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Glyphosate-based herbicide exposure: effects on gill microbiota of rainbow trout (*Oncorhynchus mykiss*) and the aquatic bacterial ecosystem

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Abstract :

The herbicide glyphosate has been widely used in the past 40 years, under the assumption that side effects were minimal. In recent years, its impact on microbial compositions and potential indirect effects on plant, animal and human health have been strongly suspected. Glyphosate and co-formulates have been detected in various water sources, but our understanding of their potential effects on aquatic animals is still in its infancy compared with mammals. In this study, we investigated the effect of chronic exposure to an environmentally relevant concentration of glyphosate on bacterial communities of rainbow trout (Oncorhynchus mykiss). Gills, gut contents and gut epithelia were then analyzed by metabarcoding targeting the 16S rRNA gene. Our results revealed that rainbow trout has its own bacterial communities that differ from their surrounding habitats and possesses microbiomes specific to these three compartments. The glyphosate-based herbicide treatment significantly affected the gill microbiome, with a decrease in diversity. Glyphosate treatments disrupted microbial taxonomic composition and some bacteria seem to be sensitive to this environmental pollutant. Lastly, co-occurrence networks showed that microbial interactions in gills tended to decrease with chemical exposure. These results demonstrate that glyphosate could affect microbiota associated with aquaculture fish.

Keywords : Microbiome, Glyphosate, Co-occurrence network, Gill, Chronic exposition, Dysbiosis

INTRODUCTION

The microbiota, i.e., all microorganisms living in a given system, is unique to an organism and specific to a system. Research on teleost microbiota is still scarce compared to studies in humans or mammal models, but it is commonly accepted that the microbiota is essential in maintaining fish health. Aquaculture is an exponentially growing sector of agriculture, with increasing global demand for fish protein that requires improvements in yield and aquaculture practices (FAO 2016). To optimize productivity in aquaculture systems, a key factor will be to better understand interactions between fish and their associated bacterial communities and potential dysbiosis (an imbalance in the microbiome) (Llewellyn *et al.* 2014; Legrand *et al.* 2019). Rainbow trout (*Oncorhynchus mykiss)* is a globally significant aquaculture fish species present on all continents except Antarctica. Its production has grown exponentially since the 1950s, especially in Europe and in Chile, and reproduction techniques are well developed (FAO 2016). A trout farm facility must have high-quality water, with low concentrations of iron, zinc, and copper, as well as a stable range of temperatures or pH, generally found in river water, ground water or in ponds with flowing water. This environmental water exposes wild or farmed rainbow trout to potential pollutants and to a variety of diseases with bacteria, viruses or protozoa as causative agents. Currently, the most studied microbiome in fish, and especially in salmonids, is the gut or gastrointestinal that plays a critical role in nutrition, development, immunity and resistance to pathogens (Tarnecki *et al.* 2017; Egerton *et al.* 2018; Wang *et al.* 2018). Certain other mucosal tissues, such as the skin or gills, are major pathways for pathogens or pollutants to enter fish, and their microbiota probably plays a role as a defense barrier (Merrifield and Rodiles 2015). Environmental factors including water quality, season, and geographic location, dietary factors such as lipo-protein ratio and plant extract, and host factors including genetics, trophic level, development, and fish treatments, have been reported to influence the composition of fish microbiomes (Merrifield and Rodiles

2015; Legrand *et al.* 2019). Water quality is an important factor, especially for aquaculture species like rainbow trout, and depends on many parameters (pH, salinity, temperature, oxygen, etc.) that could be disrupted by pollutants. Some environmental pollutants, such as heavy metals, persistent organic pollutants or pesticides, seem to be able to induce gut microbiota dysbiosis and may have effects on human health (Jin *et al.* 2017). For teleosts, research on a link between pollutants in water and fish microbiota is rare, but recent publications have suggested effects associated with pesticides like diazinon, glyphosate or carbendazim (Bao *et al.* 2020; Ding *et al.* 2021; Tang *et al.* 2021).

The broad-spectrum herbicide glyphosate and associated commercial formulations called glyphosate-based herbicides (GBHs) are the most commonly used herbicides worldwide (Benbrook 2016). They inhibit 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme of the shikimate pathway, which stops the synthesis of essential aromatic amino acids. This metabolic pathway is present in plants, fungi and some microorganisms, but is absent in vertebrates (Herrmann and Weaver 1999), which had led to the massive use of glyphosate in modern agriculture. The European Food Safety Authority has classified the impact of glyphosate on aquatic organisms as "limited" (European Food Safety Authority (EFSA) 2015). This active substance (AS) is one of the major pollutants of water (IFEN 2006). In France, glyphosate detection in surface water has doubled from 22.2 to 49.7% of sampling points analyzed in ten years (2007–2017) (Anses 2019). Furthermore, effects of chronic exposure to this AS have been found in non-target species such as aquatic organisms, Daphnia (Suppa *et al.* 2020), rainbow trout (Du-Carrée, Morin and Danion 2021) and zebrafish (Sulukan *et al.* 2017). Until recently, the presence of the shikimate pathway in most prokaryotes, like bacteria, was not taken into account, but with the exponential increase in studies showing the importance of the microbiome in fundamental functions of vertebrate

organisms like fish, it appears relevant to assess the potential impact of glyphosate and GBHs on these communities of microorganisms.

In this study, we investigated the impact of chronic exposure to glyphosate (AS alone) or two GBHs (i.e., Round Up Innovert® and Viaglif Jardin®) on the microbiota of rainbow trout and on their surrounding environments through metabarcoding approaches. The two GBHs are commercial products formulated for two different uses: professional for Round Up Innovert® and home gardens for Viaglif Jardin®. First, we investigated whether the bacterial communities of rainbow trout differ from their surrounding habitats (water and biofilm in tanks). Then, we assessed the structure, diversity and taxonomic composition of three bacterial microbiomes (gills, gut content, and epithelium of the intestines) and possible core microbiota. Lastly, we examined whether GBH exposure could affect the microbial community and interactions.

MATERIALS AND METHODS

Ethics statement

Fish experimentation was carried out in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). Experimental procedures were validated by the animal ethics committee ANSES/ENVA/UPC No. 16 and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS\2017090117104091). Euthanasia was carried out by impact to the head, then cervical dislocation (rupture of the medullar canal). Animals showing lesions (damaged fins, wounds) or abnormal behavior during the experiment were submitted to compassionate euthanasia.

Experimental design

Fish experiments were done using 48 at the start but we had one death during the experiment, so we conducted analysis on 47 specific pathogen-free (SPF) rainbow trout aged 30 months, including both males and females, from the protected and monitored fish facilities of the ANSES Plouzané Laboratory site, France. Fishes were placed in four 400 L tanks. All tanks were located in the same room and maintained identically to limit inter-tank variability as much as possible. Continuous flow-through conditions (300 L) and oxygen levels above 60% of saturation with aeration were maintained to raise fish. Trout were fed daily with appropriate food at 1.5% of the biomass (B Repro 32 ASTX semi F9, Le Gouessant, France). The natural photoperiod occurring in Brest, France was maintained throughout the experiment. In water tanks, temperature increased from 9 to 21°C between April and July and decreased from 21 to 8°C between July and November.

Fish were exposed to four conditions of chemical exposure (one tank with n = 12 per condition): AS glyphosate (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6), Round Up Innovert® (R; Agrilisa - for professional use), Viaglif Jardin® (V; Agrilisa - for home gardens), and non-exposed control. G had a purity of 98%, while the concentrations of R and V were 360 $g.L^{-1}$ and 420 $g.L^{-1}$ of glyphosate, respectively. Commercial formulations of G, R and V contained several co-formulants of unknown nature and concentration. Concentrated solutions of each product (4 $mg.L^{-1}$) were prepared and stored under appropriate conditions (darkness, controlled temperature). For 6 months, from May to November 2019, a volume of 100 mL of each of these respective solutions was added to the fish tanks every working day (generally 5 days a week), with freshwater flow stopped for one hour. Regulated water flow was set up for the rest of the day at 13.5 $L.h^{-1}$, after one hour of contact with glyphosate,

allowing its gradual dilution. The integrated mean daily expected concentration was approximately 123 ng.L^{-1} (for details see (Du-Carrée, Morin and Danion 2021)).

Sampling

All samples were collected on the same day (21 Nov 2019) under a laminar flow hood and using sterile instruments and materials. From each tank, a volume of 250 mL of water was collected in triplicate in sterile jars and was immediately filtered on 0.22 µm sterile membranes, using a sterile filtration unit connected to a vacuum pump. Filters were frozen at -80°C until DNA extraction. Biofilm samples were collected on an area of about 5 square centimeters located in the middle of the submerged area, in triplicate, using sterile swabs and frozen at -80°C until DNA extraction. After euthanasia, fish were sampled: Gills, Gut content and intestinal epithelium (Intestine). All sample details are given in **Supplementary Table 1**. For digestive tract tissues, 3 cm for microbiota analysis and 2 to 5 cm for FISH analyses, were cut from the end of the mid- and hindgut (no clear separation to allow clear distinction between them), approximately at 2 cm above the anal sphincter. For fluorescence *in situ* hybridization (FISH), the orientation of the samples was noted. On the 3 cm sample, gut content was removed by gently squeezing the tissue with sterile forceps. The fragment was then opened along its entire length with a scalpel and its surface was scraped in order to recover all the intestinal mucosa. For gills, the outermost branchial arch to the right (for molecular analysis), and to the left (for FISH analysis) of the head was sampled for all studied fish. All tissues for molecular analysis were stored at -80°C until DNA extraction. Samples dedicated to FISH microscopy were fixed in 3% formalin sterile water for 2 hours. Samples were then removed and rinsed twice in sterile 1X phosphate buffered saline (PBS) solution and stored in PBS 2X/absolute ethanol v:v at -20°C until processed.

16S rRNA bacterial diversity analyses by Illumina MiSeq

DNA from the different tissues, biofilm and water was used for amplification of prokaryotic diversity based on the 16S rRNA gene and sent to the Bordeaux Transcriptome Genome Platform (www.pgtb.cgfb.u-bordeaux.fr; Cestas, France). Total DNA was extracted from each tissue (around 250 mg) of *O. mykiss* using a Qiagen® Power Fecal Pro DNA kit, and for biofilm or water, using a Qiagen® DNeasy PowerWater kit, following the manufacturer's instructions. Negative controls (blank samples from each extraction kit) were also used for amplification. Sequencing was performed on a 450 bp fragment of the 16S rRNA gene (V3-V4 variable region, primers 341F: "CCTACGGGNGGCWGCAG" and 785R: "GACTACHVGGGTATCTAATCC") frequently used for microbial diversity analyses (Klindworth *et al.* 2013; Fadrosh *et al.* 2014) on the Illumina MiSeq platform, using 2 x 250 bp chemistry.

Bioinformatics data processing

Prokaryotic 16S rRNA paired-end reads were merged using USEARCH (Edgar and Flyvbjerg 2015) after q25 trimming of the ends. The resulting 16S reads were processed using the Find Rapidly OTU with Galaxy Solution (FROGS) pipeline (Escudié *et al.* 2018). In short, sequences were depleted of barcode, then sequences < 380 bp and those containing ambiguous bases were removed. Next, reads were clustered into *de novo* operational taxonomic units (OTUs) using Swarm (Mahé *et al.* 2014) with an aggregation distance equal to 3. Chimeras were then removed with VSEARCH (Rognes *et al.* 2016). Additionally, a filter (for abundance) was applied to the OTUs, with an optimal threshold of 0.005% (Bokulich *et al.* 2013). The OTUs finally selected were taxonomically assigned by BLASTn + (Camacho *et al.* 2009) using the Silva release 138 reference database (Quast *et al.* 2012). With Silva release 138, the Genome Taxonomy Database was adopted that prone significant

adaptations such as Burkholderiales, an order of *Gammaproteobacteria* (formerly known as *Betaproteobacteria* or *Betaproteobacteriales*) or Bacteroidota (known as Bacteroidetes) (Parks *et al.* 2018). Finally, filtrations were performed on BLAST taxonomic affiliation, with a minimum coverage of 80% and a minimum identity of 95%.

Statistical analysis

All statistical analyses and data visualizations were carried out in R (R version 4.0.2 (Team 2013) using R studio v 1.3.1093). Alpha diversity was computed using the Phyloseq v 1.32 (McMurdie and Holmes 2013) and Vegan package v2.5-7 (Oksanen *et al.* 2008). Differences in the alpha diversity indexes among conditions were tested using a Kruskal–Wallis test followed by pairwise Wilcoxon tests: $p < 0.05$ was considered the significance threshold for a difference between conditions. Beta diversity analyses were performed on Weigh Unifrac distances on a rarefied dataset and were visualized using non-metric multidimensional scaling (NMDS). Sample groups were compared by a permutational multivariable analysis of variance (999 permutations) with the adonis function of the Vegan package. Multilevel comparisons for the conditions were also performed with the pairwise adonis function (Martinez Arbizu 2017). Differences in taxon abundances associated with each tissue and treatment were studied using a model based on negative binomial distribution, as implemented by the DESeq function in the DESeq2 package v 1.28.1. An adjusted $p < 0.05$ was considered significant. Boxplots, barplots and bubbleplots were produced with ggplot2.

We also analyzed the core microbiome, here define as common groups (OTUs) of microbes found across samples (Shade and Handelsamn 2012; Risely 2020). Firstly, we determined "treatment core" representing not rare OTUs (number of reads > 0.01% of Om sequences) (Pascoal *et al.* 2021) shared across samples from the same tissue and treatment. In a second time, Venn diagrams displaying the numbers of OTU shared among the four treatments for

each tissue were performed. Ven diagrams were generated using Venny v2.1 software (Oliveros 2007) (https://bioinfogp.cnb.csic.es/tools/venny/index.html). We also compared resistance vs susceptible OTUs based on their relative abundance and their persistence (> 90% of treatment samples) in these treatment core.

Co-occurrence network analysis

Microbial co-occurrence networks for gills, control and contaminated conditions (Glyphosate + Round Up + Viaglif) were generated using SPIEC-EASI (Sparse inverse covariance estimation for ecological association inference) version 1.1.1 (Kurtz *et al.* 2015). The SPIEC-EASI statistical method combines data transformations, developed for compositional data, with a sparse graphical model inference framework, and inverse covariance selection algorithms (Kurtz *et al.* 2015). Pre-filtering of OTUs was performed, retaining only OTUs with a proportion of least 0.01% of total abundance. Network properties such as modularity, node degree, mean path distance, clustering coefficient and hub score are detailed in (Layeghifard, Hwang and Guttman 2017) and were calculated with R package igraph v 1.2.6 (Csardi and Nepusz 2006). Network modules were separated by the Louvain algorithm and microbial co-occurrence networks were visualized with the igraph package.

Fluorescence in situ *hybridization on gills*

Only part of the gills fixed for microscopy analysis were used. Four gill sub-samples (one per condition) were individually embedded in polyethylene glycol distearate/1-hexadecanol (9: 1) resin (Sigma, St. Louis, MO, USA) after being dehydrated and soaked (ethanol and resin series at 40°C) (Durand *et al.* 2010). Blocks were stored at -20°C until cutting. Semi-thin sections of 8 µm were done using an RM 2255 microtome (Leica, Wetzlar, Germany) and placed on Superfrost Plus™ adhesive slides (Menzel-Gläser, Braunschweig, Germany). Prior

to hybridization, resin was removed from sections in absolute ethanol bathes and sections were then rehydrated in ethanol 75°C. Sections were then hybridized for 3 hours at 46°C with hybridization buffer [0.9 M NaCl, 0.02 M Tris-HCl, 0.01% sodium dodecyl sulphate (SDS), 30% deionized formamide] containing 8 µM of each probe, washed at 48°C for 15 min in a washing buffer [0.9 M NaCl, 0.02 M Tris-HCl, 0.15 M EDTA, 0.01% SDS], and rinsed briefly with deionized water. Sections were dried and mounted with SlowFade Gold™ antifade reagent containing 40-6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA) and sealed with a cover slip. The probes used to validate the location of bacteria were the universal probe Eub338-I (5'-GCTGCCTCCCGTAGGAGT- 3') targeting most *Eubacteria* (Amann, Krumholz and Stahl 1990) and non-sense *Eubacteria*, Non338, (5'-ACTCCTACGGGAGGCAGC -3') (Wallner, Amann and Beisker 1993) (Eurofins Genomics, France). Subsequently, observations were made using an Imager.Z2 microscope equipped with an ApoTome.2 sliding module and Colibri.7 light technology (Zeiss, Jena, Germany). The micrographs were analyzed using Zen software (Zeiss).

Data availability

The data supporting the results presented in this article are available from the NCBI SRA repository (BioProject PRJNA784534).

RESULTS

Bacterial diversity analysis

Metabarcoding (region V3-V4 of the 16S rRNA gene) of bacterial communities associated with 124 *Oncorhynchus mykiss* (Om) samples (40 Gill, 37 Gut content and 47 Intestine), 12 water samples, nine biofilm samples, and eight negative controls produced a total of 6,062,610 reads after bioinformatics processing (**Supplementary Table 2**). Negative controls

(four extraction kit blanks and four PCR blanks) represented 0.47% of total reads after the affiliation process (28,876). We obtained 5840 reads with the extraction kit and 23 036 with the PCR amplification blanks with, for the latter, a particularly rich sample among the four tested. The reads clustered into 565 OTUs taxonomically assigned with the Silva 138 database (**Supplementary Table 3**). In the overall analysis, 32 OTUs from blanks and chloroplast were discarded from the final OTU table (533 OTUs).

Alpha diversity index values were determined for all samples (**Supplementary Table 4)**. Comparison of the three indexes (OTU, Shannon and Inverse Simpson) between environments (Biofilm, Om and Water) as well as both tissues (Gill and Intestine) and Gut content showed significant differences (**Supplementary Table 5**). The number of observed OTUs for environments were: Biofilm (329 ± 19), Water (427 ± 19) and Om (83 ± 81).At the tissue level, the highest richness observed was for the Gills (186 ± 54) in comparison with Gut content (55 ± 33) and Intestine (16 ± 6). Comparison between the different conditions of chemical exposure showed a significant difference in richness for Gill tissue (p = 0.015) and significant differences in the Shannon and Inverse Simpson indexes for Water (**Supplementary Table 5**). Mean richness values for Gill treatments were: Control (233 ± 50), Glyphosate (169 ± 50), Round Up (172 ±47), and Viaglif (164 ± 40).

Structure analyses were performed with Beta diversity indexes, thus making it possible to understand relationships between bacterial communities. An NMDS plot showed a clear separation between Om and the two other environments (Water and Biofilm) (**Figure 1A**). Permutational multivariate analysis of variance (PERMANOVA) analyses indicated significant among the three environments, suggesting that the factor "environment" could explain 22% of the total bacterial variation in this study, and all pairwise comparisons were significant (i.e., Om vs. Water; Om vs. Biofilm and Water vs. Biofilm) (**Supplementary Table 6).** The NMDS plot of Om tissue samples showed a clear separation for Gill, but less

clear scattering for the other samples (**Figure 1B**). PERMANOVA analyses indicated significant differences among tissues and Gut content, and all pairwise comparisons were significant (i.e., Gill vs. Gut content; Gill vs. Intestine and Gut content vs. Intestine) (**Supplementary Table 6**). The factor "Treatment" was significant for Biofilm, Water and Gill tissue only (**Supplementary Table 6**).

Microbial taxonomic composition

Bacterial community composition of Biofilm, Water and Gill had almost the same taxonomic pattern with three major phyla, but relative abundance was specific to each environment or tissue (**Figure 2**). Global relative abundance for Biofilm was dominated by *Bacteroidota* (37%), Proteobacteria (35%), and *Nitrospirota* (9%); Gill was dominated by *Verrucomicrobiota* (42%), *Proteobacteria* (31.5%), and *Bacteroidota* (14%); Water was dominated by *Bacteroidota* (49%), *Proteobacteria* (28%), and *Verrucomicrobiota* (7.5%). The *Proteobacteria* phylum was composed of two classes: *Alphaproteobacteria* and *Gammaproteobacteria*. Bacterial community composition at the phylum level was the same between Treatments (i.e., control and three treatments), but the relative abundance of each phylum was different. For example, for Gill, the phylum *Verrucomicrobiota* was high (> 50%) with the Round Up treatment but around 30% for the Control condition or, conversely, *Bacteroidota* was more prevalent in the Control condition than in all other treatments.

A heatmap on the top 150 abundant taxa between Gill and Water allowed to distinguish clear differences (**Figure 3**). For example, two of the most abundant families in Gill (*Burkolderiales Incertae Sedis* and *Chlamydiales Incertae Sedis*) are absent from water. In contrary, many families present in Water are absent from Gill. Specifics comparison between Gill and Water were analyzed for four major phyla/Classes. **(Supplementary Figure 1**). *Alphaproteobacteria* and *Bacteroidota* were dominated by the same families

Rhodobacteraceae and *Flavobacteriaceae*, respectively. For *Gammaproteobacteria*, one family (*Comamonadaceae*) was particularly prevalent in Water, whereas Gill samples showed more diversity. *Verrucomicrobiota* was dominated by a single family (*Chlamydiales incertae sedis*) for Gill, but this family was almost absent for Water.

Regardless of the treatment, Gut content and Intestine samples were dominated by the *Firmicutes* phylum (**Figure 2**). Among the *Firmicutes* phylum, the order *Mycoplasmatales* was almost exclusively present for all treatments of Intestine and highly abundant for Gut content (**Supplementary Figure 2**). Several other *Firmicutes* orders were identified for Gut content and some differences appeared between treatments. For example, *Lactobacillales* were present in the Control and Round Up conditions, but absent in Glyphosate-exposed fish. *Firmicutes* were much less abundant for Gill tissue and seemed more taxonomically diverse.

Core microbiome of rainbow trout tissues

To determine the core microbiome of each Om tissue, we first identified OTUs present within each treatment separately, with relative read abundance across the Om dataset > 0.01% (defined here as "treatment core") and then identified the overlap between different treatment cores as the "shared core" (**Figure 4**). The shared core included 23, 11 and 6 OTUs for Gill, Gut content and Intestine, respectively. We observed that most OTUs within each treatment core belonged to the shared core for each Om subsample, except for Gut content Round Up and Glyphosate. The taxonomic composition at three levels (phylum, family and genus) of each shared core was identified (**Supplementary Table 7**). Only OTUs affiliated with *Firmicutes* (*Mycoplasma*) composed the shared core of Intestine tissue, whereas six and five phyla were identified for Gill and Gut content samples, respectively. The shared core of Gut content was composed of four families of *Firmicutes*. The shared core of Gill was more diverse, with six families from *Gammaproteobacteria*, for example. The core microbiome of

each Om tissue appeared to be specific. Two exceptions were found: Intestine and Gut content which shared *Firmicutes* (*Mycoplasma*); Gill and Gut content which shared two genera from *Proteobacteria* (*Hyphomicrobium* and *Rickettsiella*).

Venn diagrams also revealed the resistance of the bacterial OTUs to GBHs. Interestingly, OTUs from the shared core seemed resistant to GBHs since they were found in all exposure conditions with a high persistence (> 90%). The taxonomic composition at three levels (phylum, family and genus) of "resistant" OTUs was determined (**Supplementary Table 8**). For Gill, it was notably composed of three potentials pathogenic OTU (*Flavobacterirum*, *Candidatus Branchimonas* and *Candidatus Piscichlamydia*) and the *Mycoplasma* genus (Firmicutes), which were found in all samples.

Differential abundance analysis

For the Gill versus Water bacterial community analysis, we used the total data set, meaning that all treatments were grouped together. A differential abundance analysis at the genus level showed that 68 genera were significantly different between Gill and Water samples (**Supplementary Figure 3**). The Log_2 Fold Change is the effect size estimate: Log_2 Fold Change > 0 shows how much the genus abundance seems to be different due to Gill in comparison to Water, and inversely. Gill differences were associated with the occurrence of two potentially pathogenic agents (*Candidatus Piscichlamydia* and *Candidatus Branchiomonas*), absent from the Water community. Gill lineage overabundances were mainly related to *Proteobacteria*, *Plantomycetota*, *Firmicutes* and *Actinobacteriota*. Water bacterial communities were related to *Bacteroidota* and some lineages of *Verrucomicrobiota* and *Proteobacteria*.

For Gill bacterial communities analyses, we grouped sequences of Glyphosate, Round Up and Viaglif together under the term "Contaminated". A differential taxonomic comparison

revealed that five bacterial genera were significantly differentially abundant between the Control and Contaminated samples (**Figure 5A**). Importantly, *Candidatus Branchiomonas* and *Rhodoferax* were under-expressed for the Glyphosate conditions. *Polynucleobacter* was present in all samples, but with an overabundance in the Control condition. On the contrary, the *Limnohabitans* genus was present in Contaminated samples, especially Glyphosate, but absent from the Control condition (**Figure 5B**).

The topological and taxonomic properties of the co-occurrence Gill network

Co-occurrence network analysis using SPIEC-EASI was carried out to explore Gill Control and Gill Contaminated (Glyphosate + Round Up + Viaglif) samples.

The Control and Contaminated networks were comparable in size (294 and 312 nodes, respectively) and overall topological features were identified (**Table 1**). They were both relatively poorly dense (0.02 and 0.08) and showed comparable clustering coefficients (0.07 and 0.02). The number of edges (i.e., links between each pair of nodes) was different, with 752 edges for the Control and only 367 edges for the Contaminated network, suggesting that bacterial interactions could be less numerous in Contaminated samples than in the Control (**Figure 6**).

Modularity analyses showed high values (0.48 for Control and 0.75 for Contaminated), indicating that these networks had dense connections within certain groups of nodes, but sparse connections between them. The Network community algorithm (Louvain) showed that the Control network could be divided into 12 modules (i.e., a group of OTU nodes that were interconnected more frequently among themselves than with nodes in other modules), while the Contaminated network could be divided into 19 modules (**Figure 6**). The size of the modules ranged from 10 to 34 nodes for the Control and from 2 to 28 nodes for the Contaminated network. The taxonomic compositions of the modules for both networks were

investigated and represented in a heat map (**Figure 6**). For the Control network, 17 phyla/classes were observed but only two of them were represented inside each of the 12 modules (*Alphaproteobacteria* and *Bacteroidota*). On the other hand, taxonomic composition did not seem to be a key factor in the modular structure since we observed that the number of phyla/classes was between 4 to 11 for each module. The Control network was divided into 12 modules but four accounted for 46.9% (modules 2, 5, 10 and 12). These four major modules were composed of *Alphaproteobacteria*, *Bacteroidota*, *Gammaproteobacteria*, *Planctomycetota* and *Verrucomicrobiota*. For the Contaminated network, 19 phyla/classes were observed but none were inside each module, with a range between 2 to 12. This network had more modules at 19, with less weight, as the major module represented only 9.3% of nodes. The three major phyla among all modules were *Gammaproteobacteria* (20%), *Bacteroidota* (17%) and *Alphaproteobacteria* (15%).

Fluorescence in situ *hybridization approach*

The universal Eub338-I probe was used for preliminary characterization of bacterial cell morphologies on gills in order to localize potential microbiota colonization. Specificity of Eub338-I was confirmed by the absence of a hybridization signal using a non-sense probe. Different shapes of bacteria were observed in the gill control specimen with rod single or short chains, coccoid and rod aggregates. Overall, the microbial community was not very dense and did not penetrate the gill tissues. In this control specimen, bacteria were fixed on the tissue, along the gill epithelium, rather scattered, and mainly located near the blood vessel region. In the exposed specimens (glyphosate or GBHs), different shapes of bacteria were observed scattered along the gill filament and some appeared to be in the gill epithelium tissue (**Supplementary Figure 4 A, B**). The most remarkable specimen was the Viaglif-exposed one, for which numerous and larger rod aggregates were observed (**Supplementary**

Figure 4 C). In chemically exposed trout, bacteria seem to be further away from the blood vessels (**Supplementary Figure 4 D**).

DISCUSSION

O. mykiss-associated bacteria

In this study, we show that the rainbow trout used harbor multiple bacterial communities distinct from those of the water and the biofilm of tanks inside which they were reared. Furthermore, rainbow trout have distinct microbiota according to the tissue considered (Gill or Intestine) and Gut content, suggesting the existence of specific core microbiomes. The Gill microbial taxonomic composition showed four dominant phyla/classes (*Alpha-, Gammaproteobacteria*, *Bacteroidota* and *Verrucomicrobiota*), whereas Gut content and Intestine were both dominated by *Firmicutes*.

Bacterial communities present on the gills of salmonids, and especially of *O. mykiss*, have not been widely studied, compared to the digestive tract. Despite their direct contact with the aquatic environment, they play a major role as a physical barrier and first line of immune or anti-oxidant defense against pathogens and pollutants. The fact that some culture-based studies have suggested that the gill microbiome of fish is similar to the surrounding water, but also that poor water quality and seasonality could influence microbiome composition, could explain this lack of interest and results on this key compartment (Merrifield and Rodiles 2015). Our data show that gill and water microbiomes are different in their pattern of relative abundance of phyla, as well as at a deeper taxonomic level such as the family level. Within the gill microbiota, three OTUs affiliated with three related pathogenic bacteria are dominant: *Flavobacterium psychrophilum* (*Bacteroidata*), *Candidatus* Branchiomonas cysticola (*Gammaproteobacteria*) and *Candidatus* Piscichlamydia salmonis (*Verrucomicrobiota*).

Candidatus Piscichlamydia salmonis is an intracellular Chlamydiales bacterium associated with epitheliocystis, first characterized in farmed Atlantic salmon (Draghi *et al.* 2004). Epitheliocystis in fish generally refers to a gill disease where cytoplasmic bacterial inclusions (cysts) developing in the gill epithelia can lead to respiratory distress and death (Blandford *et al.* 2018).

Flavobacterium psychrophilum is the causative agent of bacterial coldwater disease, a major threat to salmonid aquaculture, against which *O. mykiss* could be resistant or susceptible. A study on the composition of *O. mykiss* microbiomes (stool, gut and gill) between resistant and susceptible fish after infection by *F. psychrophilum* (Valdés *et al.* 2020) revealed differences in gill microbiota, but not in the other two tissues. Resistant fish harbored a greater abundance of *Proteobacteria* and a smaller proportion of *Firmicutes* than susceptible ones, as we observed for the gill microbiota of our fish for all conditions analyzed. In another study investigating the bacterial composition of gills from *O. mykiss*, distinctive genetic lines (resistant or susceptible to *F. psychrophilum)* were found, and alpha diversity metrics were similar in both lines (Brown, Wiens and Salinas 2019). The most abundant phylum was *Proteobacteria* and the most prevalent genus was *Candidatus* Branchiomonas sp.

Candidatus Branchiomonas cysticola is an agent of epitheliocystis also characterized in sea-farmed Atlantic salmon, but genetically distinct from *Candidatus* Piscichlamydia salmonis (Mitchell *et al.* 2013). In this study, fish were visually healthy, suggesting that both *Candidatus* Piscichlamydia salmonis and *Candidatus* Branchiomonas cysticola could be common members of *O. mykiss* gill microbiota on farms. Furthermore, sequences of these pathogenic bacteria were found in all conditions tested, but not in water samples. As a result, these lineages may be a specific trout microbiota component, and may not be harmful as trouts were all healthy.

A study on the microbiome of five mucosal surfaces of adult *O. mykiss* found a mean of 95 OTUs and 14 different phyla dominated by *Proteobacteria* and *Bacteroidetes* (Lowrey *et al.* 2015). We observed the same distribution but with greater diversity (180 OTUs) that could be explained by the use of two distinct regions of 16S DNA (V1-V3 versus V3-V4) and different technologies (454 pyrosequencing versus Illumina Mi Seq, where the second provides deeper analyses).

The gastrointestinal microbiota of fish plays an essential role in the immune system and nutrient acquisition, also outcompeting opportunistic pathogens. Since the advent of metagenomics, many studies of gut microbiome were carried out on economically significant species like rainbow trout (Tarnecki *et al.* 2017). To be able to compare gut microbiota results, it is important to understand the structure of the gastrointestinal tract and which kind of samples were used. In fish, two types of microbiomes are available: the autochthonous bacterial community (resident), represented by mucosal surfaces or epithelial tissues, and the allochthonous community (transient), composed of non-adherent and free-living organisms. The transient microbial community seems to be influenced mainly by environmental factors, whereas the resident one is more linked with host genotype (Legrand *et al.* 2019). Digestive tract anatomy varies according to the fish species and stage of development. For *O. mykiss* juveniles and adults, digestive organs can be divided into three parts: the fore-, mid- and hindgut (Egerton *et al.* 2018). The foregut consists of the esophagus and a U-shaped stomach. The midgut, including pyloric ceca at the anterior position, is the longest portion of the gut where the majority of digestive processes occurs. The hindgut, composed of the distal intestine and anus, is difficult to distinguish from the midgut for some species, like rainbow trout. Both types of samples, autochthonous microbiota (named Intestine), and allochthonous microbiota (Gut content), were sampled at the end of the mid- and hindgut. To gain consistency, we have chosen to compare our results only with previous publications of mid-

and hindgut microbiota obtained using NGS technologies. For example, the exploration of the distal intestine microbiome of rainbow trout by Lyons et al. showed differences in diversity, with 90 genera for the allochthonous (intestinal lumen) and 159 for the autochthonous (mucosal epithelium) communities (Lyons et al., 2017). We confirmed these differences but with a lower level of diversity, which could be explained by the strong dominance of the *Mycoplasma* genus in both microbiotas in our study. Nevertheless, our results lead to the same finding: allochthonous and autochthonous gut microbiomes of rainbow trout are different and must be studied separately in future research.

Fish used in this study were infected by *Flavobacterium psychrophilum*. Analysis of the gut and gill microbiome in resistant and susceptible lines of rainbow trout to *F. psychrophilum* (Brown, Wiens and Salinas 2019) indicated that their midgut microbiomes were dominated by *Mycoplasma*. In rainbow trout, *Mycoplasma* has been found with high prevalence in both the anterior and posterior gut samples (Lowrey *et al.* 2015), in the distal gut of farmed and aquarium fish (Lyons et al., 2017), and in the intestinal mucus (Etyemez and Balcázar 2015). *Mycoplasma* sp. was also described as a major component of the gastrointestinal microbiome of other salmonid species like Atlantic salmon (Holben *et al.* 2002) or Chinook salmon (Ciric *et al.* 2018). *Mycoplasma* spp. were reported in the distal gut of heathy salmon in a cohort of fish infected with *Tenacibaculum dicentrarchi*, with positive correlation between the relative abundance of this bacterium and fish weight (Bozzi *et al.* 2021). Despite an increase in studies, thank to NGS technologies, revealing the prevalence of *Mycoplasma* within the gut microbiota, its function is still poorly understood despite high dependency of this bacterium on its host, as it is not retrieved in the environment (Cheaib *et al.* 2021). Considering the wide range of hosts, geographic locations, farmed or wild caught, pathogen infection or pollution stress, it appears that *Mycoplasma* thrive in the digestive tract of salmonids through a strong

evolutionary force. Further studies are required to understand the nature and functional relationship with fish.

GBHs and fish microbiota

Significant effects of glyphosate or GBHs on microbiota have been observed in different terrestrial organisms, such as Honey Bees (Motta, Raymann and Moran 2018; Motta *et al.* 2020), the Colorado potato beetle (Gómez-Gallego *et al.* 2020), or mice and rats (Aitbali *et al.* 2018) (Mao *et al.* 2018; Nielsen *et al.* 2018). In aquatic organisms, only a few studies are available on the gastrointestinal microbiota of Hawaiian green turtles (Kittle *et al.* 2018) and of Zebrafish, where a decrease of the *Proteobacteria* phylum was observed in the glyphosate group compared to the controls (Ding *et al.* 2021). To our knowledge, we present the first results of the impact of chronic glyphosate or GBH exposure in rainbow trout, an economically important species and a model of interest in ecotoxicology, and their associated microbiomes. We showed that the active substance glyphosate and the GBHs tested affect the gill microbiota by decreasing bacterial diversity and microbial interactions, as suggested by the co-occurrence networks and modifications in taxonomic composition. Preliminary results of *in situ* hybridization go in the same direction, with poorer profiles of microbial morphologies and an apparent more marked distance of the bacteria from the blood vessel regions – with potential impact on exchanges - in the treated samples compared to the control. These data must be confirmed by further observations (**Supplementary Figure 4 A, B, C, D)**. Glyphosate is an herbicide that targets the key enzyme of the shikimate pathway, EPSPS. This biochemical enzyme is found in many bacteria and can be classified into four groups based on differential sensibility to glyphosate. Class I EPSP sequences are sensitive to glyphosate, whereas species with Class II sequences tend to be resistant and Classes III and IV are putatively resistant (Priestman *et al.* 2005; Funke *et al.* 2007; Light *et al.* 2016). The majority

of bacterial species (57%) have class I enzymes (sensitive to glyphosate), whereas 32% have class III enzymes (putatively resistant to glyphosate) (Leino *et al.* 2021). Despite a lack of data, a large proportion of bacteria seem to be sensitive to glyphosate in the human gut microbiome, suggesting a potential decrease in bacterial diversity (Qin *et al.* 2010; Leino *et al.* 2021). In the Gut content of *O. mykiss*, we noticed the presence of Clostridiales (order of *Firmicutes*) known to share both types of species: sensitive (class I) and resistant (class II). Interestingly, we found that one OTU of Clostridiales, *Clostridium* sensu stricto 5, was detected only in the Control condition (**Supplementary Table 8**), whereas another OTU of Clostridiales with multi affiliation (*Clostridium* sp.) was present in all conditions and notably in the Viaglif treatment (**Supplementary Table 7**). A similar observation was made for the Gill microbiota (**Supplementary Figure 2**). Lactobacillales (*Firmicutes* order), showed an intriguing pattern in the Gut content samples with different observations between treatments (**Supplementary Figure 2**). This order was composed of 7 OTUs with genera such as *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Lactococcus* or *Streptococcus*. An OTU affiliated with the *Lactobacillus* genus was present in Control, Viaglif and Round Up but absent from Glyphosate treatment. *Lactobacillus* is a genus of lactic acid bacteria (LAB) that plays a major role in human and animal intestinal health. Many studies in biomedical research have focused on LAB and *Lactobacillus* has been reported to be one of the most promising probiotic species in the prevention of degenerative diseases (Azad *et al.* 2018). An *in vitro* study of potential pathogens or beneficial members of agricultural poultry microbiota has revealed that beneficial *Lactobacillus* spp. were found to be moderate to highly susceptible to Round Up UltraMax®, whereas certain potential pathogenic bacteria such as *Clostridium perfringens* and *C. botulinum* are highly resistant (Shehata *et al.* 2013). Glyphosate also affects the gut microbiota composition of honey bees, with decreased relative abundance of core species like *Lactobacillus* (Motta, Raymann and Moran 2018). LAB identified in the

digestive tract of salmonids and precisely *O. mykiss* were derived mostly from culture-dependent studies and are affiliated with the *Carnobacterium* genus (Merrifield *et al.* 2014). An OTU affiliated with *Carnobacterium* was found in this study in Gut content under the glyphosate condition.

In this study, gill microbiota shows significant differences of relative abundance (**Figure 5**) with decreased *Proteobacteria* (*Polynucleobacter*, *Rhodoferax*). Additionally, some potentially pathogenic bacteria such as *Flavobacterium* and *Candidatus* Branchiomonas sp. seem to be more abundant in control conditions. In adult beetles, a significant increase in the relative abundance of *Agrobacterium* was found in the gut microbiome after treatment with a GBH, whereas three genera (*Acidovorax*, *Rhodobacter* and *Rhizobium*) had reduced relative abundance. In our study, we observed an OTU affiliated with *Acidoverax* in gill microbiota only in the Control treatment (**Supplementary Table 8**), whereas *Rhodobacter* sequences were found in all treatments, but were slightly more abundant in the Control (**Supplementary Table 7).**

CONCLUSION

Metabarcoding results and preliminary microscopic observations indicate that the freshwater fish *Oncorhynchus mykiss* harbors its own bacterial community, distinct from its aquatic environment, and has specific digestive and respiratory microbiotas. Chronic exposure to environmental concentrations of glyphosate or GBHs, pesticides widely used for several decades, may impact the gill microbiota of this fish species of high economic interest. These results open new perspectives for the emerging microbial ecotoxicology discipline (i.e., study of the ecological impacts of chemical pollution at the microbial scale), and raise important questions in the One Heath context for the strategic aquaculture sector. The consequences of

glyphosate-induced changes in the gill microbiota remain unknown and require further studies at the functional level.

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AUTHOR CONTRIBUTIONS

LB carried out the molecular biology experiments and bioinformatics analysis. LD performed FISH analyses. JLD-C, FA and MD carried out fish maintenance and chemical exposure. All authors helped with sampling. LB analyzed the data and wrote the paper. TM completed and corrected the paper. All the authors read, edited and approved the final manuscript.

Conflict of interest. None declared.

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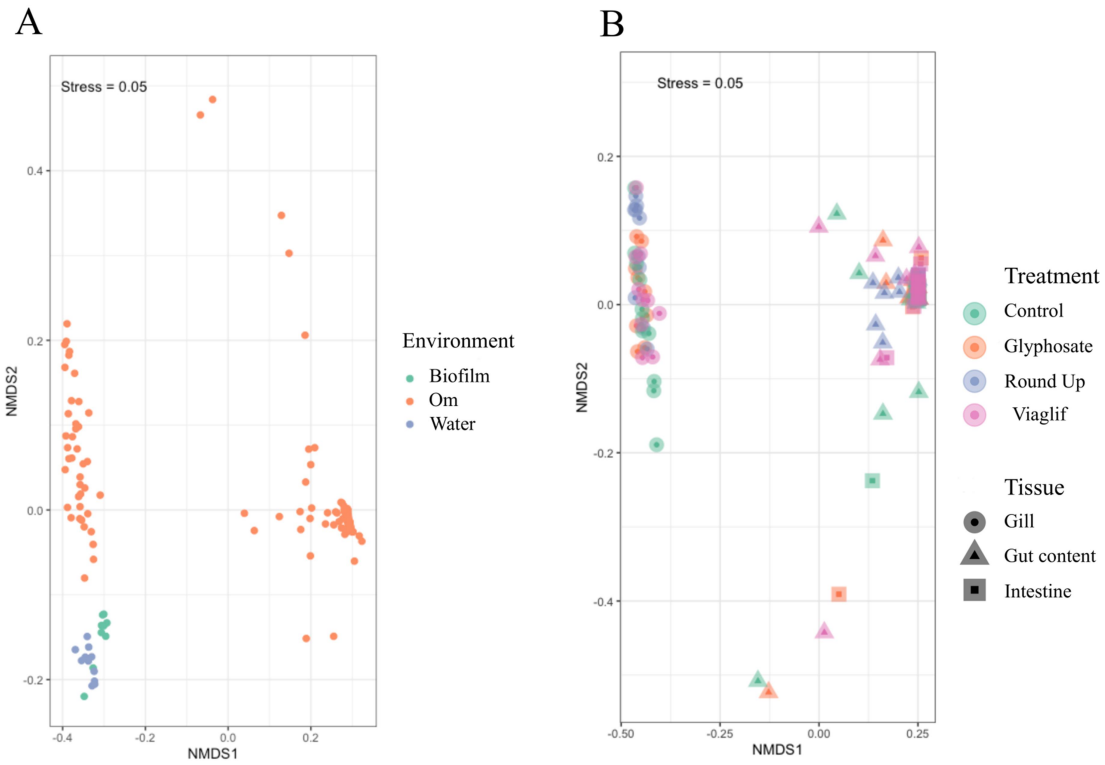

Figure 1: NMDS-plot based on Weigh Unifrac distances illustrating the similarities and differences of bacterial communities from the three environments (A) and from the three rainbow trout tissues with the four treatments (B).

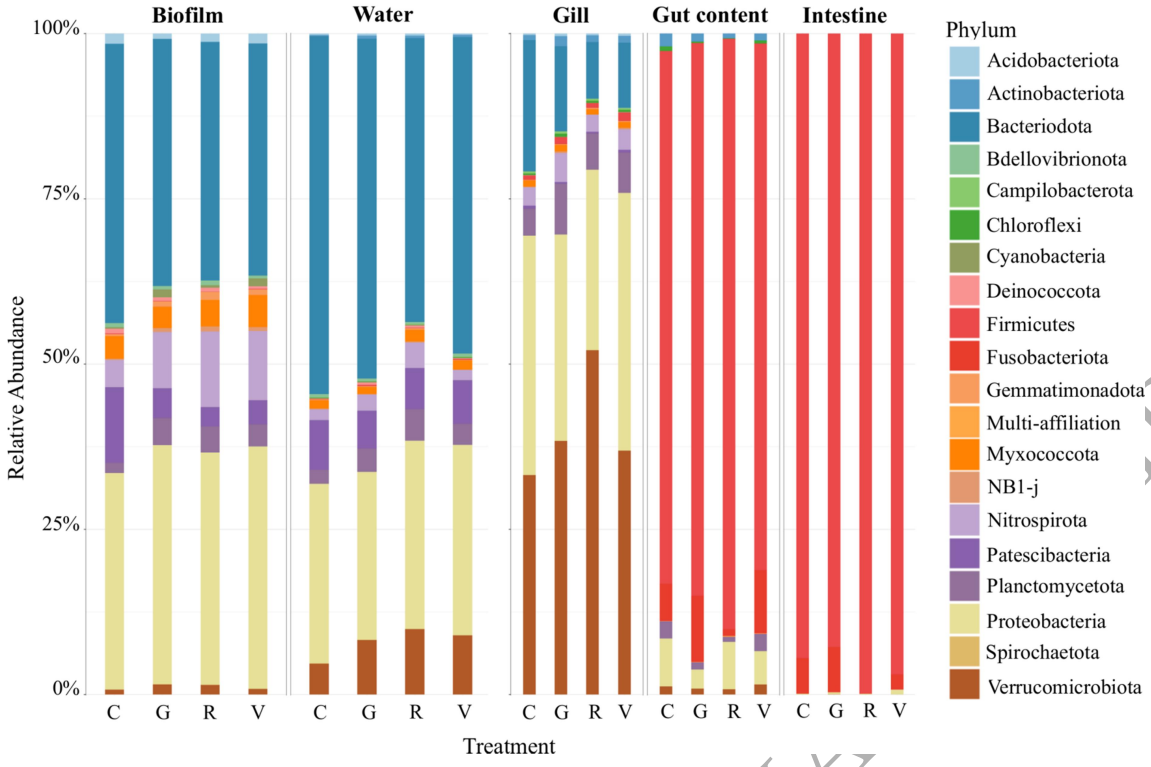

Figure 2: Bacterial community composition at the phylum level of Biofilm, Water and three subsamples of *Oncorhynchus mykiss* for each treatment (C: control; G: glyphosate; R: Round Up and V: Viaglif). Relative abundance is represented in terms of percentage of the total effective bacterial sequences per environment and treatment.

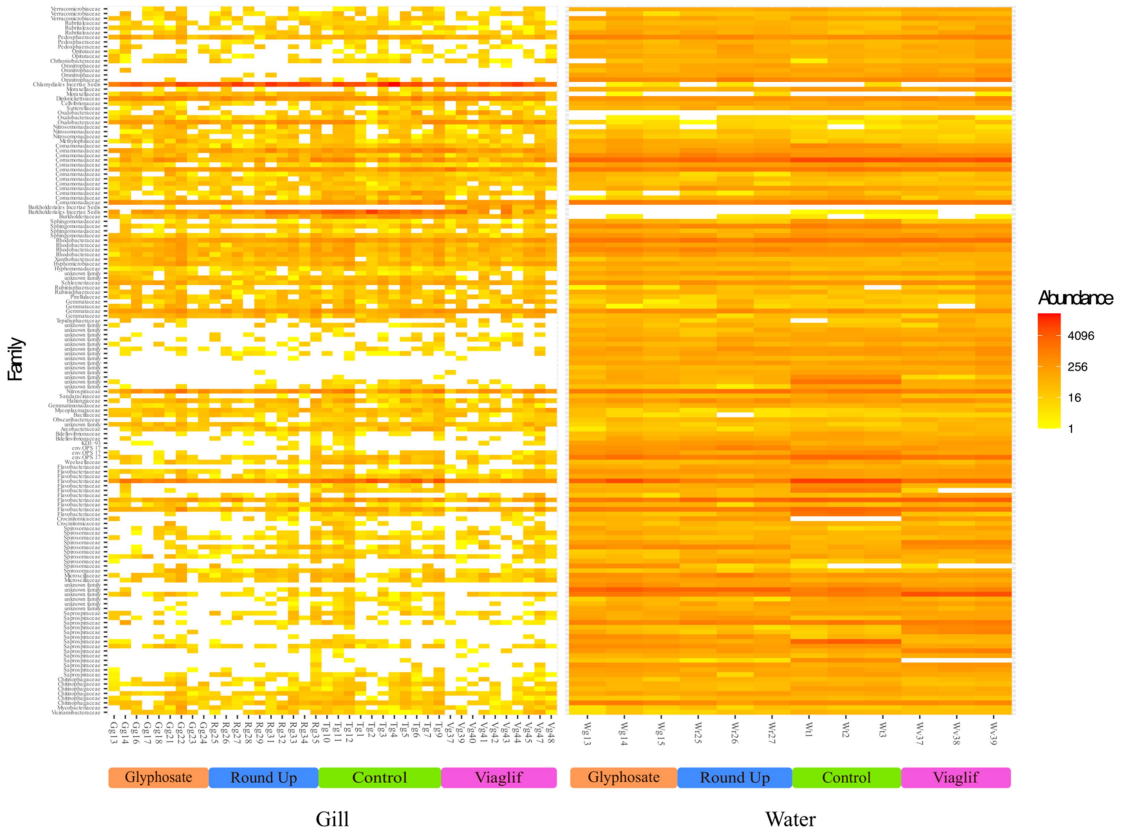

Figure 3: Heatmap of the 150 top abundant taxa between Gill and Water samples. Heatmap was clustered by Weight Unifrac distance and ordinated by MDS.

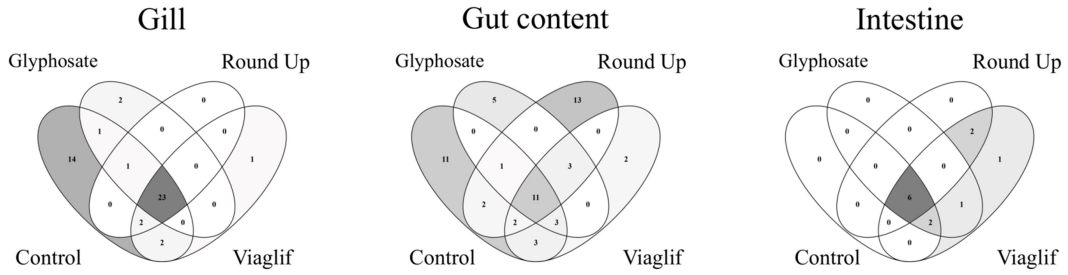

Figure 4: Venn diagrams between the four treatments (Control, Glyphosate, Round Up and Viaglif) for the three subsamples of *Oncorhynchus mykiss* (Gill, Gut content and Intestine). Only OTUs > 0.01% (relative abundance of total reads) were used.

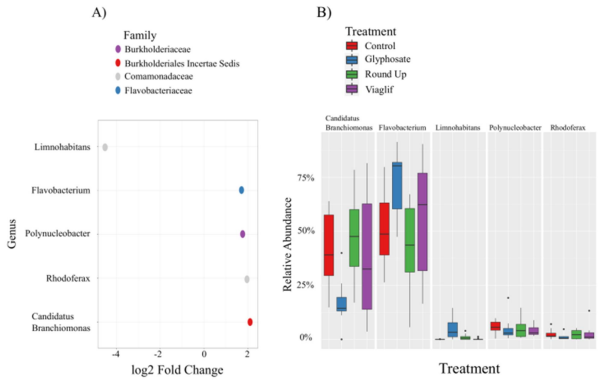

Figure 5: Differentially abundant features at the genus level for Gill. (A) Analysis was performed for Control versus Contaminated samples (Glyphosate, Round Up and Viaglif). Each circle represents a genus and each color a phylum. (B) Bacterial community distribution for the five genera for each treatment separately.

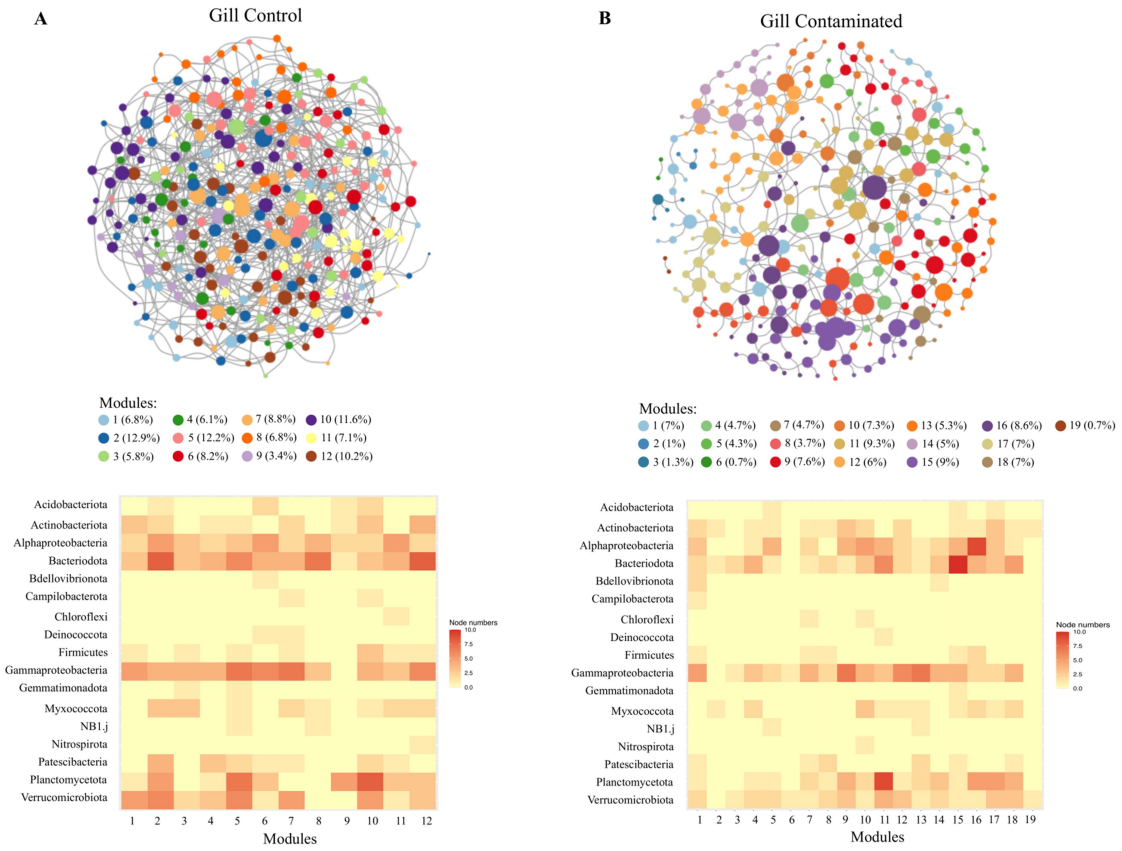

Figure 6: Network analysis showing co-occurrence patterns of bacterial communities among Gill control (A) and Gill contaminated (B), respectively. The nodes are colored according to modularity classes. The size of each node is proportional to the number of connections (i.e., degree). Components of taxonomical diversity in each module of the co-occurrence network are given at the phylum/class-level.

Table 1: Global network topologies

	Control	Contaminated
No. of samples	11	29
No. of nodes	294	312
No. of edges (+/-)	752 (393 / 359)	367 (249 / 118)
No. of modules	12	19
Modularity	0.48	0.75
Mean Node Degree	5.11	2.35
Clustering coefficient	0.07	0.02
Mean path distance	3.85	7.44
Density	0.02	0.08