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SHORT COMMUNICATION

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Preliminary insight into the fish microbiota in a small lowland river affected by municipal wastewater effluent

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Abstract – Based on 16S rRNA sequencing, the microbiomes of gudgeon, perch, and stone loach were characterized and compared between a site affected by municipal sewage discharge and an unpolluted site. *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Planctomycetes* were dominant in fish from unpolluted water, with microbial diversity varying among fish species. Shifts in *Proteobacteria* and *Bacteroidetes* were observed in stone loach and gudgeon between two studied sites. Microbiota of fish from polluted water contained more *Actinobacteria*, while from unpolluted water had more *Patescibacteria*, *Planctomycetes*, *Cyanobacteria* and *Verrucomicrobia*. The difference in bacterial families between polluted and unpolluted sites, with some bacterial taxa gained or lost were recorded and discussed as potential bioindicators of sewage pollutions.

Keywords: Microbiome / environmental pollution / freshwater fish / domestic pollution / microbial diversity

1 Introduction

The vertebrate body harbours a complex community of microorganisms (microbiota) that are critical to the host's functioning (Colston and Jackson, 2016). These microbes, found in the gut and on other organs and epithelial surfaces, influence nutrient acquisition, immunity, behaviour, development, reproduction and health (Suzuki, 2017; Sehnal *et al.*, 2021). Fish microbiomes, while less studied than those of mammals, are very diverse, and their composition and host-microbiota interactions are shaped by several factors like salinity, temperature, diet and pollution *etc.* (Sullam *et al.*, 2012; Kim *et al.*, 2021; Chen *et al.*, 2022). Microbiota associated with external mucosal surfaces of fish, such as gills and skin, have received less attention than gut microbiota, despite being the first defence barrier against pathogens (Colston and Jackson, 2016; Legrand *et al.*, 2020; Gomez and Primm, 2021; Bell *et al.*, 2024). Polluted waters can disrupt fish microbiota, as often contain pharmaceuticals, pesticides, and personal hygiene products (Giang *et al.*, 2018; Felis *et al.*, 2020). While the impact of these pollutants on fish gut microbiomes has been studied, their combined impact with wastewater bacteria on the microbiome of external mucosal surfaces in natural habitats is less understood (*e.g.*, Giang *et al.*, 2018; Restivo *et al.*, 2021; Xue *et al.*, 2021; Côte *et al.*, 2022; Bell *et al.*, 2024).

Microbiota is very sensitive to any perturbation in surrounding environment, which leads to imbalance resulting in replacement of some bacterial taxa beneficial for a host, by those more resistant or pathogenic. It suggests a potential for applying microbiota as indicator of environmental pollution and fish health. It is currently being developed in aquaculture (Xavier *et al.*, 2024, Tay et al., 2025), but much less in the wild environment (Restivo *et al.*, 2021, Côte *et al.*, 2022). Small freshwater fish species of no commercial value are seldom the target of microbiota studies (Tarnecki *et al.*, 2017), though they are often the most abundant species in natural fish assemblages. The gudgeon (*Gobio gobio*), stone loach (*Barbatula barbatula*) and perch (*Perca fluviatilis*) are common freshwater fishes of small lowland rivers in continental Europe (Fieseler and Wolter, 2006; Bergerot *et al.*, 2008). These species are known to be relatively resistant to anthropogenic stressors in the riverine environment, such as pollution, river engineering schemes, *etc.*, and their dominance is believed to indicate serious disturbance (Kruk, 2007). Thus, they occur in river sections of different water quality that enable comparisons of individuals between impacted (polluted) and reference (unpolluted) environment.

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This study aims to: 1) characterize the microbiome diversity and microbiota community of the external and internal mucosal surfaces of: gudgeon, stone loach and perch in a small stream, and to 2) examine how wastewater effluent affect their skin and gill microbiomes. We predicted that microbial communities of the three co-occurring species would be host specific but would exhibit similar changes in the taxonomic composition of commensal microorganisms under exposure to the same domestic sewage discharge. We discussed the expected microbiota alterations in the context of their possible application as indicators of fish health and the presence of domestic pollutions.

2 Material and methods

Fish samples were collected in July 2019 from two sites along the River Gać, selected based on their proximity to anthropogenic pollution from a sewage treatment plant in Spała village (Poland). The site 1, located upstream in a forest-agricultural area, was considered relatively unpolluted, while site 2, located directly downstream from the sewage discharge, was classified as polluted (see Supplementary Tab. 1). Altogether thirty fish (five by species of gudgeon, perch, and stone loach at each site) were collected via electrofishing. Following euthanasia, skin mucus, gill, and intestinal tissues were aseptically sampled and stored at −80 °C for subsequent microbial analysis. Next-generation sequencing (NGS) targeting the V3-V4 region of the 16S rRNA gene was performed on 70 samples. Following DADA2 processing, the mean sequence count per sample was 27,063 ± 17,625 (4,596–87,915). Quality filtering yielded 2,526 unique amplicon sequence variants (ASVs). All samples included in downstream analyses contained ≥9,092 high-quality sequences (see details in Supplementary Materials and Methods).

3 Results and discussion

In total, 1840281 ASVs were detected in analysed samples in three species, across four body locations (gills, skin mucus, distal and proximal intestine) and from two sampling sites. These ASVs represent 23 phyla, 54 classes, 133 orders and 266 families. The dominant bacterial phyla in microbial communities of gudgeon, perch and stone loach were similar and included *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Planctomycetes*, that all together accounted for 83 to 90 % of all sequence reads in the fishes from site 1 (unpolluted water). The order of dominance among these phyla differed depending on body location, but Proteobacteria contributed most to relative abundances of all studied mucus structure microbiota for gudgeon and stone loach, with some exceptions for perch (Supplementary material Fig. 1). These findings agreed with general microbiome characteristics of fish that harbour different bacterial communities across the major body mucus surfaces, although *Proteobacteria* remains the most abundant phylum in all of them (Llewellyn *et al.*, 2014; Chiarello *et al.*, 2018; Legrand *et al.*, 2020).

The microbiomes of distal and proximal gut, gills and skin mucus did not differ significantly in number of ASVs ($H_{7,44} = 14.31$; $p = 0.216$). Pairwise comparisons of alpha diversity measured with the Shannon diversity index did not detect any differences between microbiomes of four body locations for a given species or in beta diversity. The ANOSIM based on the Bray-Curtis similarity coefficient, calculated at the level of family, confirmed differences among microbiomes of body regions ($R = 0.161$, $p < 0.001$) and a post-hoc pairwise Bonferroni test showed significant differences between all body locations, except for skin and gills (Supplementary material Tab. 2). The average dissimilarity between microbiota of body locations was 76%. The SIMPER analysis identified families that were most responsible for differences among factors, *i.e.*, body location. Their relative abundance in the microbiota of each body location depended on species, but the families *Ilumatobacteraceae*, *Pirelluceae* and *Burkholderiaceae* tended to dominate on external surfaces such as gills and skin mucus of all species (Fig. 1). *Lachnospiraceae* and *Flavobacteriaceae* contributed more to the microbiota in the distal gut, while *Bifidobacteriaceae*, *Saccharimonadaceae* and *Acetobacteraceae* were characteristic of the proximal gut (Fig. 1). *Pirelluceae* and *Burkholderiaceae* occupy diverse ecological niches both terrestrial and aquatic also in close association with plants, animals and fungi as a component of biofilms and benthic sediment (Coenye, 2014; Farkas *et al.*, 2020) and can be easily aquisited from the surrounding environment. Families identified in our study as most abundant in the gut microbiota of all fish species, are listed among bacteria commonly found in the vertebrate gut, *e.g.*, the families *Lachnospiraceae* are well-known for their association with several herbivorous hosts, including fishes (Clements *et al.*, 2009; Vacca *et al.*, 2020; Escalas *et al.*, 2021), being particularly efficient in cellulose degradation. *Acetobacteraceae*, establish symbiotic relationships with several insects (Crotti *et al.*, 2010). It is possible that the presence of these bacterial families in fish gut microbiomes arose from their prey species. Diet shapes fish gut microbiome, not only the role of symbiotic microorganisms in the food digestion processes, but also microorganisms entering the fish gut as symbionts of their prey (Sullam *et al.*, 2012).

The microbiomes of three co-occurring fish species differed in their diversity and microbiota community structure. Among-species differences were detected in the alpha diversity measured by the Shannon diversity index ($H_{3,88} = 13.51$; $p = 0.001$) and beta diversity measured using Bray-Curtis distance (pseudo $F = 1.79$; $p = 0.001$). Pairwise comparison tests showed that alpha diversity was highest in gudgeon and was significantly different to perch ($H_{2,34} = 10.30$; $p = 0.004$) and stone loach ($H_{2,28} = 8.55$; $p = 0.005$), but there was no difference between perch and stone loach ($H_{2,26} = 0.111$; $p = 0.916$). There were differences among all species in the beta diversity: gudgeon vs. perch (pseudo $F = 1.67$; $p = 0.004$), gudgeon vs. stone loach (pseudo $F = 1.97$; $p = 0.003$) and perch vs. stone loach (pseudo $F = 1.77$; $p = 0.004$).

In the gudgeon microbiome 222 bacterial families were detected, compared to 210 families in stone loach and 206 in perch. A total of 166 families were shared by all three species, but their contribution to respective microbiota varied depending on species and body region (Fig. 1).

Comparing the microbiome composition at the level of family we found significant difference among species (ANOSIM, $R = 0.159$, $p < 0.001$) and a post-hoc pairwise Bonferroni test showed significant differences between

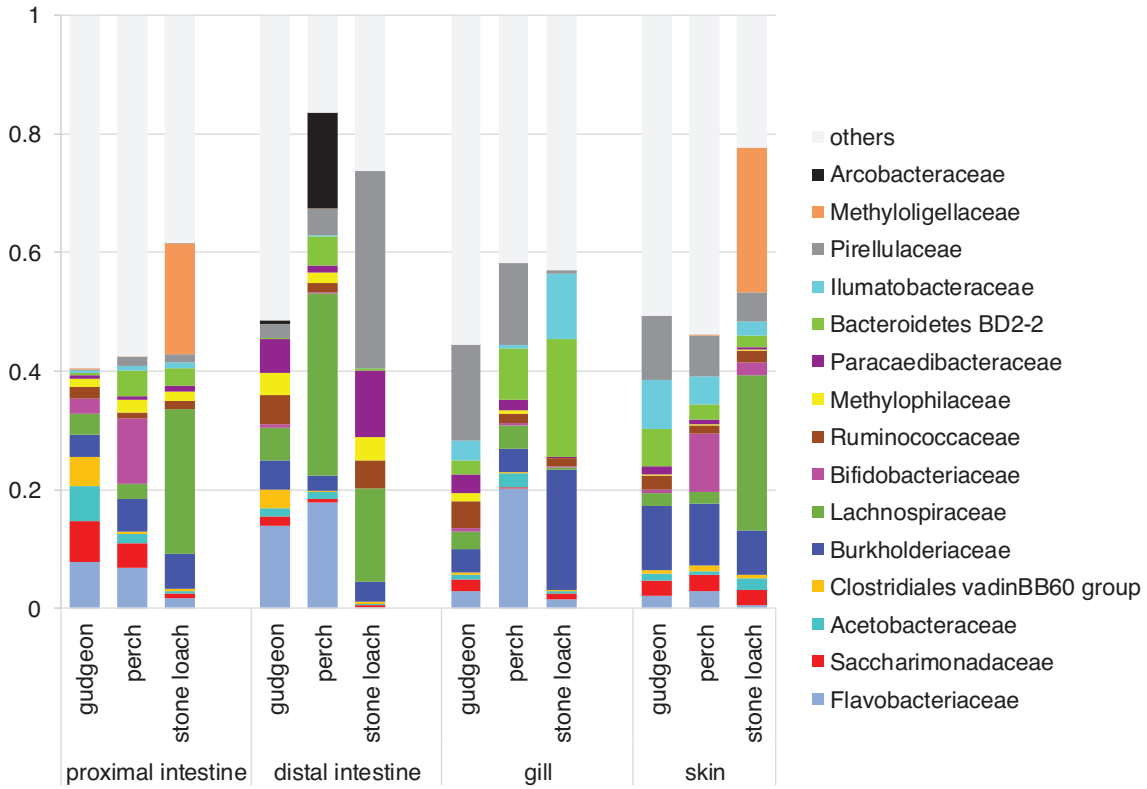

Fig. 1. Relative abundance of main microbial families detected in microbial communities of skin, gill, proximal and distal intestine of gudgeon, perch and stone loach at unpolluted site 1.

gudgeon and perch, as well as between gudgeon and stone loach (Supplementary material Tab. 2).

Diet and feeding habits influence gut microbiota composition and it causes differences in microbiome between co-occurring fish species (Sullam *et al.*, 2012; Wang *et al.*, 2018). The increasing trend in the diversity of gastrointestinal microbiota was observed following the order of carnivores, omnivores and herbivores (Liu *et al.*, 2016). Perch are more carnivorous than gudgeon and stone loach, but only becomes piscivorous after achieving a body size of *c*. 15 cm total length (L_T) (Craig, 2000), *i.e.*, much larger than the individuals included in our study, which were at the stage when benthivory dominates their feeding habits. While it can be assumed that all three study species had similar food spectra and fed mainly on macroinvertebrates, their diets may comprise different prey species because of their spatiotemporal differences in feeding habits and habitat preferences. Gudgeon and stone loach, both more associated with the river bottom in comparison with perch, differed slightly in their microhabitat preferences, activity rhythms, mode of foraging (Zweimüller, 1995; Worischka *et al.*, 2012). For example, the largest interspecific differences were found in the abundance of *Methyloligellaceae* bacteria that constituted 16.3% of the stone loach microbiome, while less than 0.05% in the case of gudgeon and perch. This outcome may relate to the stone loach habitat preference and feeding habits that are associated with sediments that are rich in decaying organic matter. *Methyloligellaceae* are methylotrophic bacteria, that are especially common in or near environments where biogenic methane is formed by conversion of organic matter decomposed in sediments by bacterial decay under anaerobic conditions.

The role of the surrounding environment in shaping fish skin microbiome was highlighted in several studies (Boutin *et al.*, 2013; Krotman *et al.*, 2020; Côte et al., 2022). The microbial communities present on external surfaces were much more influenced by the environment compared to those present in the intestinal mucus (Minich et al., 2020). Indeed, we found differences in the diversity and taxonomic composition of skin and gill microbial communities of fish between sites affected and unaffected by wastewater effluents. Microbiomes of external structures (pooled data for gills and skin mucus) did not differ in number of ASVs ($H_{5,40} = 6.25$; $p = 0.282$) or Shannon alpha diversity ($H_{5,40} = 12.38$; $p = 0.030$) for any species between sampling sites (Supplementary material Fig. 2). There were significant differences in beta diversity (*i.e.*, Bray-Curtis distance) between sites (pseudo $F = 2.41$; $p = 0.001$) for all species, gudgeon (pseudo $F = 2.47$; $p = 0.003$), perch (pseudo $F = 2.66$; $p = 0.005$) and stone loach (pseudo $F = 2.04$; $p = 0.006$) as well as among species within a site. Lack of a difference in alpha diversity of microbiomes between sites indicates that microbial species richness and their evenness remained relatively unchanged, which is quite common observation in fish microbiome studies (Tay et al., 2025). More crucial is a difference in beta diversity that means compositional dissimilarity between sites and indicates taxon turnover or replacement of taxa beneficial to fish health by more resistant to pollution or even pathogenic ones (Tay et al., 2025). Thus, it may be the first sign of

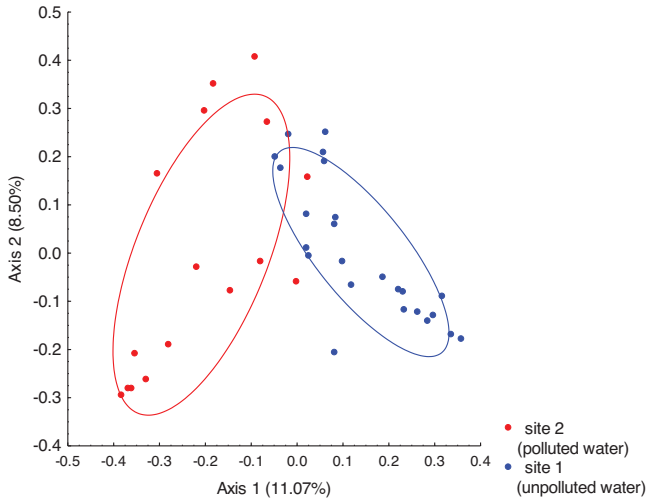

Fig. 2. PCoA ordination plot showing differences in microbiomes of fish from site 1 (unpolluted water) and site 2 (polluted water).

disturbances in environment with implication for dysbiosis and fish condition (Xavier *et al.*, 2024).

The differences in composition of microbiota rather than their richness were displayed by multidimensional scaling (PCoA) on ASVs that separated microbiomes of fish from unpolluted and polluted waters (Fig. 2).

Alterations in the skin and gill microbiome structure of the studied fish species also involved a decline in the relative abundance of some bacteria at the site affected by polluted waters. The 94–98% of bacteria identified from external surfaces (pooled skin and gills samples) of fish at two sites belong to 10 phyla (Fig. 3a), and among them, *Proteobacteria* dominated in microbial communities of all fish species regardless of site. The shift in ratio between *Proteobacteria* and *Bacteroidetes* caused by an increase in the relative abundance of *Bacteroidetes* and a decrease in *Proteobacteria* is associated with wastewater pollution and fish health (Legrand *et al.*, 2018). Fish from eutrophic water had elevated *Bacteroidetes* relative abundances and reduced *Proteobacteria* relative abundances (Krotman *et al.*, 2020). In our study such a shift in the ratio of *Proteobacteria*/*Bacteroidetes* was detected in stone loach and gudgeon microbiota. *Actinobacteria* had higher relative abundance in site 2 (polluted water) in each of the three fish species. *Patescibacteria*, *Planctomycetes*, *Cyanobacteria* and *Verrucomicrobia* were more abundant in site 1 (unpolluted water), *i.e.*, for gudgeon their abundances were respectively 2-, 4-, 3- and 5-fold higher than in site 2 (polluted water). Planctomycetes and Verrucomicrobia are typical bacteria for natural freshwaters and are sensitive to any disturbances, such as pollution of various origin, also faecal (Paruch *et al.*, 2019; Sazykina *et al.*, 2022). Thus, decrease of their abundance, like in our results, can indicate increasing anthropogenic pressure. The proportion of the most abundant classes depended on species (Fig. 3b), but Subgroup 18 (from phylum *Acidobacteria*), *Acidimicrobiia* tended to have higher proportion in site 2 (polluted water) for all fish species, and *Bacteroidia* and *Gammaproteobacteria* for gudgeon and stone loach but not perch. *Alphaproteobacteria*, *Planctomycetacia*, *Saccharimonadia*, *Oxyphotobacteria* and *Verrucomicrobiae* contributed more to overall abundance in site 1 (unpolluted water). They belonged to typical bacterial classes found in freshwaters, but their role as symbiotic bacteria of fish microbiota is unknown.

The differences between sites and among species were also found at the family level. A greater number of bacterial families was found in fish microbial communities in unpolluted than in polluted waters for gudgeon and perch (175 vs 152 and 166 vs 149 respectively), but not for stone loach, *i.e.*, 160 vs 163. There were also differences in taxonomic structure, as some families were recorded only in samples from unpolluted water, while others only occurred in samples from polluted water. The ANOSIM test confirmed separation among species from different sites ($R = 0.274$, $p = 0.001$) (Supplementary material Tab. 2). SIMPER pairwise comparison of groups, *i.e.*, fish species from two sites, revealed that the different bacterial families had the highest contribution to the average overall Bray-Curtis dissimilarity between sites depending on fish species (Supplementary material Fig. 3). The average dissimilarity between sites was 69% for gudgeon, 76% for perch and 73% for stone loach (Supplementary material Tab. 2). SIMPER analysis identified families that contributed to the overall dissimilarity of the external microbiota of each fish species between groups (polluted *vs* unpolluted sites) (Supplementary material Fig. 3). Generally, in microbial communities of all three fish species, *Ilumatobacteraceae*, *Lentimicrobiaceae*, *Paenibacillaceae* were more abundant at site 2 (polluted water), while *Pirellulaceae*, *Saccharimonadaceae* and *Paracaedibacteraceae* were more abundant at site 1 (unpolluted water). Such differences in taxonomic composition of external surface microbiota might result from water pollution. However, further studies that would include water and sediment samples are needed to find association between alteration of these microbial families abundance in fish skin and gills, and water quality. Some implications of the given taxa potential as sewage bio-indicators can be found in other studies. For example, *Ilumatobacteraceae* became dominant actinobacteria of microbial communities associated with endemic sponges during environmental crisis in Lake Baikal caused by anthropogenic stressors, such as a pollution discharge from rivers and tourism in coastal areas (Lipko *et al.*, 2020). *Lentimicrobiaceae* are strictly anaerobic bacteria, found in sediment and first isolated from methanogenic granular sludge in a reactor treating organic wastewater (Sun *et al.*, 2016), thus their higher relative abundance in the microbiome of fish from polluted site may be associated with wastewater effluent. At the site contaminated with sewage there were also some bacterial indicators traditionally used in faecal sewage detection, such as members of the family *Enterobacteraceae* (phylum *Proteobacteria*), associated with the human gut. In our study, *Enterobacteraceae* was one of the families that differentiated microbiota composition between study sites in case of stone loach, with a higher relative abundance in the polluted site than the unpolluted one. We did not notice any visible signs of infection in fish collected in the study. However, in microbial communities of perch skin and gills at the polluted site, members of the family *Mycobacteriaceae* had high relative abundance, while they were almost absent from the unpolluted site. This family includes a large number of pathogenic bacteria associated with mycobacteriosis infections

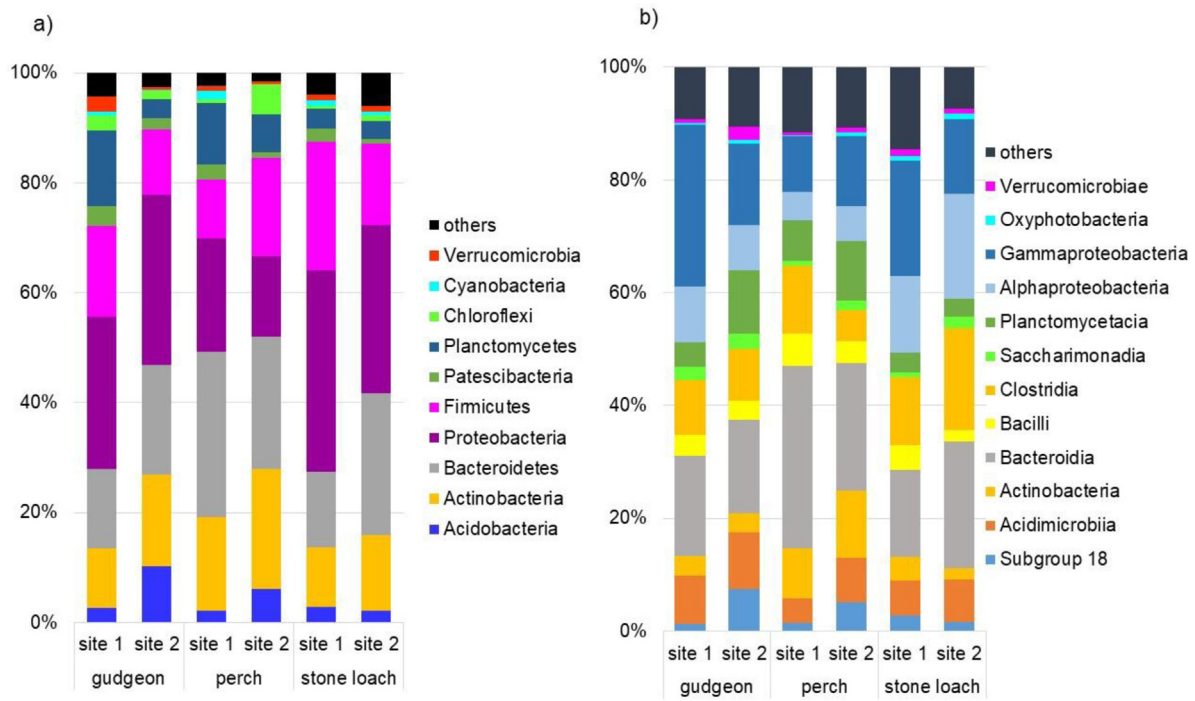

Fig. 3. Relative abundance of main microbial phyla (a) and classes detected from external mucus surface (data for gills and skin pooled) in fish species at (a) site 1 (unpolluted water) and (b) site 2 (polluted water).

in fish (Delghandi *et al.*, 2020). Mycobacterial organisms thrive under certain environmental conditions, including warm water temperatures, low dissolved oxygen levels, acidic pH and an organically rich environment (Delghandi *et al.*, 2020). Thus, waters receiving household sewage seem to be an ideal environment for them.

The above changes in the composition and relative abundance in the skin and gill microbiota that we found when comparing fish from unpolluted and polluted sites may cause dysbiosis that is defined as disruption to microbiotic homeostasis caused by an imbalance in the microflora, changes in their functional composition and metabolic activities (Mougin and Joyce 2023). Review of dysbiosis signatures in aquacultured fish (Xavier *et al.*, 2024) concluded that fish homeostasis can be effectively monitored by regularly surveying changes in bacterial community structure (beta diversity) in conjunction with the detection of increases of potential pathogenic abundance. Based on our results we cannot predict for certain how the differences found in microbial communities of three common fish species due to exposure to sewage contamination may affect their condition or functioning. This question would require further studies, which were beyond the scope of the present work. The other studies on European brook lamprey (*Lampetra planeri*) conducted at the same river showed that individuals from polluted site, below sewage discharge, had significantly worse body conditions (Zięba *et al.*, 2024) and exhibited inferior body growth (Pyrzanowski *et al.*, 2025) in comparison with relatively unpolluted river section. However, it was species-specific as co-occurring Ukrainian brook lamprey (*Eudontomyzon mariae*) responded to such environmental stressor in different way, what suggests their better resilience to household wastewater pollution (Pyrzanowski *et al.*, 2025).

To conclude, the microbiota of the three common fish species, co-occurring in unpolluted water, varied depending on body location, *i.e.*, skin and gills vs. guts, and differed in diversity and composition among fish species, what probably reflects differences in their ecology (diet, habitat preferences, feeding habits). Despite that several congruent tendencies in alteration of external mucus surfaces microbiota were found for all fish species from site exposed to wastewater pollution in comparison with those from unpolluted site. The changes included differences in beta diversity, while their alpha diversity remained unchanged, and composition of microbiota, such as a decrease in the *Proteobacteria/Bacteroidetes* ratio, increase of some bacterial families, together with decrease of others. While the application of fish microbiome as bio-indicator of fish condition and health receives a lot of interest in aquaculture, much less is known how microbiota of wild fish species of no commercial value respond to common anthropogenic stressor, like wastewater pollution. Our preliminary study gives a promising insight into this problem, although the impact of pollutants on the microbiome, and its consequences for fish health, warrants further research, including more fish individuals and water samples, as well as confrontation with other relevant scheme polluted/unpolluted site to check if the tendencies are consistent across species and sites.

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Supplementary materials

Supplementary material Fig. 1. Relative abundance of the main microbial phyla detected in skin, gill, proximal (intP) and distal intestine (intD) three fish species (G - gudgeon; P - perch; S - stone loach) site 1 (unpolluted).

Supplementary material Fig. 2. Alpha diversity of microbiomes among species (data for external body regions: gill and skin mucus pooled) in site 1 (unpolluted water) and site 2 (polluted water) measured with the Shannon index. Gsite 1 - gudgeon in site 1, Gsite 2 - gudgeon in site 2, Psite 1 - perch in site 1, Psite 2 - perch in site 2, Ssite 1 - stone loach in site 1, Ssite 2 - stone loach in site 2.

Supplementary material Fig. 3. Average relative abundance (±SE) of microbial families, identified by SIMPER analysis, with the highest contribution to the overall dissimilarity between sites for gudgeon (a), perch (b) and stone loach (c).

Supplementary material Table 1. Comparison of parameters describing the study site and water quality for the River Gać during sample collection (5th July 2019).

Supplementary material Table 2. Pairwise comparisons (Bonferroni test) of fish microbiota following one-way ANOSIM and SIMPER analysis.

The Supplementary Material is available at https://www.kmae-journal.org/10.1051/kmae/2025026/olm.

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