



Research paper

Long-term industrial metal contamination unexpectedly shaped diversity and activity response of sediment microbiome



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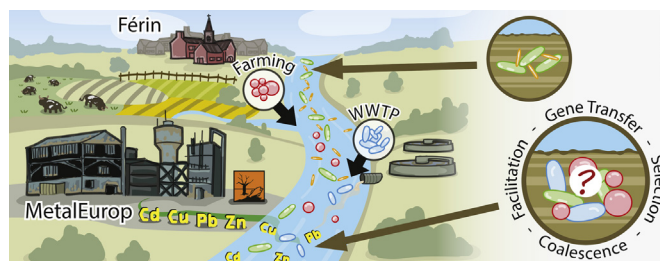
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HIGHLIGHTS

- Combined DNA/RNA sequencing and FRGs accurately predicted microbial lifestyles.
- Metal pollution in sediment resulted in unexpected higher microbial diversity.
- Community coalescence, HGT and microbial facilitation explained this higher diversity.

GRAPHICAL ABSTRACT



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ABSTRACT

Metal contamination poses serious biotoxicity and bioaccumulation issues, affecting both abiotic conditions and biological activity in ecosystem trophic levels, especially sediments. The MetalEurop foundry released metals directly into the French river “la Deûle” during a century, contaminating sediments with a 30-fold increase compared to upstream unpolluted areas (Férin, Sensée canal). Previous metaproteomic work revealed phylogenetically analogous, but functionally different microbial communities between the two locations. However, their potential activity status *in situ* remains unknown. The present study respectively compares the structures of both total and active fractions of sediment prokaryotic microbiomes by coupling DNA and RNA-based sequencing approaches at the polluted MetalEurop site and its upstream control. We applied the innovative ecological concept of Functional Response Groups (FRGs) to decipher the adaptive tolerance range of the communities through characterization of microbial lifestyles and strategists. The complementing use of DNA and RNA sequencing revealed indications that metals selected for mechanisms such as microbial facilitation *via* “public-good” providing bacteria, Horizontal Gene Transfer (HGT) and community coalescence, overall resulting in an unexpected higher microbial diversity at the polluted site.

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1. Introduction

Although known to naturally occur due to particular geological context, metal contamination of soils and sediments often origi-

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nates from anthropogenic activities such as mining activities [1,2], wood processing [3], shipping, dredging [4], urbanization [5] and industrial processes [6,7]. These metals constitute a serious risk because of their biotoxicity and bioaccumulation in the environment [8].

The peculiar characteristics of sedimentary environments, such as redox potential, pH, organic matter as well as biological activity, turn them into natural accumulation hotspots adsorbing and precipitating up to 90% of soluble metals and metalloids from water compartments [2,9]. Fresh water sediments are hosting thousands of microbial species [10], seating at the bottom of the trophic levels and often actively involved in metal movements in the biosphere [11]. Therefore, many studies have analyzed the link between metal accumulation and microbial communities in sediments. Investigating sediment microbiomes represents a good opportunity to understand resistance/tolerance adaptation and molecular mechanisms involved, with important applications in the field of bioremediation and biostimulation [12]. Metal-contaminated sediment bacteria highlighted by previous studies were mainly affiliated to Proteobacteria [1,7,13,14], Bacteroidetes [13,14], Firmicutes [12,13] and Actinobacteria [5,7]. Beta- and Gammaproteobacteria are the essential Proteobacterial classes, including respectively microbial members from Burkholderiales [2,7,13,14] and Pseudomonadales/Xanthomonadales [5,7,12–14].

Total microbial biomass, activity and phylogeny are the most studied traits when investigating the response of environmental microorganisms to metals. For instance, Gillan et al. [7] found no changes in biomass and activity in an 80-years metal contaminated fjord, but revealed community structure variations *via* DGGE fingerprints, implying long-term adaptation and functional recovery over time [15]. These observations were also reported for other systems, including river-connected lakes in the Rouyn-Noranda region, Canada [16], as well as long-term copper polluted grasslands, for which the microbial community composition was altered at DNA level with no consequences on species richness [17]. Conversely, Nayar and colleagues used mesocosms to show the negative impact of metals on crucial ecosystem components, such as primary producers (*e.g.* phytoplankton and autotrophic bacterial activity) [4]. They also pointed out that heterotrophic bacteria seem to be less affected by metals as a short-term stress [4]. Conversely, some recent studies have revealed strong negative impact of metals on microbial diversity in terms of richness and evenness [1,3,5]. These contrasting observations imply that other factors are involved in structuring the microbial response to metals. These might include site-specific physicochemical differences (*e.g.* pH, organic matter...), sediment sampling depth and associated metal bioavailability (*e.g.* anoxic gradient, ecosystem temporal dynamics...), the nature of social interactions between microbiome members (*e.g.* facilitation, exclusion, priority, biofilms, keystone species...) and genetic modalities of metal resistance/tolerance (chromosomes and/or plasmids) [18]. In addition, differences between molecular markers have been reported. For instance, Berg et al. found no richness loss after long-term copper pollution in grassland soil at the DNA level [17], while a recent study on the same site reported a clear diversity loss in the potentially active microbial fraction at the RNA level [3], both based on 16S rRNA amplicon sequencing. This suggests that some community members might display low activity profiles, explaining the maintained DNA diversity levels unlike RNA. This also implies that genetic diversity may not be lost due to metal pollution *per se*, but will still be present under latent state, suggesting potential reactivation of dormant ecosystem functions in case of disturbance removal. Overall, these contrasting observations call for better alternatives to investigate and understand the ecology and modalities of microbial adaptation to metals.

In the present study, we have revisited the sediments from the Deûle river sites in northern France, previously investigated by a metaproteogenomic approach [7]. Sediments were exposed to long-term metal releases from the industrial site of MetalEurop, a former foundry operating from 1893 to 2003 in Noyelles-Godault [19]. Metal concentrations in these sediments are currently up to 30-fold higher compared to control upstream locations at the Sensée canal in Férin. Sediments are mainly contaminated with cadmium, copper, lead and zinc that respectively reach 38.1, 100, 913.8 and 3218.5 mg/kg (Table S1) [6,7,19]. Gillan et al. showed by shotgun metagenomics that microbiomes from Férin (FER) and MetalEurop (MET) were phylogenetically analogous but functionally different [7]. In this study, we aimed to investigate the sediment prokaryotic communities with a refined complementing approach using both DNA and RNA (cDNA) molecular levels by means of high throughput sequencing of the 16S rRNA gene. We hypothesized that the long-term pollution has impacted the prokaryote diversity and selected for different microbial strategies and lifestyles. We applied the ecological concept of functional response groups (FRGs), which aims to classify the response of microorganisms “as a function of” environmental parameters [3,20,21]. FRGs should be clearly differentiated from Functional Effect Groups (*aka* guilds), which are groups of organisms contributing to the same ecosystem function (*e.g.* nitrogen cycling or cellulose degradation). Defining response groups based on RNA/DNA abundance patterns in relation to environmental variables and without any phylogenetic *a priori* is a powerful method in ecology for detecting niches occupied by specific microbial strategists [3,20]. Although communities are sharing similarities as previously reported, the resolution of our analysis allowed identification of six microbial response groups with specific DNA and RNA molecular signatures linked to metal sensitivity/tolerance after long-term exposure. Our study adds a decisive and innovative contribution to the current knowledge regarding microbial adaptation to metals in sediments, with regards to the contrasting results often reported in the literature.

2. Materials and methods

2.1. Sampling, DNA and RNA extraction, cDNA synthesis

Sediments were sampled in May 2016 from the Sensée Canal and the Deûle river sediment in Férin (FER) and Noyelles-Godault next to MetalEurop (MET) in France, respectively. Three sediment cores were collected at each station and two samples of 2 g were taken from the upper part of each core, representing a total of 12 samples (3 cores \times 2 samples at FER and MET, respectively, Table S2). Samples were stored in Life-Guard RNA blocking solution (Mobio) at 4 °C during transport and –20 °C in the laboratory. For DNA/RNA extraction, 6 \times 2 g of sediments per station were washed using the Fortin et al. (2004) procedure in order to remove potential PCR inhibitors [22]. From the 2 mL re-suspended and washed sediment, 500 μ L were used for total DNA extraction (FastDNA[®] SPIN Kit for Soil, MP Biomedicals, Santa Ana, CA, USA) and 800 μ L were used for total RNA extraction (FastRNA[™] Pro Soil-Direct Kit, MP Biomedicals, Santa Ana, CA, USA). DNA was removed from the RNA solution with a DNaseI treatment using the Ambion[®] DNA-free[™] DNase Treatment and Removal Reagents kit (ThermoFisher Scientific, Waltham, MA, USA). cDNA synthesis was performed using 10 ng of DNaseI treated RNA as template with Random Hexamer primers (Sigma, St. Louis, MO, USA) using the Roche Expand[™] Reverse Transcriptase kit (Roche, Basel, Switzerland), according to manufacturer's instruction. Generated cDNA samples were stored at –20 °C until further processing.

2.2. High throughput 16S rRNA gene sequencing

Amplicon sequencing was realized according to acknowledged best practices guidelines [23]. An initial PCR amplification step was performed, using a set of primers, 341F: 5'-CCTAYGGGRBGCASCAG-3 and 806R: 5'-GGACTACNNGGTATCTAAT-3 [3], which flank the approx. 460 bp variable V3–V4 region of the Prokaryote 16S rRNA gene, including domains of Archaea and Bacteria. Tagging and adding sequencing adapters to amplified DNA was done in a second amplification step using fusion primers that have adaptor barcode tags and spacers as previously described [3]. Purification and size-selection (removal of products less than 200 bp) of the approx. 620 bp PCR amplicon products was performed using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) according to manufacturer's instructions. The samples were pooled and adjusted to equimolar concentrations, concentrated using the DNA Clean and Concentrator™ -5 kit (Zymo Research, Irvine, CA, USA). Finally, they were subjected to 2 × 250 bp paired-end high-throughput sequencing on an Illumina® MiSeq® platform (Illumina, San Diego, CA, USA) according to manufacturer's instructions. Unassembled raw amplicon data were deposited at the Sequence Read Archive public repository (SRA, <https://www.ncbi.nlm.nih.gov/sra>) under the accession number SRP112522 (<https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP112522>).

2.3. Annotation and generation of the contingency table

Amplicon analysis was realized according to acknowledged best practices guidelines [23]. Generated amplicon sequences were analyzed using qiime_pipe (https://github.com/maasha/qiime_pipe) as previously described [3]. Sequence demultiplexing was done using the MiSeq Controller Software and diversity spacers were trimmed using biopieces (www.biopieces.org). Sequence mate-pairing and filtering was done using *usearch* v7.0.1090 [24]. OTU clustering, dereplication and singleton removal was performed using *uparse* [25]. Paired-end mating was applied with a minimum overlap of 50 bp, maximum mismatches of 15 and a minimum quality of 30. Criteria for sequence trimming were based on: (1) reads shorter than 200 bp, (2) average quality scores lower than 25, (3) maximum number of ambiguous bases and (4) six as maximum lengths of homopolymers. Chimera checking and removal was performed using *usearch* and the *ChimeraSlayer* package [26]. Operational Taxonomic Units (OTUs) were picked at 97% sequence identity level using *Mothur* v.1.25.0 [27]. An UniFrac phylogenetic tree was built using *Greengenes* [28] with QIIME wrappers for *PyNAST* [29], *Fast-Tree* [30], and alignment filtering [31]. A read contingency table was exported at species level. Samples with less than 2,000 sequences were not considered, as they barely provide enough coverage for further diversity analysis [32]. Information regarding the sequence counts for each sample is provided in the Supporting Table S2.

2.4. Alpha-diversity analysis

Alpha-diversity analysis was carried out as previously described [20,33]. The raw sequencing counts were used directly to estimate the sequencing depth completeness via rarefaction curves (Table S3) using the *PAST* software [34]. Diversity indices were calculated on rarefied data at 8,900 counts per sample. Samples below 8,900 counts were not included for this particular analysis (Table S2), leaving each condition with at least four biological replicates. Venn diagrams were established to display the richness distribution of rarefied data among the different tested conditions (Fig. S1) using the *Rgui* package *limma* [35]. The following indices were used to assess the diversity: the sample richness, the Shannon (H), the Chao-1 and ACE indices (Fig. 1). Statistical analysis was done with

Table 1

PERMANOVA analysis based on Bray–Curtis dissimilarity with 10,000 permutations. The top panel shows a three-way PERMANOVA on the following factors: 1) Sediment core, 2) DNA/RNA level and 3) Site (Férin/MetalEurop). The bottom panel is showing refined one-way PERMANOVAs showing the nested differences between sites according to 1) DNA and 2) RNA levels, followed by the differences between RNA/DNA levels within each site, namely 3) Férin and 4) MetalEurop. Significance: ***if $p < 0.001$; **if $p < 0.01$; *if $p < 0.05$.

Factors tested	r ²	p	Signif.
Three-way permanova (Bray–Curtis, 10000 perms.)			
1: Sediment core	0.048	0.105	–
2: DNA/RNA level	0.142	1.7E–4	***
3: Site (Férin/Metal)	0.224	1.0E–5	***
One-way permanovas (Bray–Curtis, 10000 perms.)			
1: DNA level: Férin vs Metal	0.361	8.2E–3	**
2: RNA level: Férin vs Metal	0.321	3.3E–4	***
3: Férin: DNA vs RNA	0.224	0.015	*
4: Metal: DNA vs RNA	0.291	8.1E–4	***

the *Rgui* software version 3.0.2 [36] using the *multcomp* package with ANOVA and a post-hoc Tukey HSD correction test ($p < 0.05$).

2.5. Beta-diversity analysis

The dissimilarity and multivariate analysis were done with the *Rgui* software [36] using the *Rgui* package *vegan* [37] as previously described [3,20,33]. As the OTU contingency tables have features with abundance variation higher than 1000-folds, a log₁₀ transformation was applied. A cluster dendrogram based on Bray–Curtis dissimilarity was established on OTU profiles using 1,000 permutations (Fig. 2). PERMANOVA tests were performed on the Bray–Curtis dissimilarity profiles using 10,000 permutations (Table 1) to assess the significance of the tested factors (sampling site, nucleotide material, sampling core). Major relative abundance phylogenetic changes in the datasets were investigated by means of ANOVA with a false discovery rate correction test (FDR, $p < 0.05$). Changes attributed to sediment site-specific differences are presented in Supporting Table S3. Differences related to nucleotide levels (DNA and RNA) are presented in Supporting Table S4.

2.6. Identification and validation of functional response groups (FRGs)

The identification and validation of FRGs was adapted from a previously described procedure [3,20]. OTUs significantly affected between sites were identified with an analysis of deviance on the raw non-rarefied counts under negative binomial distribution and generalized linear model, corrected by 1,000 resampling iterations of the residual variance (nbGLM, $p < 0.05$) using the *Rgui* package *mvabund* [38]. The 139 significantly affected OTUs were plotted in a generalized heatmap, and response groups were defined with a hierarchical cluster dendrogram (Euclidean distance and average clustering) using the *Rgui* package *vegan* (Fig. S4) [37]. Statistical validity of the so-obtained six FRGs was tested against a null-model by Monte-Carlo simulation with all OTUs to reinforce randomization power and avoid a priori effects during group determination (Fig. S5). A summary showing FRGs abundance patterns and phylogenetic composition is shown in Fig. 3.

Phylogenetic structure in FRGs was tested using relatedness indices [39]. The unweighted UniFrac 16S rRNA amplicon tree produced by *QIIME* was used with the *Rgui* package *ape* [40]. The *RGui* package *picante* was used to calculate relatedness [41] via the Mean Nearest Taxon Distance index (MNTD, aka -1^*NTI for Nearest Taxon Index) [42]. Significance of the FRGs relatedness indices was tested using a simulated null model with random mock groups of the same size (Z-scores, 10,000 permutations, 95% confidence interval, $p < 0.05$, Table 2).

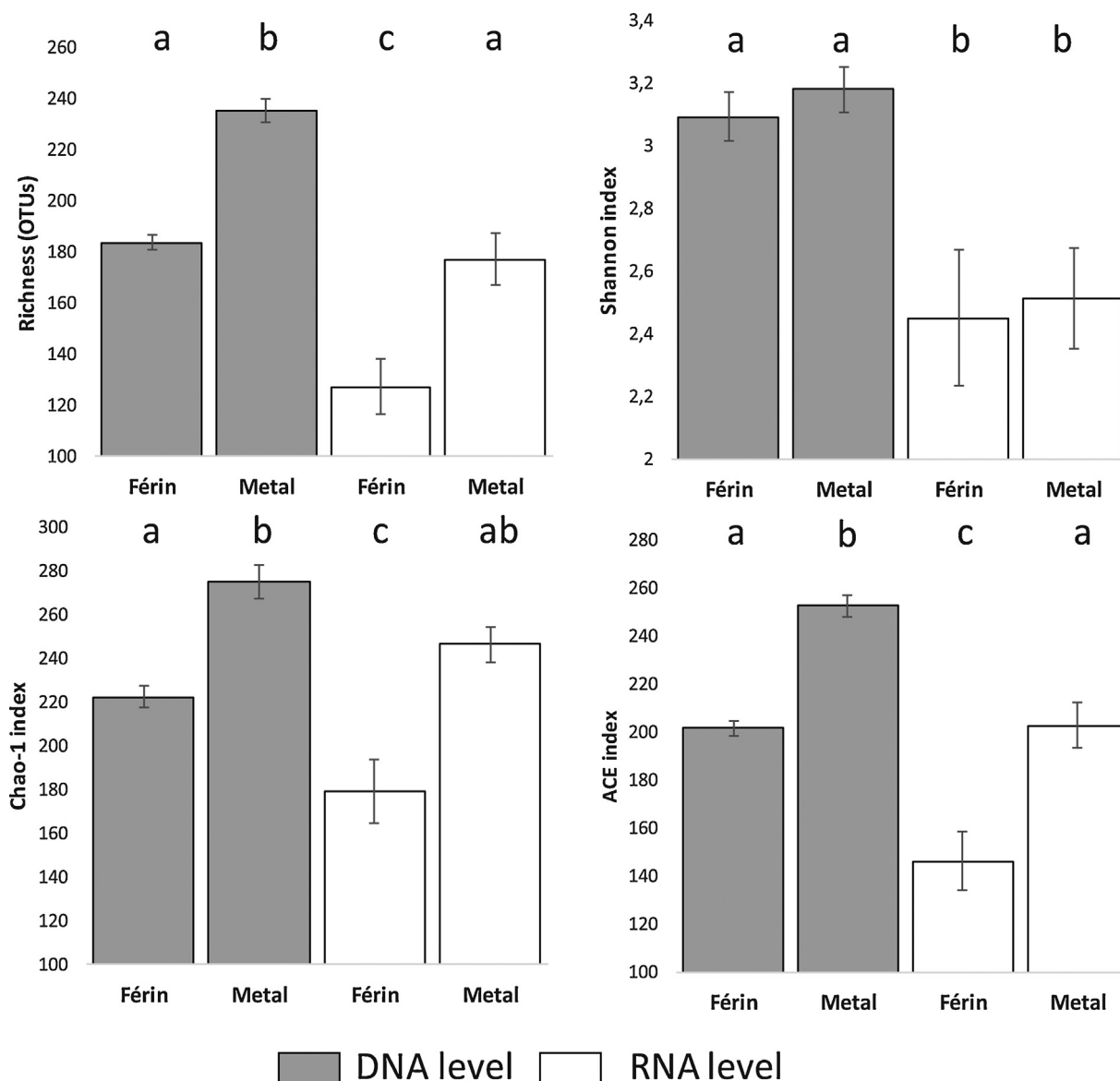


Fig. 1. Diversity indices in each sediment sites and according to DNA and RNA levels. Statistical significance was inferred by ANOVA with a Tukey post-hoc correction test ($p < 0.05$). The letters indicate statistically significant differences between conditions.

Table 2
Phylogenetic relatedness of the Functional Response Groups (FRGs) and overall sediment communities (Férin and MetalEurop) and the associated statistical analysis. The Mean Nearest Taxon Distance (MNTD obs.) was calculated for each FRG and their mock version based on the UniFrac distance in the OTU tree, and compared to their respective simulated null model counterpart (MNTD null \pm SEM) using a Z-test (Z-scores after a null model simulation with 1,000 permutations, $p < 0.05$). Significance: ***if $p < 0.001$; **if $p < 0.01$; *if $p < 0.05$.

FRGs	OTUs (n)	MNTD (obs.)	MNTD (null)	MNTD (z-score)	MNTD (p)
FRG1	36	0.174	0.236 \pm 0.001	-1.841	0.028*
FRG2	29	0.245	0.254 \pm 0.001	-0.228	0.437
FRG3	39	0.229	0.229 \pm 0.001	-0.005	0.496
FRG4	13	0.16	0.335 \pm 0.002	-92.303	0.003**
FRG5	11	0.275	0.36 \pm 0.003	-0.955	0.161
FRG6	11	0.292	0.359 \pm 0.003	-0.763	0.243
Férin	314	0.105	0.106 \pm 0.003	-0.349	0.353
MetalEurop	360	0.097	0.101 \pm 0.002	-2.783	0.003**

3. Results and discussion

Studies focused on microbial ecology of long-term environmental metal contaminations have reported contrasting observations in terms of adaptation strategies and community diversity status. Although part of these discrepancies could be explained by

ecosystem types and site specificities [3,16,17,43], some reported cases for similar locations still revealed interpellant observations depending on applied experimental design, especially the molecular methodology used (e.g. DNA [17] or RNA [3]). In this study, the investigated model sediment sites were previously studied *via* metagenomics [7], revealing taxonomically analogous communi-

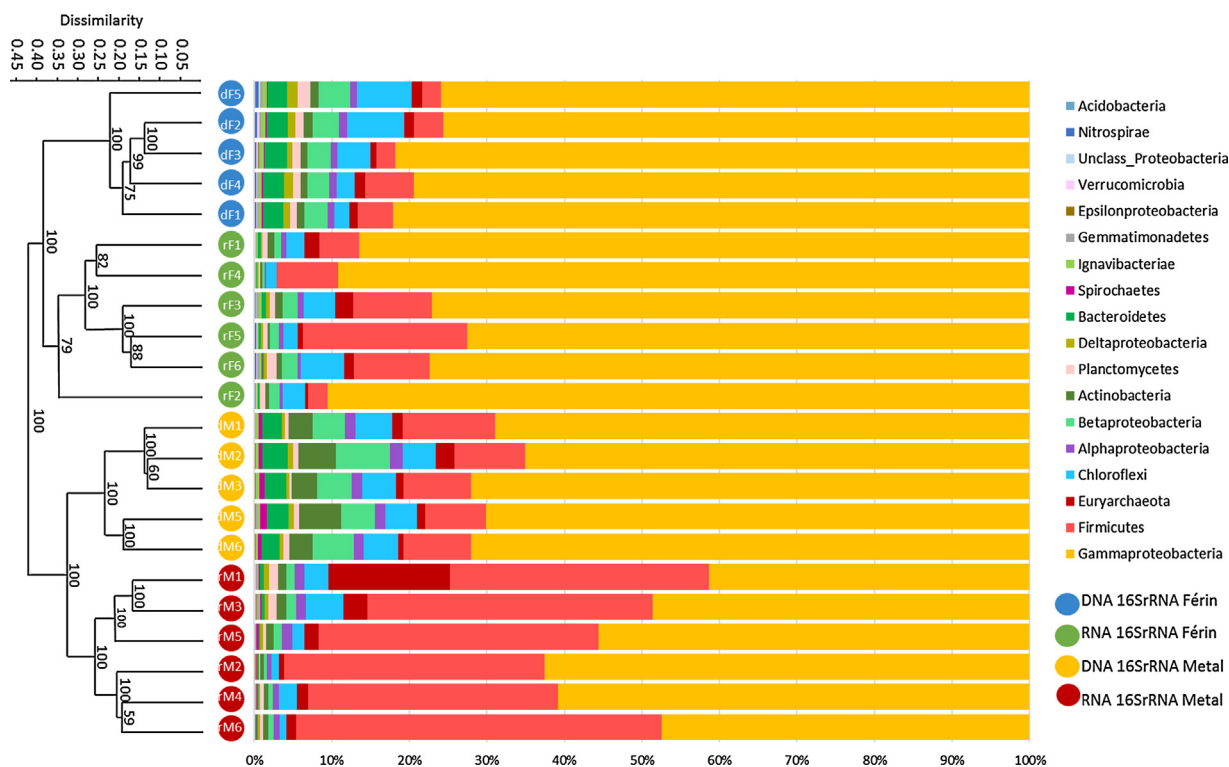


Fig. 2. Cluster dendrogram analysis of the taxonomic profiles at OTU level based on the Bray-Curtis dissimilarity index. The grouping coherency was assessed by 1,000 bootstraps retaining the highest cophenic correlation index possible (0.78).

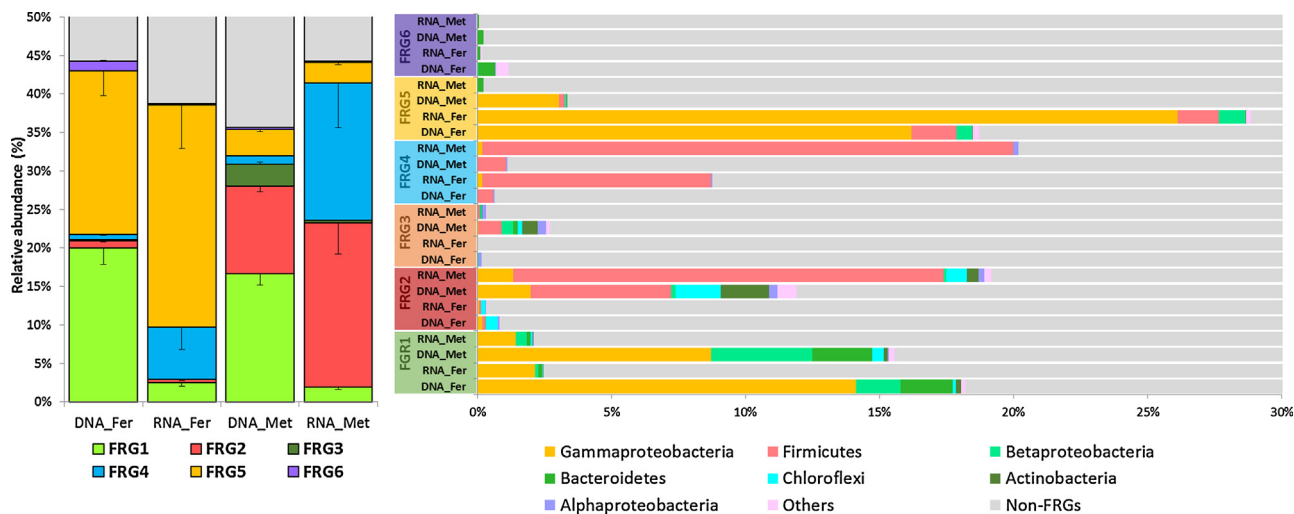


Fig. 3. Composition and abundance of the six Functional Response Groups identified in this study. Panel A represents the abundance patterns of the six FRGs across the sites (Férin and MetalEurop) and RNA/DNA levels. Panel B represents the weighted abundance of phylogenetic groups in each FRG for each compartment. The grey color in the right barchart represents all remaining sequences not included in the particular FRG displayed.

ties between foundry-contaminated sediments (MetalEurop) and a control upstream location. This work goes a step further using 16S rRNA gene sequencing at DNA and RNA (cDNA) molecular levels to precisely characterize community diversity and structure via identification of FRGs for better interpretations of previous observations and pinpoint microbial strategies.

3.1. RNA and DNA description of sediment microbiomes

Despite significant differences seen on richness between Férin and MetalEurop (Fig. 1), taxonomic profiling confirmed previously observed analogies between sites, sharing 70% OTUs at the DNA

level (Fig. S2) and with an overall high beta-diversity similarity level between profiles (Table 1). Indeed, the PERMANOVA on the Bray-Curtis dissimilarity index with 10,000 permutations (Table 1), revealed significant but moderate site ($r^2 = 0.23$, $p = 1.0E-5$) and DNA/RNA effects ($r^2 = 0.14$, $p = 2.9E-4$), followed by a minor but significant interaction between the two factors ($r^2 = 0.06$, $p = 0.04$). No effect of the sampling core was detected, indicating overruling site and DNA/RNA effects compared to biological sampling heterogeneity ($r^2 = 0.05$, $p = 0.11$). Nested analyses at the nucleotide level (DNA vs RNA; Table 1) revealed a quasi-similar and significant discriminative power for both DNA ($r^2 = 0.36$, $p = 8.2E-3$) and RNA ($r^2 = 0.37$, $p = 2.2E-3$) in differentiating the two sites. This is coherent with

evenness analysis showing similar Shannon index levels between DNA and RNA profiles (Fig. 1). On the other hand, the DNA/RNA differences at each site seemed to be more explanatory and significant at MetalEurop ($r^2 = 0.38$, $p = 2.2E-3$) compared to the Férin site ($r^2 = 0.22$, $p = 0.01$). This makes sense as the river dynamic flow between the upstream control site and the downstream polluted one would continuously contribute in maintaining the microbial diversity longitudinally with new inoculum material carried by the water stream [44].

Proteobacteria was very prominent, which is expected in these ecosystems [1,7,14]. Gammaproteobacteria (Enterobacteriales, Pseudomonadales and Aeromonadales) clearly dominated all samples, (Fig. 2) although they significantly dropped in metal contaminated samples (Table S3) as previously observed [17]. Conversely, Alpha- and Betaproteobacteria classes were enhanced in the metal-contaminated sediments (Table S3), but only Alphaproteobacteria were enriched at the RNA level (Table S4), implying potentially enhanced activity. This is in agreement with previous observations showing some Alphaproteobacteria species reacting to metallic contaminants as potential bioindicators in a positive (e.g. members from Sphingomonadales) [45,46], but also negative manner (e.g. members from Rhizobiales) [47]. The second dominant phylum in metal-contaminated sediments was Firmicutes (Fig. 2), as previously described [7,12]. This group is particularly active in metal contaminated sediments and includes mainly known metal-coping bacteria from Clostridiales and Lactobacilliales [48,49].

Bacteroidetes were mainly represented in the passive part of the community (Fig. 2) and are known to be impacted by metals [17]. Although often associated with anaerobic niches, some members are affiliated to oxygenated environments like representatives from *Flavobacterium* [50], which were indeed detected here in the oxic upper sediment parts. In agreement with previous DNA-based analysis of polluted sediments [51], Actinobacteria were more represented at MetalEurop (Fig. 2) although their RNA levels were lowered, indicating some sensitivity and/or activity decrease (e.g. sporulation/dormancy [52]). Most low-abundance phyla seemed significantly impacted by the anthropogenic pollution at the DNA level, including Deltaproteobacteria, Gemmatimonadetes, Ignavibacteriae or Verrucomicrobia (Table S3). This highlights the negative effect of metals on rare members, with potential losses of important ecological functions associated with these groups in aquatic ecosystems [53]. On the other hand, when comparing the average OTU RNA/DNA ratio (Fig. S3), only Actinobacteria OTUs were significantly decreased in activity in MetalEurop, confirming their sensitivity. Conversely, Acidobacteria, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gemmatimonadetes, Nitrospirae, Planctomycetes and Verrucomicrobia OTUs unexpectedly had significantly increased activity ratios at the contaminated station. This increase could be linked to altered behavior *via* active resistance and/or stress reaction due to metal pollution, but also through changes in social interactions between sediment microbiome members.

3.2. Deciphering the community tolerance and sensitivity range

In order to determine microbial strategies and adaptation to anthropogenic metal contamination, we applied the concept of Functional Response Group, also known as groups of organisms responding similarly to environmental clues with no phylogenetic a priori [3,20]. OTU response patterns were extracted by means of generalized linear models under negative binomial distribution, and significance inferred by likelihood ratio tests corrected with residual deviance resampling with 1000 iterations (nbGLM, $p < 0.05$). This procedure is one of the most reliable way to find significantly responding OTUs by minimizing the risk of error [54], enabling better modelling of variable inter-dependency and

mean-to-variance relationship [37]. A total of 139 OTUs responding significantly were extracted and represented in a heatmap for pattern detection (Fig. S4). The constrained ordination of all OTUs found in this study under Monte-Carlo simulation revealed a very significant and non-random clustering into six distinct response groups (called FRG1-6, $p < 1.0E-08$, Fig. S5) displaying peculiar molecular patterns and phylogenetic signatures (Fig. 3, Table 2).

The dormant “seed bank” (FRG1) represents an ubiquitous fraction of the community, characterized by a significant phylogenetic signal (Table 2) from metal-tolerant but slow-growing and/or inactive members, mainly from Gamma-, Betaproteobacteria and Bacteroidetes. It features fresh-water bacteria associated to metal-tolerance, like *Aeromonas* and *Shewanella* [55], *Leadbetterella*, *Halicomenobacter* and *Anaerolinea* [2]. Some were previously identified in metal contaminated sediments and soil like *Flavobacterium* [50], *Methylobacter* [56] and *Arenimonas* [57]. Some members from *Sideroxydans*, *Thiobacillus* or *Spirochaeta* have already been identified as typical slow-growing chemo-autotrophic bacteria [58–61], reinforcing the idea that the low RNA representation might be a characteristic lifestyle feature of this group.

The groups characterized as “upcoming bacteria” were recruited in the polluted site for their obvious tolerance to metals (Fig. 3), either under potentially active (FRG2) or passive (FRG3) states. Many active Firmicutes members from FRG2 have reported metal-resistance, like *Lactobacillus* [49,62], *Clostridium* [48], *Proteiniclasticum* [63] and *Turicibacter* [64]. Overall, Firmicutes are prone to acquire novel beneficial genetic traits increasing their fitness [65], and some members are well-known for their notable natural competence capacities (e.g. *Bacillus* and *Streptococcus*) [66–68]. These characteristics may explain their high activity and adaptive success in MetalEurop sediments. Some active Proteobacterial members were also described as harboring metal-resistant representatives, like *Rhodobacter* [69], *Pseudomonas* [13,70], *Raoultella* [71] and *Acinetobacter* [72]. The lack of phylogenetic signal inside FRG2 and FRG3 (Table 2) imply that their adaptive response to metal selection is either due to genetic parallel evolution, co-evolution/facilitation processes, or the involvement of Horizontal Gene Transfer *via* broad host range mobile genetic elements spreading across phylogenetic barriers, as previously highlighted with conjugative plasmids [20,73].

“Fecal-related bacteria” (FRG4) has a pronounced RNA representation in the polluted site (Fig. 3), with a significant phylogenetic relatedness between the main constituting members: *Clostridium* and Enterobacteriaceae (Table 2, Fig. S2). Presence of *Clostridium* members in polluted and pristine sediments was previously observed [74–76], likewise for metal-resistance [48]. They also have the capacity to form endospores that can, at low temperature, produce a high amount of rRNA [77] which may explain their RNA prevalence [52]. Both *Clostridium* and Enterobacteriaceae are typical bioindicators of fecal contamination in water ecosystems [20,78,79]. Our results suggest that fecal-related microorganisms could tolerate metal pollution in sediments, representing an intermediate environmental niche where they could potentially thrive after environmental release from wastewater treatment plants (WWTPs) and/or agricultural sources. This might be related to their ability to cope with antibiotic residues often found in wastewater [20]. Indeed, bacterial resistance strategies against antibiotics and metals are known to be similar and correlated [80,81]. Resistant wastewater bacteria exposed to residual antimicrobial agents from diverse origin as WWTPs [82–84] or organic fertilizers (e.g. manure) [85] will potentially have a selective advantage to cope with metals in sediments.

The group labelled as “Dominant metal sensitive bacteria” (FRG5) includes mainly prominent sediment Gammaproteobacteria members (e.g. *Pseudomonas* sp.) with significantly reduced representation at both RNA and DNA level in the contaminated

site (Fig. 3). While this significant drop is likely driven by metals as previously reported [17], the significant uprising of FRG3-4 at the contaminated site could partly explain this observation. This would indicate that dominant sediment bacteria are being challenged by both metal pollution and potential strong niche competitors. Finally, the group labelled as “Rare metal sensitive bacteria” (FRG6) mostly contained dim archetypical oligotrophs and specialist members from Bacteroidetes [86], Acidobacteria [87], and Deltaproteobacteria [88], which were significantly impacted by metals. Due to the rarity and significance of this trend, these members could be relevant bioindicator candidates of sediment pollution.

3.3. Significant increase in richness in the contaminated site

Richness index and tested estimators (ACE and Chao-1 indices) highlighted the higher diversity levels observed in the polluted site compared to Férin at both DNA and RNA level (Fig. 1). This was not seen previously with 454 Roche metagenomic sequencing due to inaccurate taxonomic affiliation of short metagenomic reads [33,89,90], but also because of sequencing depth limitations associated with the technology and lack of replicates [7]. This finding does not support our initial postulate on putative diversity loss driven by pollution, and conversely suggests that metals altered the community composition in an unexpected manner. One possibility to explain this observation may be linked to the toning down of some dominant sediment Gammaproteobacteria members (Fig. 2). Gammaproteobacteria are often characterized as fast growing copiotrophs [91,92], and some of them, like members from *Pseudomonas*, are well-characterized strong competitors (aka weed-species), prone to dominate [20,93]. Nevertheless, the effect of metals on *Pseudomonas* members (FRG5) and also competition with better-fitted groups (FRG3 and 4) supports the idea of a microbial competition for niche occupation. Reduction of dominant *Pseudomonas* members allowed novel, metal-tolerant, slow-growing/specialist bacteria to thrive better, resulting in an overall diversity increase. Identification of slow-growing oligotrophic microbes in the polluted sediments is supporting this assertion, like Sphingomonadales members from “upcoming bacteria” (FRG2-3), involved in biodegradation of metal associated compounds [91]. Moreover, other oligotrophic specialists were detected with higher RNA prevalence in the metal contaminated sediments, including Acidobacteria, Verrucomicrobia and Planctomycetes (Fig. S3), which is coherent with previous observations [94,95].

Another phenomenon potentially participating to the increased richness at the polluted site is the extra-cellular metal precipitation ability carried by specific bacteria like *Pseudomonas* and *Bacillus* members via metallophores, EPS, biogenic sulphides or calcite [96]. These bacteria may be considered as providers of so-called “public goods”, benefiting the whole community by creating local safe micro-niches for sensitive bacteria to thrive [62,60]. Indeed, *Lactobacillus* members, which are present in our sediment communities and in FRG2, have this ability to bind and sequester metals, providing local safe spots where other sensitive community members could be protected [62]. Other members from FRG2 and FRG4 are known to be able to ensure metal removal like *Clostridium* [48], *Rhodobacter* [69] and *Acinetobacter* [97]. Our results based on FRGs strongly suggest that such local microscale facilitation mechanisms could indeed enhance the survival of sensitive and/or new comers toward metals, contributing to an overall increase in diversity.

In relation to this aspect, recruitment of new species coming from different inlet sources along the water stream (e.g. WWTP outlets, tributary creeks, agricultural sources. . .) may also contribute to richness increase. Indeed, a WWTP is actually located upstream from MetalEurop in Douai (France), releasing specific types of

microbes, which may explain the specific enrichment in Actinobacteria, Betaproteobacteria and Firmicutes [20,98–100]. The presence of fecal-related bacteria in the metal-contaminated sediments (FRG4) likely originates from the WWTP located between the two sites, and/or potentially organic fertilizers (e.g. manure) coming from nearby agricultural fields. Furthermore, as several WWTPs are also located upstream Férin (Goeluzin and Estrée, France), it becomes more likely that the selection of these microbes could be directly linked to metal pollution itself, or indirectly via the local safe micro-niches created by resistant strains. These observations relate to the concept of bacterial coalescence, where microbiomes of different ecosystems meet and intermix, which may result in higher richness [101]. Furthermore, as described above, the known co-selection for antibiotic and metal resistance could also contribute in increasing the richness, as wastewater and organic fertilizers bacteria harboring antimicrobial resistance genes may have benefited from a selective advantage in metal polluted sediments. This idea is reinforced by the fact that WWTPs bacteria are known to potentially act as conjugative plasmid shuttles from the sewage to the environment via freshwater microbial copiotrophs [20]. As a consequence, metal contaminated sediments may be seen as an intermediate environmental hotspot contributing to the maintenance and spreading of antibiotic/metal resistance genes located on mobile genetic elements (MGEs) such as conjugative plasmids. This may be achieved either via hosting the surviving and resistant microbes from effluent sources, or through freshwater bacteria that acquired these genes via HGTs [20].

Finally, it is important to emphasize that the higher OTU richness observed at the polluted site (Fig. 1) did not result in higher gene diversity in metagenomes (Fig. S2), which were mostly driven by a high functional redundancy between sites. Nevertheless, the metaproteogenomics comparison of Gillan et al. [7] highlights the specific higher proportion of metal-resistance genes at the polluted site, as well as the presence of mobile genetic elements [7]. While no direct causal evidences could be established due to differences between techniques, the enrichment of these metagenomic functions could likely be associated with the specific microbial response groups selected at the contaminated site (e.g. FRGs 2, 3 and 4). Nevertheless, these separate observations would support the presence of a metal-driven selection in favor of phylogenetically close organisms sharing similar enriched genomic features and/or mobile genetic elements. Our results tend to support this assertion, as an overall significant phylogenetic relatedness signal was seen at the metal contaminated sediments compared to the control (Table 2). Both observations indicate a positive selection for phylogenetically close organisms at the polluted site, implying that the specific metagenomic enrichment in metal resistance genes and mobile elements could be linked to this phylogenetic signal as a characteristic signature of selected microbes. These assertions support the idea that resistance mechanisms could be located on narrow host-range mobile genetic elements, like conjugative plasmids from specific incompatibility groups.

Conflict of interests

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jhazmat.2017.09.046>.

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