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Research Article

Changes in fish skin microbiota along gradients of eutrophication in human-altered rivers

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One sentence summary: Eutrophication, due to anthropogenic pressures, is related to changes in skin microbiota composition and diversity in a freshwater fish species.
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Abstract

The skin microbiota plays a major role in health of organisms but it is still unclear how such bacterial assemblages respond to changes in environmental conditions and anthropogenic perturbations. In this study, we investigated the effects of the eutrophication of freshwater ecosystems on the skin microbiota of fish. We sampled wild gudgeon *Gobio occitaniae* from 17 river sites along an eutrophication gradient and compared their skin microbiota diversity and composition, using a 16s rRNA gene metabarcoding approach. Results showed a tendency for higher taxonomic and phylogenetic diversity in highly eutrophic sites linked to the presence of suspended organic matters. We also highlighted significant links between eutrophication and skin microbiota taxonomic composition and beta-diversity. In contrast, skin microbiota characteristics did not correlate with host factors such as age or sex, although microbiota beta-diversity did vary significantly according to host parasite load. To conclude, our study highlights the importance of environmental factors, especially eutrophication, on the diversity and composition of skin mucus bacterial communities. Because changes in the skin microbiota may induce potential deleterious consequences on host health and population persistence, our results confirm the importance of accounting for host-microbiota interactions when examining the consequences of anthropogenic activities on aquatic fauna.

Keywords: bacteria, disturbances, fish, microbiota, agriculture, urbanization

Introduction

In freshwater ecosystems, one of the main consequences of human urban and agricultural activities is eutrophication. Eutrophication is characterized by an increase of nutrients such as phosphorus, nitrites, and suspended organic matters (SOM) which leads to a decrease of oxygen and light availability (Nasir Khan 2014). Thus, eutrophication can strongly impact freshwater fauna and especially fish by modifying habitat quality, energy flow, and food webs (Weisse 1991). Accordingly, fish living in eutrophicated rivers display significant alterations in morphology, physiology, with consequences for fitness and population dynamics (Tammi *et al.* 1999, Vonlanthen *et al.* 2012, Alexander, Vonlanthen and Seehausen 2017). Alongside these direct effects, eutrophication may also impact fish through the modifications of microbial communities that are hosted in the gills, the gastrointestinal tract, and skin mucus. The skin mucus microbiota (SMM) is of particular interest in the context of eutrophication, first because it is in direct contact with the surrounding water and is likely to be particularly influenced by changes in the environment (Callewaert, Ravard Helffer and Lebaron 2020, Krotman *et al.* 2020) and second, because it plays a pivotal role in host health and fitness (Ángeles Esteban 2012, Saleh *et al.* 2019). In fish, the skin mucus constitutes an important protective barrier composed of glycoproteins or mucins, innate immune cells, lipids, and microbial communities (Ángeles Esteban 2012) that play a major role in fish resistance to pathogens and diseases (Galindo-Villegas *et al.* 2012, Mohammed andArias 2015). The protection conferred by SMM against pathogens may occur passively by competition with pathogens for space and nutrients (Balcazar *et al.* 2006) or actively through the production of inhibitory compounds (Boutin *et al.* 2012, 2013).

Like all host-associated microbiota, previous studies suggest that fish SMM is influenced both by individual characteristics of the host (species identity, morphology, physiology, or behavior) (Larsen *et al.* 2013, Boutin *et al.* 2014), and environmental characteristics (Chiarello *et al.* 2019), including environmental parameters such as water temperature, pH, and conductivity (Sylvain *et al.* 2019, Krotman *et al.* 2020) and anthropogenic disturbances such as urbanization (Colin *et al.* 2021). For instance, environmental perturbations can alter the relative abundance of some phyla such as Proteobacteria, Actinobacteria, and Bacteroidetes in fish thereby altering the ratio Proteobacteria/ Bacteroidetes which is

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associated with dysbiosis and decreased fish health (Krotman *et al.* 2020). The ratio between potentially pathogenic bacteria compared to non-pathogenic bacteria could also potentially be altered by environmental stress (Chowdhury, Sahu and Das 1996, Fang *et al.* 2016), although this question remains unclear. Taxonomic composition is thus likely to be strongly impacted in the context of anthropogenic pollution (Mlejnková and Sovová 2010). Accordingly, previous studies thus suggest that there is a complex interplay between host and environmental characteristics in shaping the SMM of freshwater fish (Llewellyn *et al.* 2014, Lokesh and Kiron 2016), but their relative roles remain poorly understood. There is thus an urgent need to further examine and disentangle the relative effects of host and environmental factors on the diversity and composition of skin mucosal bacterial communities in human-altered environments. This fundamental question is all the more important in the context of the rapid degradation of water quality and eutrophication, because eutrophication of aquatic systems strongly alters both environmental quality and host physiology and health (Budria 2017, Côte *et al.* 2021), which may alter the relative influence of these factors on the SMM. Assessing the impact of eutrophication on the SMM of fish is also important to better predict its repercussion on health because skin microbiota may either mitigate, through plastic and adaptive processes (Alberdi *et al.* 2016) or on the contrary further enhance detrimental effects (O'Sullivan *et al.* 2019, Grisnik *et al.* 2020). However, there is currently a lack of large-scale studies comparing skin microbiota composition along gradients of eutrophication within the same host species, thereby hindering our understanding of the effect of anthropogenic perturbations on fish-microbiota interactions.

In this study, we sampled the SMM of 17 populations of gudgeon *Gobio occitaniae* living in habitats showing different levels of eutrophication and used a metabarcoding approach to describe SMM diversity and composition. We chose this fish model species because it is widely exposed to human-driven alterations in rivers, and shows a high level of intraspecific variability in the study area (South-West of France). First, we examined the effects of eutrophication and host traits on SMM taxonomic and phylogenetic alpha-diversity. Second, we tested the impact of eutrophication and host traits on SMM beta-diversity, i.e. community composition. Third, we compared the bacterial communities of fish skin with bacterial communities found in the water, to test the specificity of skin microbiota compared to the surrounding environment.

Material and methods

Study populations

Seventeen sampling sites sharing similar climatic and geological characteristics were selected in the Adour-Garonne watershed (South-Western France). These sites were chosen along gradients of water quality and land use (urban, agricultural, and natural) (Fig. 1), based on a previously published study (Côte *et al.* 2021). Land use was characterized using the CORINE Land cover database (2012) (https://land.copernicus.eu/pan-european/corine-land-cover/clc-2012/), with circular buffers of 1 km around the capture site representative of the habitat range of the species. We then focused on physicochemical variables characterizing local variations in hydrology and water quality, based on their ecological relevance and on previous studies in the same study area (Côte *et al.* 2021). In particular, we extracted temperature, SOM, dissolved oxygen, nitrites, and phosphorus from the SIEAG database of the Adour-Garonne Water French Agency (http://adour-garonne.eaufrance.fr/), and water flow regime from the RHT database (Pella *et al.* 2012). Data from year 2012 to year 2016 were used to reflect an average value based on the average lifespan of *Gobio sp.* (4 years). We then computed a multivariate PCA analysis (R library *ade4*) and extracted the first axis (41% of the variance) as a synthetic environmental variable reflecting an 'eutrophication gradient': highly eutrophic sites corresponded to agricultural sites with high levels of phosphorus, SOM, nitrites, warm water, and low oxygen levels. Coordinates of sites on this axis were extracted and used as an index of eutrophication level throughout the manuscript (see also (Côte *et al.* 2021; Fig. S1, See online supplementary material for a color version of this figure).

Fish sampling and measurements

Up to 20 gudgeon fish *Gobio occitaniae* per population were caught by electrofishing after authorization by the local authorities (Fig. S1, See online supplementary material for a color version of this figure). All procedures were conducted in compliance with European (2010/63/UE) and national legislation for animal experimentation (art L. 436–9 and 432–6). Fish capture was conducted under the authorization of local authorities ('Direction Départementale des Territoires') and approved by the ethical committee n°073 (authorization n°853). Individuals were anaesthetized with eugenol (0.1 ml^{-1}), their fork length measured to the nearest mm and weighed to the nearest mg. Fulton condition index was also calculated as: mass (mg)/ $size^3$ (mm) (Froese 2006). On each individual, we counted the number of *Gyrodactylus* ectoparasites on the pelvic fin in order to estimate parasite load. Such parasites, when abundant, can affect fish health and microbiota composition as they attach to the host body and fins and feed on mucus. We also sampled three water samples for each site and samples of skin microbiota (see below).

Microbiota sampling, PCR amplification, and high-throughput sequencing

To sample the skin microbiota of the fish, we used a sterile swab (Dutscher, Brumath, France) applied on the mucus of the left side of the fish. The same manipulation was performed on all fish in order to standardize the measure and the quantity of mucus collected to the best of our ability. For each site, water samples were collected by using a sterile tip soaked for 3 minutes in water in three different areas within the site. Although this is not the standard procedure to characterize bacterioplankton communities, our aim here was to compare the SMM composition with bacterioplankton sampled using the exact same sampling procedure (Fig. S2, See online supplementary material for a color version of this figure). Moreover, we also opened sterile swabs with which no sampling was performed so as to evaluate potential contamination during the manipulation of the swab. Non-used swab controls were also collected. Samples were then stored at –20°C for further molecular analyses. We extracted bacterial DNA using the QiagenDNeasy® Blood & Tissue Kit and the standard protocol designed for purification of DNA from Gram-positive bacteria (Qiagen, Venlo, Netherlands). The V5-V6 region of the bacterial 16S rRNA gene was amplified by PCR using the following universal primers: Forward: 5′-GGATTAGATACCCTGGTAGT-3′ and reverse: 5′CACGACACGAGCTGACG-3′ (Fliegerova *et al.* 2014). We performed the PCR amplification in a 20 μL mixture including 2μL of 1/10 diluted DNA, 1U of AmpliTaq Gold DNA Polymerase, 0.4 μM of each primer, 1 × of Taq Buffer, 0.16 μL of Bovine Serum Albumin, 0.2 mM of each dNTP, 2.5 mM$MgCl_2$ and 12.06 μL water and following this program: initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 30 seconds, hybridation at 57°C

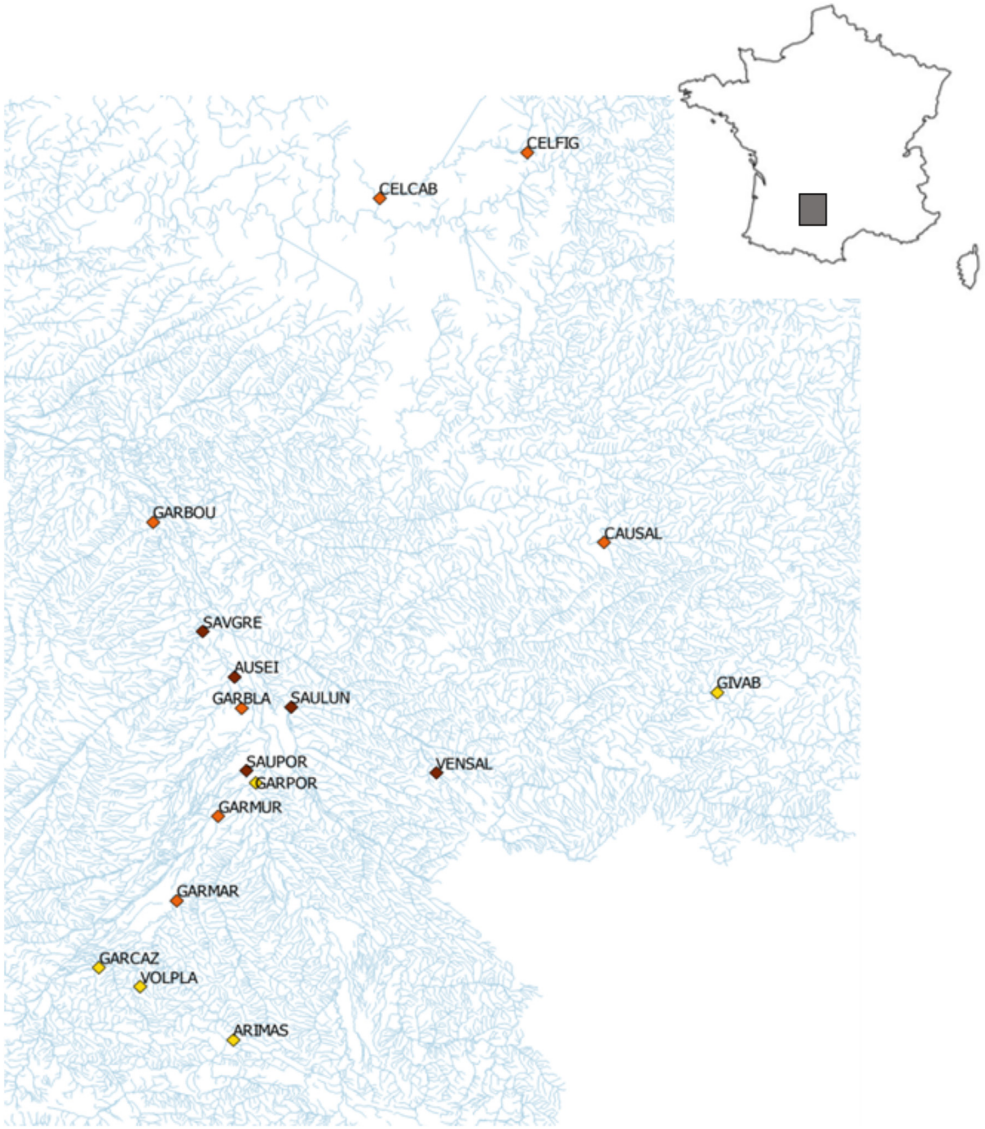

Figure 1. Sample sites. Yellow points represent the sites with low level of eutrophication, orange points represent the sites with medium level of eutrophication and the red points represent sites with high levels of eutrophication.

for 30 seconds and elongation at 72°C for 90 seconds (Fliegerova *et al.* 2014). Some combinations of 8 bp tags were also added at the 5' end of forward and reverse primers so as to identify samples after sequencing part. Positive and negative controls but also replicated PCRs of samples were also included in the analysis so as to evaluate PCR effectiveness. We collected 4 μL of amplicons per sample and pooled them with other samples. The library construction and the sequencing by Illumina MiSeq 250 bp paired-end V3 were performed at the Genotoul-GeT-PlaGe core facility, Toulouse, France.

Bioinformatic analysis

We used the OBITools package developed by Boyer *et al.* 2016 to process and filter the Illumina sequences. First, we kept consensus reads with quality upper 50 and aligned paired-end reads in consensus sequences. Then, we assigned reads to their samples. Then, we dereplicated reads and we removed the reads appearing only once in the data set (called singleton). We then clustered reads into OTUs (Operational taxonomic units) using the *Sumaclust* algorithm (https://git.metabarcoding.org/obitools/sumatra/wikis/home) based on a similarity threshold of 97%. The taxonomic assignation was then performed using RDP classifier software based on the taxonomy proposed in Bergey's Taxonomic outline of the Prokaryotes (Wang *et al.* 2007).

After data set curation, we obtained a data set with a total 8716 OTUS for 338 samples. We first removed OTUs with less than 10 sequences and OTU with a total abundance lower than 0.005% of the total database abundance, following the procedure from Bokulich *et al.* (2013) as they are most likely sequencing errors. Then, we removed OTUs presenting higher mean abundances in negative controls than in biological samples (144 OTUs considered as contaminants and removed from the dataset). So as to take into account mistagging or tag-switching induced by PCR chimera, we substracted the mean abundance of blanks from the OTUs in biological samples. The final data set consisted of 293057 reads and 532 OTUs for 338 samples. Finally, we standardized the sequencing depth of each sample by rarefying to 1000 reads.

Alpha diversity analyses

We used the R software and the *Vegan* package (Oksanen *et al.* 2009) to compute most analyses. Alpha diversity was characterized by the calculation of taxonomic and phylogenetic indices. Among taxonomic indices, we calculated the Shannon index reflecting the overall taxa diversity, the Chao richness index as an abundance-based estimator of richness to take into account rare taxa, and Pielou's evenness index which compare the relative abundance of each taxa in a given sample to measure the aggregation of species (from 0: the community is dominated by one taxon in very high relative abundance 1: all taxa are in similar relative abundance in the community) (Oksanen *et al.* 2009).

Phylogenetic Diversity (PD) was also calculated to take into account phylogenetic differences and account for shared evolutionary history between bacterial taxa, while the phylogenetic evenness (PSE) index was calculated as measure of the distribution of OTUs among branches in a phylogenetic tree (Webb and Pitman 2002). These indices were calculated using the *Picante* package (Kembel *et al.* 2010). The phylogenetic tree of OTUs used for the calculation of phylogenetic indices was generated by aligning OTUs representative sequences using Seaview software. Then, we used RAxML and the GTRCAT substitution model for nucleotide sequences to build the phylogenetic tree.

First, we tested the link between alpha-diversity indices and eutrophication level using linear mixed models. Eutrophication (coordinates along the first axis of PCA, Principal Component Analysis) was included as a fixed environmental effect, while fish size, sex, and parasite load as fixed covariable effects. The number of reads was also included as a fixed effect in all models. The site identity nested within the upstream–downstream section and the river size was included as random effects to take into account shared conditions within sites. To test detailed effects of specific physicochemical variables linked to eutrophication, we also ran complementary additional mixed models with SOM, temperature, or oxygen rate separately when necessary.

Taxonomic composition

We compared the taxonomic composition of the SMM among sites with varying degrees of eutrophication because the relative abundance of some phyla such as Proteobacteria, Actinobacteria, and Bacteroidetes can change in altered environments (Krotman *et al.* 2020). We especially focused on the different classes within Proteobacteria, which have been shown to respond to anthropogenic pollution (Mlejnková and Sovová 2010). The Proteobacteria/Bacteroidetes relative abundance ratio was also investigated since it is known to be associated with dysbiosis and fish health, a high Bacteroidetes/Proteobacteria ratio suggesting reduced health for individuals (Legrand *et al.* 2018, Krotman *et al.* 2020).

Bacterial taxonomic composition was studied using the *Phyloseq* package in R and compositional barplots were obtained. So as to determine which taxonomic phylum was the most discriminant among the different groups of eutrophic sites (low, medium, or high levels of eutrophication), we used a linear discriminant analysis (LDA) effect size (*Lefse* analysis, Segata *et al.* 2011). The *Lefse* method is used so as to identify the OTUs presenting the highest differences in abundances among groups. Lefse analyses were computed using the galaxy software using LDA scores > 2 and a *P*-value < 0.005 as thresholds (https://huttenhower.sph.harvard.edu/galaxy/).

In addition, we tested the relative abundance of potentially pathogenic and non-pathogenic genera. The pathogenicity status of each OTU was determined according to the genus to which it had been assigned. Our database does not identify the OTU until the species level. Consequently, based on the literature, we considered the following genera as potential pathogens for fish organisms (Chiarello *et al.* 2019), although all the species from these genera are not pathogens : *Flavobacterium*, *Aeromonas*, *Pseudomonas*, *Acinetobacter* (Austin and Austin 2016), and *Vibrio* (Frans *et al.* 2011).

Beta diversity

To compare SMM composition across sites and decipher the respective role of eutrophication and individual variables on microbiota β-diversity, we used the *Vegan* package (Oksanen, Kindt and Legendre 2013) using Bray–Curtis dissimilarity based on abundance community matrices, but also UNIFRAC distances. We used a permutational multivariate ADONIS analysis to test the variance partitioning due to the effects of eutrophication on beta-diversity. ADONIS was computed with 1000 permutations with the option 'margin' to test the marginal effect of each variable accounting for the effect of the other variables of the model, with the identity of the site included. We performed non metric multidimensional scaling (NMDS) ordination to graphically visualize microbiota β-diversity. We then tested whether NMDS scores (coordinates of points on axes of NMDS) were correlated to environmental (eutrophication) and individual factors (body size, sex, and parasite load), by computing an *Envfit* analysis. We also tested the homogeneity of dispersion among groups of eutrophication using the *Betadisper* function. *Betadisper* enables to compare the variance heterogeneity among levels of eutrophication (distance to centroid). Finally, we analyzed water microbiota using the same methods as described above, and compared water and skin microbiota, using Venn diagrams to describe the shared bacterial OTUs between water and mucus samples.

Results

SMM alpha diversity

Taxonomic and PD was marginally linked to eutrophication level (Table 1). Additional analyses on environmental variables analyzed separately showed that taxonomic and PD indices were significantly linked to the concentration of SOM, especially Shannon (Linear mixed models: $X^2 = 4.06$, $P = 0.04$), Chao (Linear mixed models: $X^2 = 5.08$, $P = 0.024$) and PD (Linear mixed models: $X^2 = 3.79$, $P = 0.05$) (Fig. 2). Fish body size had a significant effect on Chao and PD indices (Table 1). No effect of sex or parasite load was observed on alpha-diversity indices (Table 1).

SMM taxonomic composition

The taxonomic composition of the SMM was characterized by a predominance of Proteobacteria (relative abundance: $0.65 \pm 0.16\%$), Actinobacteria ($0.14 \pm 0.10\%$), and Bacteroidetes ($0.15 \pm 0.12\%$, Fig. 3). There was a significant effect of eutrophication on Proteobacteria ($X^2 = 3.87$, $P = 0.049$) and Actinobacteria ($X^2 = 5.50$, $P = 0.019$) with lower relative abundances of Proteobacteria and higher abundances of Actinobacteria in highly eutrophic sites (Fig. 3). Proteobacteria, Actinobacteria, and Bacteroidetes were the Phyla whose abundances best discriminated SMM of low and high levels of eutrophication, with a significantly higher abundance of Proteobacteria in low eutrophic mucus samples and significantly higher abundances of Actinobacteria and Bacteroidetes in highly eutrophic mucus samples (LEfSE analysis, Fig. 3; Fig S3, See online supplementary material for a color version of this figure). In contrast, results did not show any effect of eutrophication on the

Table 1. Results of linear mixed models testing the effect of eutrophication and fish traits (size, sex, and parasite load) on several indices of alpha-diversity for skin and water samples.

		Shannon			Chao			Evenness			PD			Phylogenetic evenness (PSE)		
	Variable	χ^2	df	P-value	χ^2	Df	P-value	χ^2	df	P-value	χ^2	df	P-value	χ^2	df	P-value
Skin	Eutrophication gradient	5.10	1	0.08	4.95	1	0.08	3.45	1	0.18	5.24	1	0.07	4.00	1	0.13
	Body size	0.52	1	0.47	5.66	1	0.02	1.59	1	0.21	5.44	1	0.02	1.57	1	0.21
	Sex	0.52	1	0.47	1.00	1	0.32	0.003	1	0.96	0.09	1	0.76	0.05	1	0.83
	Parasite load	2.32	1	0.13	0.39	1	0.53	0.16	1	0.68	0.34	1	0.56	1.08	1	0.30
Water	Eutrophication gradient	0.12	1	0.73	0.18	1	0.67	0.14	1	0.70	0.35	1	0.55	0.008	1	0.93

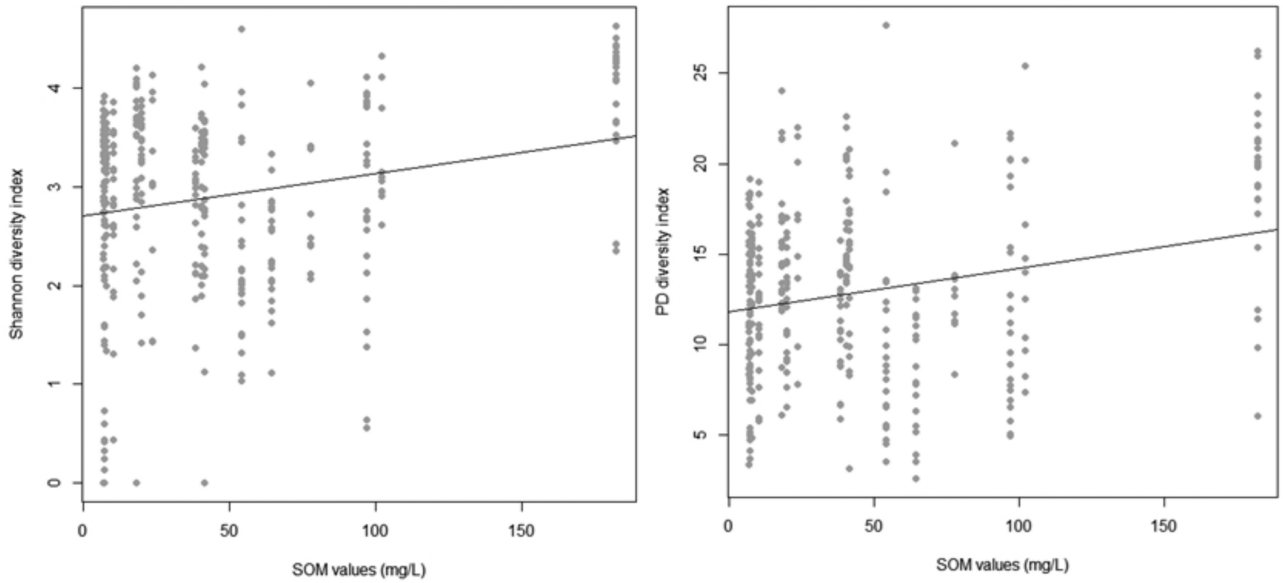

Figure 2. Relation between SOM concentration in the water and alpha-diversity of fish skin microbiota: Shannon diversity (left) and PD (right) index

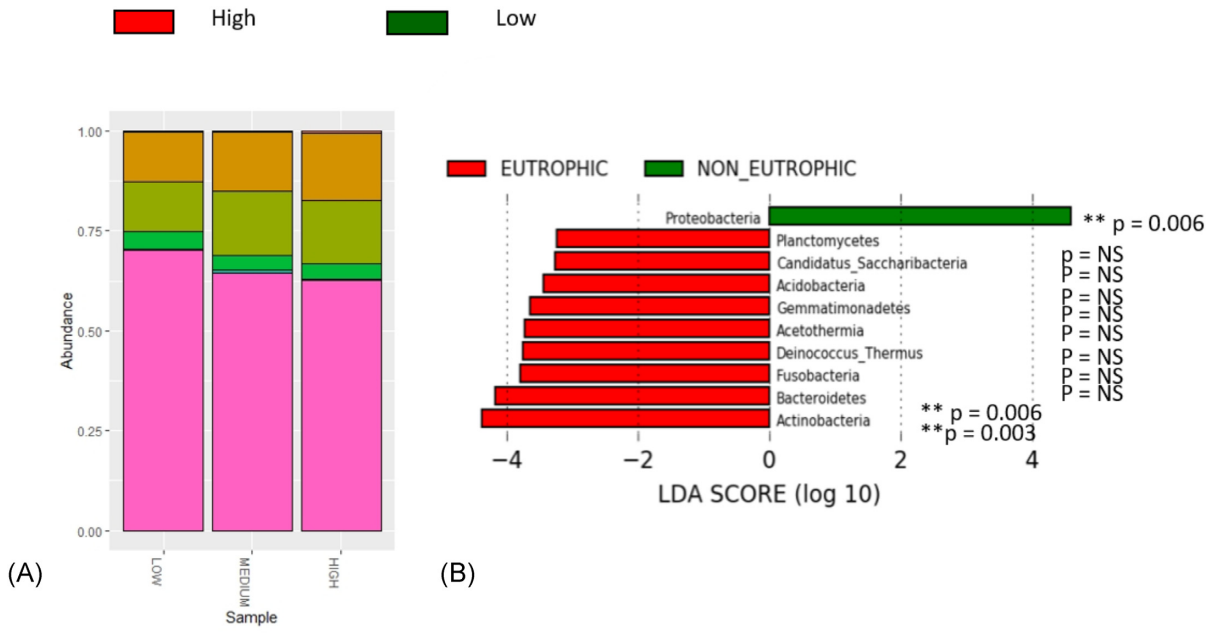

Figure 3. (A), Relative abundance of main bacterial phyla in skin samples according to the level of eutrophication (low, medium or high). **(B)**, LDA effect sizes representing families which significantly differ in abundance according to the levels of eutrophication.

Table 2. Results of permutational manova (ADONIS) testing the effect of eutrophication on beta diversity. Table also presents the betadisper analysis to test the homogeneity of variance among groups of eutrophication and land cover on mucus and water samples.

		Permutational analysis		Betadisper analysis	
	Variable	R^2	P-value	F	P-value
Skin	Eutrophication level	**10.16**	**0.001**	**4.12**	**0.017**
	Site	**6.23**	**0.001**	**4.11**	**<0.001**
Water	Eutrophication level	**3.79**	**0.001**	0.37	0.69
	Site	**2.32**	**0.001**	0.75	0.72

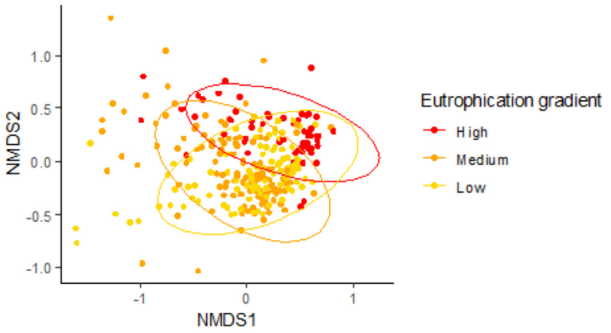

Figure 4. NMDS based on an abundance dissimilarity matrix (Bray–curtis distances) of the skin samples across the eutrophication gradient. Each color represents the level of eutrophication. Adonis analyses are significant ($P < 0.001$).

Table 3. Results of envfit models testing the effect of eutrophication and fish traits (body size, sex, and parasite load) on microbiota community dissimilarity in mucus samples.

Variable	R^2	P-value
Eutrophication gradient	**0.11**	**0.002**
Body size	0.009	0.48
Sex	0.007	0.34
Parasite load	**0.082**	**0.013**

relative abundance of potentially pathogenic and non-pathogenic OTUs ($X^2 = 2.36$, P = NS).

SMM beta diversity (community dissimilarities)

SMM dissimilarity was significantly influenced by the level of eutrophication of the environment (Table 2) with NMDS ordinations showing a strong discrimination in SMM composition in the three levels of eutrophication (Fig. 4; Fig S5, See online supplementary material for a color version of this figure). There was also a significant effect of site identity on SMM dissimilarities (Table 2). *Envfit* analyses showed a significant effect of eutrophication and parasite load on community dissimilarity but no effect of other traits such as body size and sex (Table 3). We also considered the degree of clustering according to the levels of eutrophication (distance to centroid, *betadisper* analyses) and showed a difference of variance among eutrophication levels (Table 2).

Comparison of water bacterial communities and SMM

First, we examined the bacterial communities of the water. The alpha diversity indices in water communities were not significantly related to eutrophication but were positively related to SOM, especially for the PD index ($X^2 = 4.81$, $P = 0.028$, Table 1). The taxonomic composition of water communities was characterized by a predominance of Proteobacteria (relative abundance: 0.65% ± 0.15%), Actinobacteria: (0.20% ± 0.10%) and Bacteroidetes (0.10% ± 0.07%), but did not vary significantly according to eutrophication (Fig. 5; Lefse analysis Fig. S4, See online supplementary material for a color version of this figure). Water community composition (dissimilarity), however, did differ significantly depending on eutrophication and site identity (Table 2, Fig. 6).

We then compared water and skin bacterial communities. Venn diagrams showed that the majority of OTU present in water samples were observed in skin samples, but fish skin included several specific OTU that were not found in the water (Fig. S6, See online supplementary material for a color version of this figure). The proportion of shared OTUs did not differ between the three levels of eutrophication (Fig. S6, See online supplementary material for a color version of this figure). We also tested the correlations between alpha diversity in skin and water samples and we found significant relations for Chao index (Pearson correlation: $r = 0.51$, $P = 0.03$) and PD index (Pearson correlation: $r = 0.60$, $P = 0.04$). Nevertheless, water and skin samples differed significantly in terms of community composition (PERMANOVA, Adonis analysis: $r^2 = 0.060$, $P = 0.001$, Fig. 6).

Discussion

Our results showed that environmental factors were key determinants of skin bacterial assemblages in the freshwater fish species *Gobio occitaniae*. Among environmental factors, eutrophication seemed to play a central role in the variation of fish skin microbiota, while host traits such as fish sex, size, and parasite load, were less linked to the variation observed in microbial communities.

Variation in the SMM along the eutrophication gradient

The level of eutrophication was associated to changes in skin microbiota alpha-diversity and beta-diversity. This is an interesting result since previous studies bring mixed results on the effects of environmental disturbances on microbial diversity in fish. For instance, Colin *et al.* 2021 showed a decrease in taxonomic and PD of fish skin microbiota along an urbanization gradient. In contrast, another study showed no effect of eutrophication compared to temperature and salinity on taxonomic and PD of fish skin (Krotman *et al.* 2020). In our study, sites with high levels of eutrophication showed a tendency to a higher taxonomic and PD of skin microbiota. Additional analyses showed further that there was a strong correlation between skin microbial diversity and the level of SOM in the water, suggesting that SOM is an important factor to account for when investigating determinants of mucus bacterial diversity. One potential explanation is that the presence of suspended particles in eutrophicated rivers could increase the presence of bacteria which aggregates on them and/or constitutes a source of nutrients for bacteria (Simon *et al.* 2002), although the underlying mechanisms explaining the increase in fish skin microbiota diversity remain unknown.

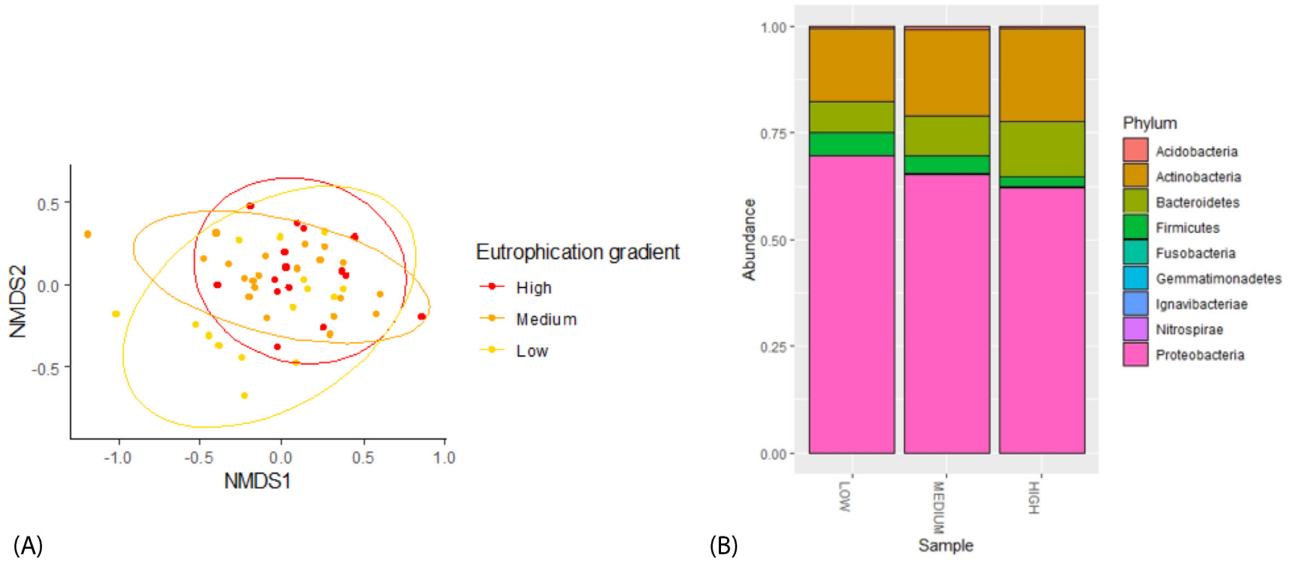

Figure 5. Analyses of water microbial communities **(A)**. NMDS based on an abundance similarity matrix (Bray–Curtis distances) of the water samples according to the level of eutrophication **(B)**. Water taxonomic composition according to the level of eutrophication.

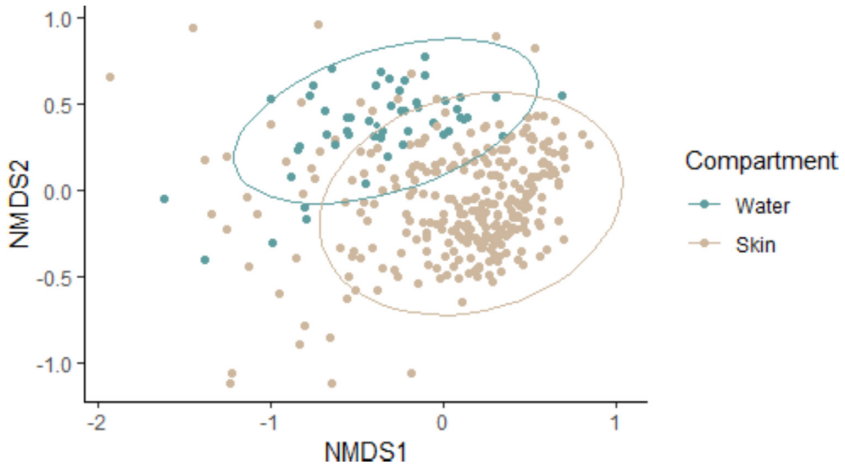

Figure 6. Comparison of skin microbiota and water microbial communities using NMDS based on an abundance similarity matrix (Bray–Curtis distances). Each color represents a type of compartment (Skin in grey or water in blue).

Environmental factors are key determinants of mucosal assemblages

Our data showed evidence of a strong link between environmental factors and mucosal assemblages especially concerning beta-diversity, suggesting that microbial skin composition dissimilarity among individuals was mainly due to environmental contrasts among sites. These results are in agreement with Chiarello *et al.* (2019) who found similar results in the European catfish (*Silurus glanis*) and showed that geographic location was the best predictor of skin microbiota composition. Another study also showed a strong correlation between the physicochemical gradient (river acidity) and taxonomic diversity in Amazonian fishes (Sylvain *et al.* 2019).

Concerning the composition of bacterial communities, we highlighted that Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes were the most represented phyla in gudgeon skin, which is consistent with previous studies (Kueneman *et al.* 2013, Larsen *et al.* 2013, Lokesh and Kiron 2016, Reverter *et al.* 2017). We also revealed a decrease of Proteobacteria in skin and water in the most eutrophicated environments. This result is in agreement with a study led on freshwater ecosystems showing that Alpha-Proteobacteria families strongly decreased along an urbanization gradient (Simonin *et al.* 2019). In addition, we showed that fish living in highly eutrophic sites displayed a decreased Proteobacteria/Bacteroidetes ratio, indicating a potential dysbiosis of the fish skin microbiota with implications for fish health (Krotman *et al.* 2020). More generally, several studies highlighted a perturbation in skin bacterial composition and dysbiosis in perturbated habitats (Sylvain *et al.* 2016, Krotman *et al.* 2020), likely because skin microbiota varies rapidly with its environment and presents a lower resilience than the gut microbiota for instance (Sylvain *et al.* 2016). Previous studies also showed significant effects of stress exposure or environmental conditions on the proportion of pathogenic bacteria on fish skin (Boutin *et al.* 2013, Chiarello *et al.* 2019). In our study, however, there was no significant increase in the proportion of potentially pathogenic taxa with eutrophication, despite an increase in bacterial diversity in high eutrophic sites. The reason for this lack of trend remains to be investigated, although it

should be noted that very few of these pathogenic taxa were found in our host species regardless of the site considered, suggesting either that gudgeon are not very prone to the skin mucus bacterial infections or that the pathogens considered are not relevant in this species.

Comparison of water microbial communities and skin microbiota

We found a fairly high level of similarity between skin and water bacterial communities, which corroborates previous evidence showing that microbial assemblages present on external surfaces were much more influenced by the environment compared to that of internal surfaces such as the intestinal mucus (Minich *et al.* 2020). However, many OTUs, including some dominants ones were specific to the skin and only observed in the skin compartment, which is consistent with previous studies in fish (Boutin *et al.* 2013, 2014). Although the results regarding alpha-diversity in water samples should be treated with caution as our water sampling method likely underestimated diversity, we found that phylogenetic and taxonomic bacterial richness were higher in skin samples than water samples. This is in agreement with previous studies in marine species (Chiarello *et al.* 2015, Minich *et al.* 2020) showing higher bacterial diversity in fish skin compared to water and sediments. Overall, our results contribute to the evidence that although the skin mucus harbors specific and diverse bacterial communities, indicating a certain level of host control/filtering (Adair and Douglas 2017, Foster *et al.* 2017). the SMM is strongly influenced by the surrounding aquatic environment (more so than the gut microbiota for instance, e.g. (Sehnal *et al.* 2021)).

Effect of host traits on skin microbiota

Of the various host traits examined, only fish size, a proxy of fish age, had an effect on SMM OTU richness and PD (but not Shannon diversity), with larger fish hosting a higher diversity of bacteria. This result could potentially be explained by the fact that a larger quantity of mucus was sampled on bigger fish, despite our efforts for standardization, or be linked to a potential age effect (e.g. Minich *et al.* 2020). SMM composition (beta-diversity) on the other hand was weakly but significantly explained by ectoparasite load (*Gyrodactylus sp.*) but not by other host traits. *Gyrodactylus* parasites, which graze on skin mucus and cells, could potentially alter skin microbiota composition, through changes in mucus composition (Buchmann and Bresciani 1997) and/or local inflammation as shown with other ectoparasites (*Tracheliastes polycolpus*, (Mathieu-Bégné 2020)). These results are promising and corroborate findings in other fish species (e.g. Llewellyn *et al.* 2014), but given the low number of infected fish caught during this study, further experimental studies manipulating microbiota composition and/or pathogen load are now needed to disentangle the underlying mechanisms and consequences for fish health and fitness.

Overall, individual host traits appeared to contribute less to explaining variation in SMM alpha- and beta-diversity than environmental factors. This contrasts with what is usually observed in terrestrial organisms in which host traits play a central role (e.g. SanMiguel and Grice 2015, Moitinho-Silva *et al.* 2021) and is likely explained by the fact that aquatic environments teem with microorganisms and expose hosts to many more potential bacterial colonists than terrestrial habitats do (Sehnal *et al.* 2021).

To conclude, our study highlights the importance of environmental factors such as eutrophication, on the diversity and composition of the skin mucosal microbiota. On the contrary, individual factors were less linked to microbiota changes in richness and composition, although beta-diversity composition was related to parasite load in *Gyrodactylus sp.* ectoparasites. This suggests that human activities causing eutrophication appears to clearly influence microbial community in skin mucus, with potential consequences for fish fitness and defense against pathogens and parasites.

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Supplementary data

Supplementary data are available at FEMSEC online.

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