA multivariate test to create a pairwise Bray–Curtis comparison with Bonferroni-corrected *P* values.

Results

Water quality measurements taken during the experiment were used to determine the actual concentration of nitrite in the water to ensure the exposure concentration aligned with our desired treatment concentrations. The increasing concentrations of nitrite significantly impacted ($P < 0.05$) the microbial composition of the nose, skin, and gills in at least one treatment (Table 1). However, the composition of the water microbiome did not significantly change in any of the treatments. The change in composition in the tissues represents a shift in the relative abundance of the present taxa of the microbiome. As a result, significant microbiome shifts do not reflect significant changes in individual taxa.

Nasal Microbiome Composition

The relative abundance of each treatment was then assessed for each tissue. The changes in relative abundance reflect trends seen in the number of ASVs relative to each other. These ASVs refer to single DNA sequences which then could be identified to different bacterial taxa. The composition of the nasal microbiome was significantly different from the control in both the 0.1 mM and 1.0 mM treatments (Table 1). At the class level, as the concentration of nitrite increased, there appeared to be a decrease in the relative abundance of Gammaproteobacteria when compared to the control. Inversely, there was an increase in the relative abundance of Planctomycetes, Bacilli, and Bacteroidia in the 0.1 mM treatments and Alphaproteobacteria and some other classes in smaller relative abundances (Fig. 1A). Further examination of the genus showed there was a decrease in the relative abundance of *Pseudomonas* and *Yersinia* in the nitrite treatments. New genera were also identified that were not observed in the control as the concentration of nitrite increased (Fig. 2). Additionally, there was a significant decrease in *Luteolibacter* which was found in the control and not in either of the nitrite treatments (Fig. 5).

Table 1 Comparison of the tissue microbiomes between nitrite treatments. Numbers indicate the pseudo-*F* and *P* values for a one-way PERMANOVA of tissue microbiomes exposed to various concentrations of nitrite and significant differences are marked with an asterisk. Significance was determined using a Bray–Curtis with *P* values corrected using Bonferroni-corrected values ($P < 0.05$) with $n = 8$ for each tissue-treatment pair. The microbiome composition was analyzed from ASVs for each tissue at each nitrite concentration

Treatment	Nose		Skin		Gill		Water	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Control VS. 0.1 MM	3.338	0.0018*	1.223	0.4446	1.623	0.1944	1.524	0.3354
Control VS. 1.0 MM	3.315	0.0027*	1.322	0.0213*	2.565	0.006*	1.187	0.6990
0.1 mM VS. 1.0 mM	2.111	0.147	0.525	1	1.604	0.1071	0.1716	1.680

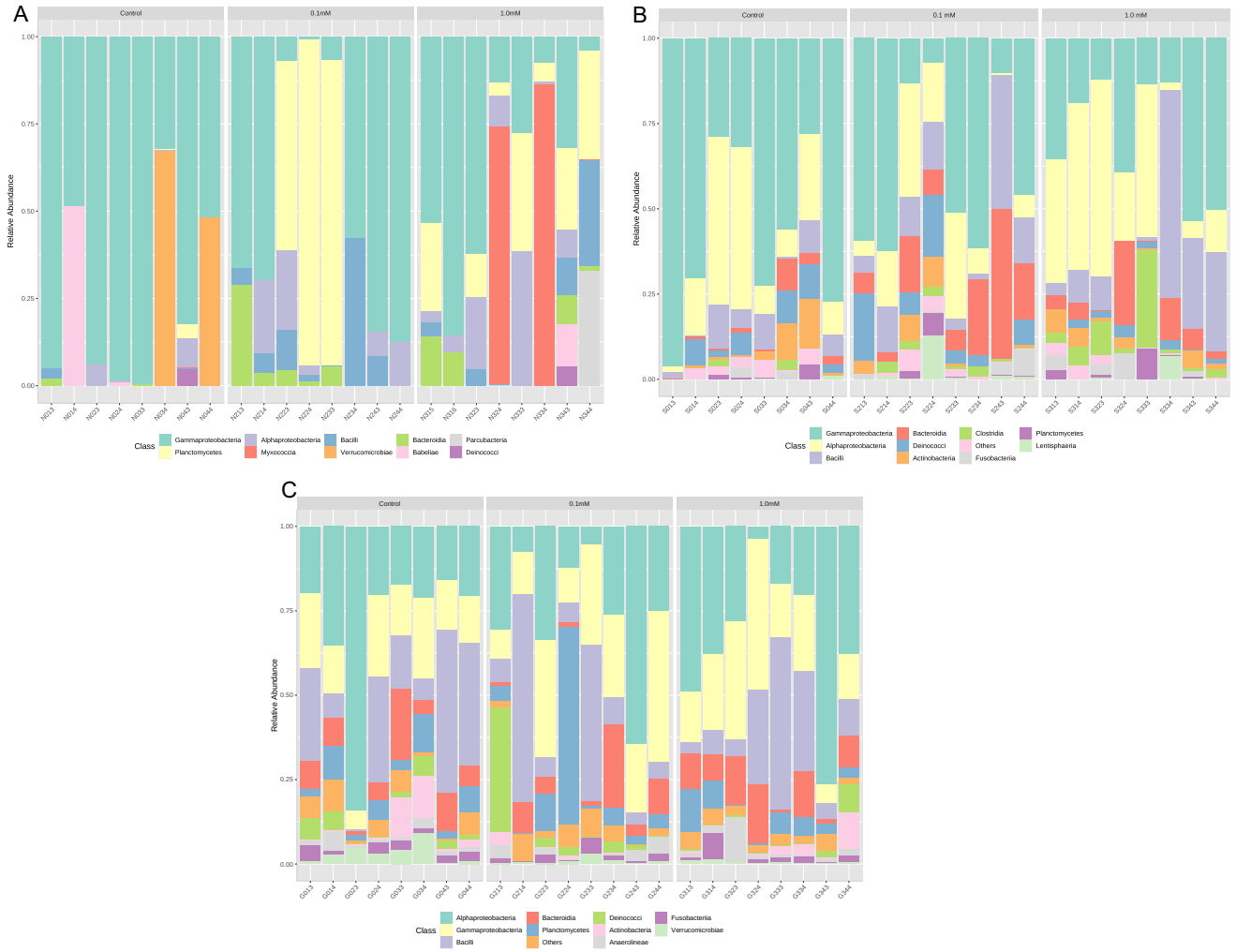

Fig. 1 Comparison of the relative abundances of the classes in the goldfish nose (**A**), skin (**B**), and gill (**C**) tissues at the 0.0 mM, 0.1 mM, and 1.0 mM treatments. Each number in the X-axis represents a different individual

Skin Microbiome Composition

The composition of the skin microbiome was only significantly different between the control and the 1.0 mM treatment (Table 1). The skin microbiome is predominantly Gammaproteobacteria and Alphaproteobacteria, but as the concentration of nitrite increase, Alphaproteobacteria increased in relative abundance over Gammaproteobacteria (Fig. 1B). The relative abundance of the less abundant taxa also increased, although variably between individuals (Fig. 1B). The composition of the skin microbiome appeared to be more variable within treatments than the other tissues examined although the treatments did show some variation in relative abundance. As the concentration of nitrite increased, there was an increase in the relative abundance of other groups and a general decrease in *Yersinia*, *Pseudomonas*, *Massilia*, and *Branchiomonas* (Fig. 3). However, no ASVs were determined to be significantly different between treatments.

Gill Microbiome Composition

The composition of the gill microbiome also only showed significant differences between the control and 1.0 mM treatment, similar to the skin microbiome (Table 1). The relative abundance of Alphaproteobacteria also increased in the gills as the concentration of nitrite increased (Fig. 1C). Bacilli was the only group to noticeably decrease in relative abundance at the class taxonomy (Fig. 4). Examining the genus, *Hyphomicrobium* and *Acidovorax* increased in relative abundance (Fig. 5). There also appeared to be more variability between individuals within the nitrite treatments. The gills had the most ASVs that were determined to be significantly different between treatments (Fig. 5). There was a small increase in *Nitrobacter vulgaris*, and *Hyphomicrobium* species, *Emticicia aquatilis*, and *Rickettsiales* in the 1.0 mM

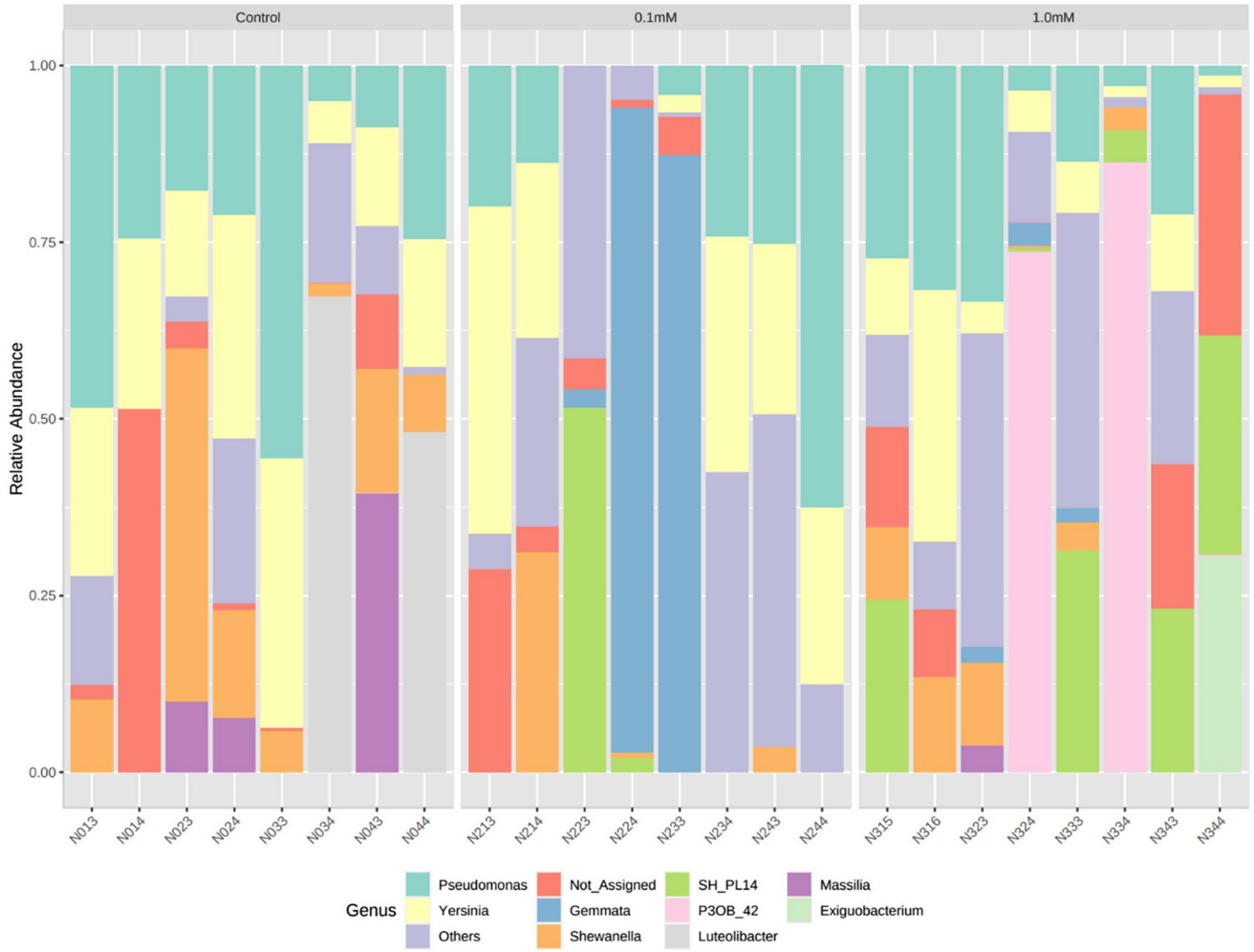

Fig. 2 Relative abundance of nasal microbiome genera. The top ten genera represented in the nasal microbiome were examined across various nitrite treatments (0.0 mM, 0.1 mM, and 1.0 mM). As the concentration of nitrite increased, new genera were introduced into the microbiome; more classes of bacteria were incorporated into the microbiome. The control is significantly different from the 0.1 mM and 1.0 mM treatments as determined using a Bray–Curtis one-way PERMANOVA with P values corrected using Bonferroni-corrected values ($P < 0.05$). On the X-axis, each sample is identified by a letter indicating the tissue it was isolated from and the numbers indicating the treatment, tank, and fish number, respectively

treatment. A few ASVs also decreased in concentration, including *Gammaproteobacteria Incertae Sedis*.

Discussion

The increase in nitrite significantly changed the composition of each of the tissue microbiomes in at least one of the treatments. The change in composition included shifts in the relative abundance of bacterial taxa, the disappearance of taxa normally present in the microbiome, or the observation of new taxa not identified in the control. The water microbiome was the only one that did not significantly change when exposed to elevated concentrations of nitrite. The detection of new genera in nitrite expose tissues may be due to growth of these communities to detectable levels or the loss of some communities due to the nitrite treatment that allowed other bacteria genera in the water to colonize the tissues.

The sublethal concentrations selected are found in natural waterways and aquaculture systems which fish would realistically encounter [9, 14]. Thus, environmental changes due to nitrogenous pollution in natural waters can affect the microbiomes of natural populations. These concentrations were also found to cause behavioral changes, damage tissue, and impact olfaction in previous research in the Huertas Lab, although no other major signs of stress were found, including lack of appetite and erratic swimming [13, 34]. Therefore, the association between changes in the microbiome and sublethal concentrations of nitrite in fish physiology needs to be further explored. For instance, if disturbances to microbiomes due to nitrite

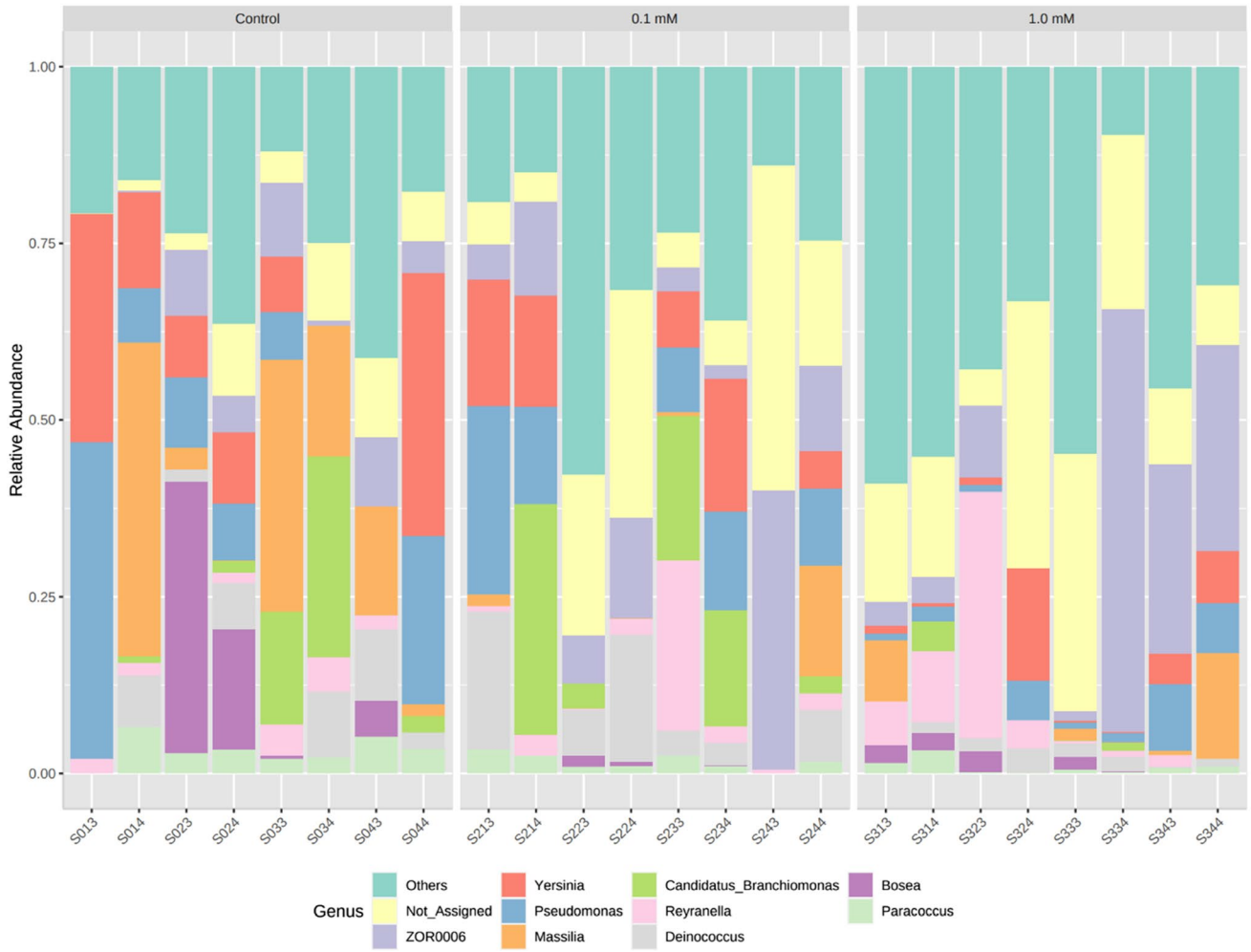

Fig. 3 Relative abundance of skin microbiome genera. The top ten most abundant genera represented in the skin microbiome were examined across various nitrite treatments. The skin microbiome is variable both within and between treatments. The control is significantly different from the 1.0 mM treatment as determined using a Bray–Curtis one-way PERMANOVA with P values corrected using Bonferroni-corrected values ($P < 0.05$). On the X-axis, each sample is identified by a letter indicating the tissue it was isolated from and the numbers indicating the treatment, tank, and fish number, respectively

are connected to physiological dysfunction, the use probiotic treatments may be beneficial in fish aquaculture.

The dominant classes of bacteria in the water microbiome are Alphaproteobacteria and Bacteroidia. Many species of Alphaproteobacteria are known to metabolize nitrite, usually specializing at utilizing either high or low environmental concentrations [35]. A member of Bacteroidia, *Sediminibacterium* species, were identified in all water treatments and some species are known to use a variety nitrogen sources [36]. As a result, the water microbiome might have already had a large population of bacteria capable of fixing nitrite, so there would have been less selective pressure on the overall microbial composition as the concentration of nitrite increased. Additionally, the continuous flow of the water in the setup may have made it more difficult for selective pressures to influence the composition of the bacterial communities in the water.

The normal nasal microbiome in fish is not well studied. However, the predominance of Gammaproteobacteria seen in the goldfish nasal microbiome has also been found in zebrafish and rainbow trout. This indicates that the untreated nasal microbiome in the goldfish is similar to other fish species [25, 37]. At the genus level, the control microbiome was mainly composed of *Pseudomonas* and *Yersinia*. *Yersinia* species are usually pathogenic to fish; however, despite composing about 25% of the microbiome the fish exhibited no symptoms of disease, including enteric redmouth disease [38]. Additionally, the fish did not show signs of stress, including loss of appetite, erratic swimming, or increased respiration. The nasal microbiome had the smallest number of genera which could indicate that these genera were selected to play a specific role in the tissue, including supporting the

Fig. 4 Relative abundance of gill microbiome genera. The top ten genera represented in the gill microbiome were examined across various nitrite treatments (0.0 mM, 0.1 mM, and 1.0 mM). The control is significantly different from the 1.0 mM treatment as determined using a Bray–Curtis one-way PERMANOVA with *P* values corrected using Bonferroni-corrected values ($P < 0.05$). On the X-axis, each sample is identified by a letter indicating the tissue it was isolated from and the numbers indicating the treatment, tank, and fish number, respectively

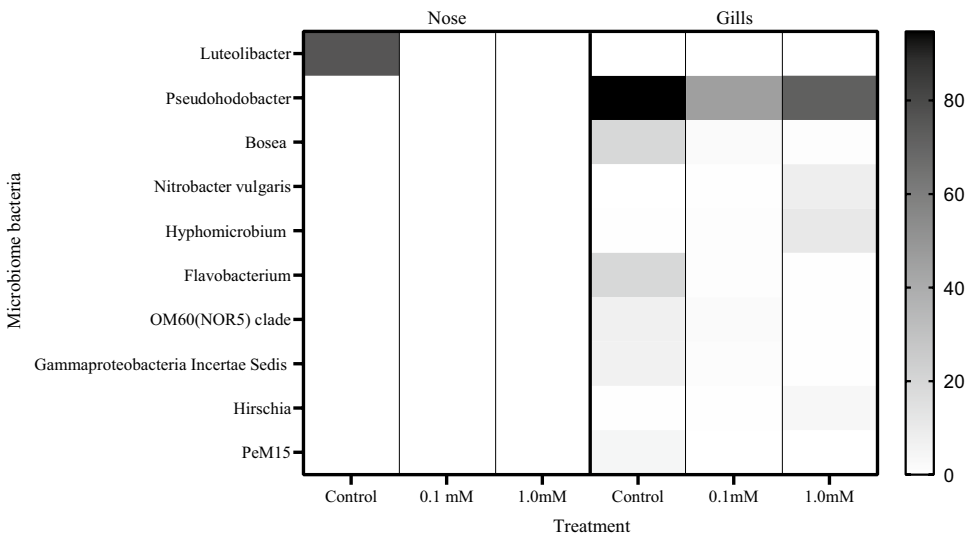

Fig. 5 Normalized changes in counts of bacterial species between nitrite treatments for the nose and gills. Top 10 most abundant ASVs that were significantly different between at least two treatments had normalized counts across the tissues. The counts were normalized by averaging the counts for each treatment and setting the smallest mean as 0% and the largest mean as 100% and using the resulting percentages as normalized values. The ASVs were then identified to their most specific classification and graphed to show the changes across treatments

immune system or mediating the cells associated with the nasal epithelium [37]. Bacteria have also been shown to directly influence behavior by releasing metabolites that are smelled by the host; however, this has not been confirmed in fish [39, 40]. No other work has described the basic structure of the goldfish microbiome and future work is needed to determine the role and importance of these bacteria in fish health and olfaction.

With regard to nitrite exposure, our results confirm the hypothesis that environmental nitrite pressures fish microbiomes and can significantly change its composition. The composition of the nasal microbiome changed significantly with increasing nitrite concentration in the water. As a result, the 0.1 mM and 1.0 mM treatments both had a significantly different microbiome composition than the control. These results show that the nose microbiome is affected at lower nitrite concentrations than the gill or skin microbiomes, significantly changing in the 0.1 mM treatment. As the concentration of nitrite increased, the microbiome shifted from mainly Gammaproteobacteria to having a larger relative abundance of other classes. When looking at the genus level, there was an increase in other groups within the microbiome. Factors that cause changes to the relative abundance of bacteria found in the microbiome can increase the chance for opportunistic pathogens [41]. Additional genera, which may increase diversity, can indicate that the microbiome is being disrupted and allowing other bacteria to colonize the tissue which are more likely to be pathogenic. There was also a decrease in *Pseudomonas* and *Shewanella* as the concentration of nitrite increased. Several *Shewanella* species can metabolize nitrite, but this usually occurs as a stress response and negatively impacts other pathways [42–44]. *Shewanella* likely decreased in relative abundance because of the stress of prolonged nitrite exposure. A similar affect may be occurring in *Pseudomonas* as some species experience nitrite toxicity as nitrite accumulates [45]. *Luteolibacter* was found only in the control and disappeared in both treatments. Some studies have shown that *Luteolibacter* can serve as a bacterial indicator of good water quality in aquatic systems [46, 47]. They were found in clean water systems and to return when nitrogenous compounds were removed from previously contaminated systems. The absence of this genus in the nose during both treatments indicates a decrease in water quality resulting from added nitrite.

Examining the skin microbiome, the 1.0 mM treatment had a significantly different composition than the control. There was a lot of variability between individuals of the same treatment. Increased variability within treatments has been seen in other studies investigating the skin microbiome possibly indicating it is less stable, although this has not been studied explicitly in fish [26, 27, 48, 49]. Since the skin is in direct contact with the water, changes to the microbiome are more likely to reflect changes occurring in the water microbiome than the other tissues. There was an increase in the relative abundance of an others group which indicates an increase in diversity within the microbiome and a general decrease in Gammaproteobacteria similar to what was seen in other tissues. However, no amplicon sequence variants (ASVs) were found to be significantly different between treatments. This means that while there were significant shifts in the overall composition at higher nitrite treatments, no individual genera were selected for or against. Nitrite has less of an impact on the skin microbiome than the other tissues, which may reflect what was seen in the water microbiome.

Like the skin microbiome, the composition of the gill microbiome was only significantly different between the control and the 1.0 mM treatment; however, the gills had the most ASVs that were significantly different between treatments. The gills create a unique water environment to facilitate the movement of ions by reducing the pH in the immediate filament interspace (also called the ammonium trap mechanism [50]); this environment is likely being changed with the addition of nitrite [51]. Unlike other tissues, individual ASVs were impacted by the nitrite more than the overall composition of the gill microbiome. Most of the changes did not seem to be related to the group's ability to metabolize nitrite. *Pseudorhodobacter* are denitrifying bacteria which initially decreased in relative abundance and then increased in the 1.0 mM treatment [52, 53]. *Bosea* and *Flavobacterium* have nitrifying species; however, the genus disappeared when nitrite was added [54, 55]. Other genera that increased or decreased in relative abundance did not appear to be highly linked to nitrite or nitrogen oxidation. However, the exception to this is the increase in relative abundance of *Nitrobacter vulgaris* seen in the gills. This bacteria is common in freshwater systems and is known to metabolize nitrite [56]. It likely increased in relative abundance due to the increased concentration of nitrite since it was most prominent in the 1.0 mM treatment of the gill's microbiome.

Exposure to nitrite changed the composition of each of the tissue microbiomes, although the changes vary depending on the physiology of the tissue. Significant changes of the microbiome can lead to an increased risk of disease and death in fish. Given that there are high mortality rates and high concentrations of nitrite in aquaculture, more research is needed to determine if the changes to the tissue microbiomes lead to disease and eventually death [2]. Moreover, most aquaculture systems are maintained at sublethal nitrite concentrations between 0.1 and 3 mM. These concentrations usually

do not cause any visual stress in fish so no preventive measures are taken; however, they can lead to hidden deleterious effects [13, 34]. Our results show that even these lower concentrations can impact the composition of the fish microbiomes which could lead to chronic stress and disease. Gaining a better understanding about how nitrite impacts the tissue microbiomes can help with nitrite management strategies in settings where elevated levels of nitrite are unavoidable.

Nitrite is not only an aquaculture byproduct, but is also found as a common environmental pollutant resulting from agricultural pollution and nutrient buildup [15]. Thus, the effects of nitrite on the microbiomes of other aquatic animals and at which point of development or physiological stages are most affected by nitrite-induced changes to the microbiome need to be explored in future studies. The changes seen in the goldfish could be negatively impacting the microbiomes, and overall health, of numbers of aquatic and land organisms. The concentration of nitrite in our natural waterways can range between concentrations lower than 0.001 mM to higher than 1 mM [14, 57]. Thus, nitrite pollution may be having a more significant impact on the greater microbiome ecosystem and human health, since nitrite can accumulate in drinking water, than previously known. The broader impact of nitrite on vertebrate microbiomes and, subsequently, physiology has not been studied. More research in this area is needed to properly determine the broader impact of nitrite exposure on vertebrate microbiomes, the subsequent physiological impact, and ways to mitigate the negative effects. Future directions include investigating the role of the impacted bacteria on the microbiome and tissues, determining the impact of microbiome changes to fish fitness, and researching ways to counter these impacts including the use of pre- and probiotics.

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Declarations

Ethics Approval IACUC #7074.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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