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Lucie Laffet, Muriel Joly, Louis Carles, Florence Donnadiou-Bernard, Anna Romani, et al.. Comparison of microbial colonization between natural and plastic substrata in a polluted watershed. *FEMS Microbiology Ecology*, 2023, 99 (8), pp.fiad062. 10.1093/femsec/fiad062 . hal-04165758

HAL Id: hal-04165758

<https://hal.science/hal-04165758v1>

Submitted on 8 Dec 2023

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Comparison of microbial colonization between natural and plastic substrata in a polluted watershed

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1. Abstract

Plastic pollution represents a threat for biological communities and the ecological functions they provide in river ecosystems. In this study, we compared the microbial colonization of two plastics (biodegradable and non-biodegradable) and three natural substrata (leaves, sediment and rocks) in two study sites of an urbanized watershed differing in their plastic-contamination degree (upstream and downstream). The density and diversity of bacterial, fungal and algal communities, as well as the extracellular enzymatic activities β -glucosidase (GLU), N-acetyl-glucosaminidase (NAG) and phosphatase (PHO), were analyzed in each substrata and site over a four-week colonization experiment. Results showed higher microbial densities and enzymatic activities in leaves and sediment compared to plastics and rocks, probably due to the greater availability of organic carbon and nutrients in the former substrata. However, the microbial colonization of the two plastics was only different in the downstream site where bacterial density and enzymatic activities were higher in the biodegradable compared to the non-biodegradable plastic. Accordingly, the presence of biodegradable plastics would enhance the heterotrophic metabolism in plastic-polluted rivers.

Keywords: macroplastics, biodegradable plastic, polyethylene, enzymatic activities, microbial heterotrophs, river

2. Introduction

Since the discovery and mass production of plastic polymers in the first half of the 20th century, the plastic production in the world has never ceased to increase (Thompson *et al.* 2009). Between the 1950s and 2015, ca. 6300 million tons of plastic waste had been generated, about 9% was recycled, 12% incinerated and 79% accumulated in landfills or the natural environment (Geyer, Jambeck and Law 2017). Today plastic pollution is present in all the environmental compartments (water, air and soil), but river ecosystems are considered as sites of particular concern due to their role as sinks of plastic pollution as well as vectors of this pollution to the ocean (Windsor *et al.* 2019).

Strong correlations have been observed between plastic quantities and human population density, urbanization, and waste management in river basins (Best 2019). Plastic waste enters river systems through either natural transport processes (runoff or wind action) or through direct dumping. Plastics polymers of different nature (polyethylene, polystyrene, polypropylene, polyamide, among others) and sizes (nanoplastics (< 0.1 μm), microplastics (0.1 μm -5 mm), mesoplastics (5 mm – 5 cm), macroplastics (> 5 cm)) are present in rivers worldwide (van Emmerik and Schwarz 2019). The nature of the plastic found depends mainly on the anthropogenic activities in the watershed while the size depends mainly on the degradation state of the polymer. Currently, microplastics pollution has been a more widely studied topic compared with macro- and mesoplastics pollution in aquatic environments (Blettler *et al.* 2018). This bias can be partly explained by the fact that macroplastic debris are efficiently removed in waste water treatment plants after the initial sieving step, thus consistently reducing the amount of macroplastic mass of wastewaters (Rasmussen *et al.* 2021). However, the increased runoff during heavy storm events in the frame of global climate change can cause important macroplastic pollution in rivers. Macroplastics transits through wastewater outflows and combined sewage overflows which discharge untreated wastewater directly into the rivers (Horton and Dixon 2017). A study including different watersheds around the world showed a strong seasonality in the amount of macroplastic items in transport in flowing waters and also that the mean of macroplastic items per hour was higher in Southeast Asian (7.1×10^3 items h^{-1}) than in European (2.5×10^2 items h^{-1}) rivers (van Calcar and van Emmerik 2019).

The dominant type of plastic pollution in rivers is soft polyolefins (including polyethylene and polypropylene) with a mean of 47% and 37% from total plastic litter in rivers from Europe and Southeast Asia, respectively (van Calcar and van Emmerik 2019). A

study of macroplastics transport in the Seine river basin (NE of France) revealed that the percentage of soft polyolefin items was dominant (60-80%) in the mid and downstream sites, whereas polystyrene and soft polyolefin items were equally dominant in upstream sites (van Emmerik *et al.* 2019). To our knowledge, macroplastics contamination has not been characterized in small rivers and streams despite the strong interaction between the terrestrial and aquatic environments in these small watersheds. The extensive contamination of natural environments by plastics has prompted countries and states to address laws in banning plastic grocery bags (oil-based plastics) responsible for so-called “white pollution” around the world. Instead, the demand for the production of bio-based plastics, based on renewable resources and susceptible to microbial decomposition, is increasing progressively (Narancic *et al.* 2020). Different types of biodegradable plastics are commercially available, such as polyhydroxyalcanoates, polylactides, polycaprolactone, aliphatic polyesters, polysaccharides and copolymer or blend of these (Shah *et al.* 2008). The present experiment aims to compare the microbial colonization of a biodegradable bioplastic (potato-starch blend) and a non-biodegradable plastic (low-density polyethylene) in the river ecosystem.

Once macroplastics reach river waters, a biofilm starts to develop. Biofilms are defined as matrix-enclosed microbial populations adherent to each other and/or to surfaces and interfaces (Costerton 1995). The so-called *plastisphere* is the microbial community colonizing plastic debris and its ecology has been well described in the marine environment (Amaral-Zettler *et al.* 2020). The *plastisphere*, as common aquatic biofilms, is characterized by an assemblage of phototrophs (diatoms, cyanobacteria), heterotrophs (bacteria), saprotrophs (fungi) and predators (protozoa) communities. Until now, the major studies of macroplastic pollution and *plastisphere* development have been performed in marine ecosystems. Although specific factors may differ from both environments, *plastisphere* development is likely to be similar. Studies from marine ecosystems concluded that microbial community composition on plastics is both driven by environmental features and biofilm formation processes, and that a small proportion of taxa are polymer-specific (Harrison *et al.* 2018; Barros and Seena 2021). When comparing microbial colonization and degradation in non-biodegradable and biodegradable plastic polymers, it was observed that benthic microbes settled in higher densities on the biodegradable plastic (corn-starch blend) than on the non-biodegradable plastic (polyethylene) placed in temperate fine-grained organic-rich marine sediments from the Western Baltic Sea (Nauendorf *et al.* 2016). By also comparing non-biodegradable and biodegradable plastic microbial colonization but including the pelagic and benthic habitat at the Mediterranean Sea, Eich *et al.* (2015) found that the habitat had a strong

influence on diatom density and diversity whereas the type of polymer influenced temporarily diatom community succession during colonization. A laboratory experiment showed a higher bacterial colonization on biodegradable polymers (*i. e.* artificially aged polyethylene with a pro-oxidant additive) compared to the non-biodegradable one (*i. e.* polyethylene, Dussud *et al.* (2018)). It is important to remark that ageing of plastics, often simulated by the increased exposure of plastics to UV radiation in the laboratory, resulted in higher weight loss and hydrophilicity after being inoculated with microorganisms (Taghavi, Zhuang and Baroutian 2021). Although much less research has been performed in river ecosystems, recent investigations indicate that plastic microbial colonization may imply enhanced metal biofilm adsorption as well as acting as reservoirs and transport of antibiotic resistance genes (Liu *et al.* 2021; Martínez-Campos *et al.* 2023). The age and weathering of plastics, the successional stage of biofilms, and the local environmental conditions (*i. e.* salinity, light, pressure, current velocity, etc...) are the factors that most likely differ on biofilm formation and taxonomic composition on plastic between freshwater and marine habitats (Harrison *et al.* 2018).

However, to our knowledge, functional changes during the microbial colonization of plastics in aquatic ecosystems have been poorly examined and, as indicated by Amaral-Zettler, Zettler and Mincer (2020) functional diversity and metabolic capacity of microorganisms found in the plastisphere are not well understood (Amaral-Zettler, Zettler and Mincer 2020). In marine environments, recent research indicate that plastics debris may affect nitrogen cycling processes; the presence of microbeads of polyurethane foam and polylactic acid promoted nitrification and denitrification, while polyvinyl chloride inhibited these processes in marine sediments (Seeley *et al.* 2020). The nitrogen cycling processes in marine sediments can therefore be significantly affected by different microplastics, which may serve as organic carbon substrates for microbial communities. Tu *et al.* (2021) observed that marine biofilms harbor plastic degrading bacteria, leading to the changes of functional groups and surface hydrophobicity, and thereby enhancing the biodegradation of microplastics. In rivers and streams the benthic biofilm developing on inorganic and organic substrata play a key biogeochemical role for the whole ecosystem functioning (Battin *et al.* 2016). Thus it is expected that if input of plastic determines a distinct biofilm community developing on them, also showing distinct functions, this may affect river ecosystem functioning.

In the present study, we examine how different is the structural and functional diversity between biofilms growing on plastics (non-biodegradable and biodegradable) and natural substrata (leaves, sediment and rocks) and whether non-biodegradable plastics might host communities more similar to natural substrata such as leaves. With this aim, two

different study sites (upstream and downstream) of the same urbanized watershed were selected to run comparative colonization experiments of the plastics and natural substrata in contrasting environmental settings. We expected higher microbial biomass, diversity and activities on substrata rich in carbon and nutrients (*i. e.* leaves and sediments) than on artificial substrata (*i. e.* rocks and plastics) where resources are mainly available from the water column. When comparing the two plastics, we expected a higher microbial biomass, diversity and activity in the biodegradable plastic than in the non-biodegradable plastic because microorganisms could metabolize biodegradable molecules contained in the biodegradable plastic and increase their abundance. The colonization of substrata will probably be higher in the downstream site than in the upstream site, since downstream sections tend to accumulate more dissolved nutrients and plastic pollution than upstream sections. Finally, the adaptation of microbial communities to plastic pollution entering the stream and the greater resources availability in the downstream site could also favor the early degradation of the biodegradable plastic.

3. Materials and methods

3.1. Experimental design

This study compared the microbial colonisation in natural substrata versus plastic substrata in two sites of a French river with contrasting plastic pollution. Natural substrata included sediments, frosted glass slides (considered as rocky substrata surrogates, Artigas *et al.* (2012)) and leaves (*Alnus glutinosa*).

Plastic substrata included a slow-degradable polymer namely Low-Density Polyethylene (LDPE, non-biodegradable plastic), which is commonly used as plastic bag and packaging, and a fast-degradable polymer namely Starch-Based Biodegradable Plastic (BDBP, biodegradable plastic). The starch-based polymer is manufactured by the firm Végéos (Le Bignon, France) and composed of 30% of bio-sourced material (potato starch) according to the ISO 16620-2: 2015 and commonly used as compostable bag. 70% of the remaining composition of BDBP bags is copolyester whose exact composition is not specified by the manufacturer.

The microbial colonization study was performed at two sites of the Artière river (Auvergne Rhône-Alpes region, France): upstream (45°43'11.0"N 3°01'20.7"E) in Saint-Genès-Champanelle (low plastic contamination site) and downstream (45°47'44.7"N 3°10'26.8"E) in Aulnat (high plastic contamination site). The upstream site (25 m reach) is located within a forested area mainly composed by *Alnus glutinosa*, *Corylus avellana*, and *Fraxinus excelsior* tree species. The riverbed is 1.53 ± 0.20 m width and the streambed is composed by around 80 ± 5.77 % of rocks, 20 ± 5.77 % of sediments and 30 ± 17.32 % of mosses (measurements from three transects in the 25 m reach). The downstream site (25 m reach) is located downstream of the city of Clermont-Ferrand and is essentially surrounded by herbaceous plants such as nettles and the riverbank protected by riprap (Figure S1). The riverbed is 5.75 ± 0.25 m width and composed by around 26.66 ± 6.66 % of rocks and 73.33 ± 6.66 % of sediments and 70 ± 10 % of macrophytes (measurements from three transects in the 25 m reach). Note that the downstream site is located approximately 1.5 km after a waste water treatment plant (WWTP) collecting waters from the Clermont-Ferrand city and 19 other municipalites and a capacity of 425,000 equivalent habitants. The WWTP is equipped with a secondary treatment including two biological basins of 80000 m³ each with activated sludge. The WWTP effluent volume is ca. 2100 m³ / day which represents 2.76 % of the discharge of the Artière river.

The colonization experiment was performed in late autumn early winter (November-December 2017). Prior to the colonization, sediments were collected from the two study sites, sieved through 2 mm and autoclaved at 121 °C during 20 minutes. Autoclaved sediment was placed in perforated sterile conical tubes of 50 mL and buried ca. 3-4 cm depth in river sediment for colonization. Frosted glass slides (2.85 x 8.25 cm) were glued onto concrete slabs and placed in the riverbed for colonization. *Alnus glutinosa* leaves (5 leaves air-dried at room temperature), LDPE and BDBP (5 pieces of 4 x 7 cm each) were placed in nylon bags (15 x 20 cm, 1 cm mesh size, 1 type of substratum per bag) and attached to a metal rod immersed in the water column for colonization. Samplings were performed weekly for 4 weeks, and five replicates of each substratum were collected from each site and transported into an icebox to the laboratory. The sediment was sampled to 1-2 cm depth directly from the conical tubes using a stainless steel spatula. The frosted glass slides were scraped with a sterile scalpel and re-suspended in 7 mL of physiological Ringer solution (6.5 g L⁻¹ NaCl, 0.12 g L⁻¹ CaCl₂, 0.14 g L⁻¹ KCl) while leaves, LDPE and BDBP were directly cut with a sterile scalpel and dispatched into the tubes corresponding to the different microbial analyses. Note that leaves were completely degraded in the downstream site at week 4. Initial

measurements (at time 0) were performed on each substratum before placed in the stream (Table S3).

3.2. Water physicochemical characteristics

Physicochemical parameters were measured weekly at both sites. Water conductivity, temperature, pH and dissolved oxygen concentration were determined with portable probes (Orion 4-star, Thermo Fisher Scientific, and ProODO, YSI, respectively). Pre-filtered (0.45 μm pore size) water samples from each site and sampling time were collected in sterile tubes and preserved frozen (-20 °C) until analyses of total dissolved carbon (TC), total nitrogen (TN) and total organic carbon (TOC) concentration by using a Total Organic Carbon Analyzer (TOC-V_{CPH}, Shimadzu, Japan). Total phosphorus concentrations in water were measured according to Murphy and Riley (1962).

3.3. Plastics pollution characterization

Macroplastics (> 5 cm) and mesoplastics (0.5 cm \leq 5 cm) *sensu* Emmerik and Schwarz (2020) were sampled, characterized and quantified in the riverside and in the river channel from upstream and downstream sites of the Artière river. The sampling at the riverside was performed in a section of 100 m in length by 20 m in width. Three transects perpendicular to the river were established (0 m, 50 m and 100 m of the section) and each transect was divided in three zones: 1) the shoreline (half in the water and half inland), 2) the intermediate (10 m from the river) and 3) the distal (20 m from the river). The macroplastics and mesoplastics sampling were performed in 40 x 40 cm squares in each zone of the three transects.

Macroplastics and mesoplastics in each square were sampled manually using sterile forceps and placed in bags. Plastic transported by the water column was determined using a manta net (0.5 mm) placed in the middle of the riverbed during 30 minutes. The quantity of plastics transported by river water was corrected by the water flow. Macroplastics quantification and characterization was based on the PLASTICOPYR project protocol (<https://plastic0pyr.wordpress.com/actions>). Once in the laboratory, the macroplastics were identified using TSG_ML (Technical Subgroup on Marine Litter) General Name according to the Guidance on Monitoring for Marine Litter in European Seas (European Commission 2013), weighed and results were expressed as plastic litter abundance (items m⁻²) or density (g

m⁻²) for each of the three zones analysed in the river bank. We focused on macroplastics contamination in order to compare with LDPE and BDBP colonization experiment.

3.4. Microbial density

Bacterial density was measured by flow cytometric counts on five replicates per site and sampling time. Fragments of 5 cm² for leaves, BDBP and LDPE or suspension equivalent to 2 cm² for sediments and 7 cm² for glass were mixed with sodium pyrophosphate (10 mM in Tris-EDTA buffer pH 8.0, Sigma-Aldrich®). Samples were gently agitated for 30 minutes, sonicated for 1 + 1 minute in a sonication bath (f: 50/60 Hz, Fisher Scientific FB15048, Germany) to detach cells from substrata. Then, samples were vortexed for 1 minute and centrifuged for 1 minute at 800 g. Finally, 500 µL of supernatant were fixed with formaldehyde (final concentration: 4 %). Bacterial suspensions were diluted 100-fold with Tris-EDTA buffer and stained with SYBR Green I (Molecular Probes) before counting cells with a BD FACS Calibur flow cytometer (15 mW at 488 nm, Becton Dickinson, USA). The results were expressed as cells per cm² for each substratum.

Fungal density was estimated by the number of 18S rRNA gene copies by quantitative PCR (qPCR) on three replicates per site and sampling time. Total DNA was extracted with the FastDNATM SPIN kit (MP BiomedicalsTM, Irvine, CA) and amplified using the FR1/FF390 primer set (5'-AICCATTC AATCGGTAIT-3' and 5'-CGATAACGAACGAGACCT-3', respectively) Prévost-Bouré *et al.* (2011) and the MESA green qPCR Master mix plus for SYBR® assay (Eurogentec). qPCR runs were performed with a BioRad CFX96TM Real-Time System (Bio-Rad laboratories, Inc., Hercules) as follow: a 5 min denaturation step at 95 °C, followed by 40 cycles consisting of denaturation (45 s at 95 °C), annealing (45 s at 48 °C), extension (70 s at 72 °C) and finally a specific denaturation curve from 70 °C to 95 °C. Standard curves were prepared by doing a ten-fold serial dilutions (from 10⁷ to 1 copies µL⁻¹) of linearized plasmid containing a 18S rRNA gene insert from fungi. Quantifications were performed as analytical triplicates. Results were expressed as the number of 18S rRNA gene copies per cm² of substratum.

Algal biomass was estimated through chlorophyll-*a* concentration determination on three replicates per site and sampling time, excepting leaves. The chlorophyll-*a* concentration was determined after lyophilisation and extraction in acetone 90 % in the dark at 4°C under

inverse stirring agitation overnight. To improve chlorophyll-*a* extraction, the extracts were sonicated 4 minutes (Fisher Scientific FB15048, f: 50/60 Hz) and centrifuged 5 minutes at 10.000 g. The chlorophyll-*a* concentration was measured on supernatants by spectrophotometry (Thermo Fisher scientific, Genesys 30, USA) according to the method of (Jeffrey and Humphrey 1975). Results were expressed as μg chlorophyll-*a* per cm^2 .

3.5. Microbial diversity

The diversity of prokaryotic and eukaryotic communities was analysed at week 3. We selected samples of week 3 given that stable and mature microbial community was expected at that time in agreement with other studies in the literature Montuelle *et al.* (2010) and in the same watershed (Joly, Mallet and Artigas 2019).

Aliquots from DNA extracted for fungal density measurements (three replicates per substrata, site and time) were used for microbial diversity analyses. The V4 region of 16S SSU rRNA gene (for prokaryotes) and V9 region of 18S SSU rRNA gene (for eukaryotes) were sequenced using the primer sets 515F/806R (5'-GTGYCAGCMGCCGCGGTAA/5'-GGACTACNVGGGTWTCTAAT, Caporaso *et al.* 2011) and Euk_1391f-7-for/EukBr-7-rev (5'-GTACACACCGCCCGTC/5'-TGATCCTTCTGCAGGTTACCTAC, Gilbert *et al.* 2014). Libraries were constructed with Fluidigm Access Array (Fluidigm, San Francisco, California, USA) and sequenced on a MiSeq flowcell (Illumina, 2 × 250 bp, 10 M–20 M paired reads) for 251 cycles from each end of the fragments using a MiSeq 500-cycle sequencing kit version 2. Fastq files were generated and demultiplexed with the bcl2fastq v2.20 Conversion Software (Illumina). Sequencing yielded a total of 2,963,827 reads (prokaryotes) and 903,893 reads (eukaryotes) which were further processed using the DADA2 pipeline (Callahan *et al.* 2016). After chloroplast and mitochondria sequences removal, rarefaction curves were performed and prokaryotes and eukaryotes data were resampled down to the lowest number of reads covering the maximal diversity (38000 and 11400 reads for prokaryotes and eukaryotes, respectively). Eukaryotic phyla, in downstream, the sediments and rocks substrata had only two replicates because the third replicates were removed during standardization because sediments and rocks had 234 sequences and 3399 sequences respectively. Taxonomic classification was carried out via a native implementation of the Ribosomal Database Project naive Bayesian classifier, and species-level assignment to 16 S and 18 S rRNA gene fragments by exact matching. The sequencing data have been deposited in NCBI's Sequence Read Archive with the following number PRJNA950420.

3.6. Extracellular Enzyme Activities

The activity of three extracellular hydrolytic enzymes β -D-glucosidase (GLU, EC 3.2.1.21), N-acetyl-glucosaminidase (NAG, EC 3.2.1.14), and phosphatase (PHO, EC 3.1.3.1-2) involved respectively in carbon, nitrogen and phosphorus acquisition were measured. Activities were analysed from five replicates per substratum, site and time. Samples (equivalents to 1 cm² of surface of each substrata) were placed in 800 μ L of Ringer solution (6.5 g L⁻¹ NaCl, 0.12 g L⁻¹ CaCl₂, 0.14 g L⁻¹ KCl) and activities were measured using fluorescent substrates analogues (4-Methylumbelliferyl- β -D-glucopyranoside, 4-Methylumbelliferyl-N-acetyl- β -D-glucosaminide and 4-Methylumbelliferyl-phosphate (Sigma-Aldrich)) at substrate-saturating concentration (0.3 mM) for 1 h at 20°C under agitation (100 rpm) in the dark according to (Romaní *et al.* 2004). The enzymatic reaction was stopped by adding a glycine buffer solution (0.05 M, pH 10.4). The measure of Methylumbelliferyl (MUF) substrate production was determined fluorometrically using a spectrofluorimeter (Fluoroskan Ascent FL, ThermoFisher Scientific, USA) set up at a $\lambda_{\text{excitation}} = 355$ nm, $\lambda_{\text{emission}} = 460$ nm. MUF concentrations were determined from MUF-standard curve ranging 0 to 12.5 μ M and after correction by Ringer's media controls. Enzymatic activities results were expressed as nmol MUF h⁻¹ cm⁻².

3.7. Data analyses

Data from the three extracellular enzyme activities and the three microbial densities did not fit conditions for statistical parametric testing, therefore a non-parametric test equivalent to three-way ANOVA (Scheirer-Ray-Hare test, $P < 0.05$) was applied using R version 4.0.5 (R Core Team (2021)) and rcompanion package version 2.4.1 (Mangiafico 2016). The three factors tested were time, substrata and site, as well as their corresponding interactions. Pairwise comparisons among substrata and sampling times were tested using the Wilcoxon test, $P < 0.05$. Data from water physicochemical descriptors followed a normal distribution and therefore two-way ANOVA ($P < 0.05$) was tested. The factors time and site were tested for physicochemical data. Statistical analyses of the three extracellular enzyme activities and the three microbial densities were performed excluding the time 0 (before placed in the stream).

Sequencing results on prokaryotes and eukaryotes at the amplicon sequence variant (ASVs) level was analysed for alpha-diversity (Chao 1 and Shannon indices) and beta-diversity using non-metric multidimensional scaling (NMDS) analysis based on a Bray-Curtis dissimilarity matrix using the package Phyloseq in R. A stress function (ranging from 0 to 1) was used to assess the goodness of fit between computed and original data. The relative abundances of phyla for prokaryotes and eukaryotes (*i.e.* mean abundance < 1 % across all samples) for the two sites and five substrata were analysed with the R package Phyloseq v1.42 according to McMurdie and Holmes (2013). Differences in microbial community composition between substrata and sites at week 3 were estimated through permutational multivariate analysis of variance (PERMANOVA) based on 9999 permutations to avoid potential autocorrelation among samples using PRIMER6 version 6.1.11. Differences in prokaryotes and eukaryotes phyla relative abundances between sites and among substrata were assessed through Scheirer-Ray-Hare test.

Relationships between structural (alpha diversity indices) and functional (extracellular enzymatic activities; GLU, NAG, PHO) descriptors of microbial communities colonizing the different substrata and sites were analysed through linear correlation analysis using the Pearson coefficient. The correlation analyses were performed for prokaryotes and eukaryotes, separately, and considering i) all the substrata and sites together and ii) separating substrata per study site.

4. Results

4.1. Water physicochemical characteristics

Water conductivity, temperature and concentrations of total dissolved carbon, dissolved organic carbon, and soluble reactive phosphorus were higher at the downstream site than in the upstream site, whereas dissolved oxygen was higher in the upstream site (Table 1). pH and concentrations of total dissolved nitrogen and total dissolved phosphorus were not statistically different between study sites, although average nutrient concentrations values were consistently higher downstream compared to upstream.

The factors time and site had an interactive effect on total dissolved carbon in water (Table S1). Indeed, stronger variations in total dissolved carbon concentrations were measured at the

downstream site (ranging from 48.72 to 65.32 mg L⁻¹) than at the upstream site (ranging from 20.2 to 23.82 mg L⁻¹) during the length of the experiment.

4.2. Characterization of plastics pollution

Macroplastics debris were not found in the upstream section, neither transported by the water column nor in the riparian area. Accordingly, we consider that the upstream section was exempt from macroplastics pollution. In the downstream section, macroplastics debris were not found in the water column but they strongly accumulated in the riparian area (Table 2). The most polluted zone is the shoreline (at the riverside) comparing to the intermediate area (10 m from the riverside) and the distal area (20 m from the riverside). Gradually, the abundance and density of plastic contamination progressively decreased when moving away from the shoreline.

Plastic debris were identified and classified in Table 2. For some plastic fragments, we were not able to identify its origin and nature due to strong deterioration of materials (*i.e.* code G121 “Other plastic or polystyrene items not identifiable”). Five types of plastics were found in the shoreline, five types in the intermediate area and one type in the distal area (Table 2). There was presence of polypropylene (*i.e.* candies packaging found at the shoreline and intermediate areas), polyethylene (*i.e.* construction tape, found at the intermediate area), and low- and high-density polyethylene (*i.e.* bags and hunting cartridge shell found at the shoreline and intermediate areas). Note that the extremely heavy item (G120) found in the shoreline corresponded to the plastic protection of a bicycle lock safety. No plastic items of biodegradable nature (*i. e.* polyhydroxyalcanoates, polylactides, aliphatic polyesters ...) were found in the downstream section, including those tested in this experiment (potato-starch copolymer).

4.3. Microbial densities

Bacterial density: bacteria increased significantly over time in the different substrata immersed in the two studied sites, with a strong increase during the first week of colonization followed by weaker variations until the end of experiment (Figure 1A, Table S2). An interactive effect between the factors substrata and site was observed (Figure 1A, Table S2).

In the upstream site, bacterial densities were significantly higher in the sediment, followed by leaves, the two plastics (BDBP and LDPE), and finally rocks (Wilcoxon test, $P < 0.05$). Conversely, bacterial densities in the downstream site were significantly higher in leaves, followed by sediment and BDBP, and finally LDPE and rocks (Wilcoxon test, $P < 0.05$). No statistical differences were observed for bacterial accumulation between study sites (Table S2).

Fungal density: differences in density values were observed among sampling times ($P < 0.0005$, Table S2) especially for leaves and sediment compared to the other substrata (Figure 1B). An interactive effect was observed between the factors site and substrata ($P < 0.05$, Table S2). In the upstream site, fungal density was higher in leaves and sediment compared to BDBP and rocks while no fungi were detected in LDPE (Figure 1B). In the downstream site, the highest fungal density was measured in leaves, followed by sediment and LDPE, and finally rocks and BDBP. The factors substrata and site had each significant effects on fungal density values ($P < 0.0005$ and $P < 0.05$, respectively, Table S2). Overall, leaves and sediments were more colonized by fungi than plastics and rocks (except for a higher variation of the fungal density in week 4 downstream). And, fungal density was higher in the plastics colonized downstream than in those colonized upstream (Figure 1B, Table S2).

Algal biomass: the factors substrata and time had a significant effect on algal density ($P < 0.0005$ and $P < 0.05$, respectively). The algal accumulation was higher in sediments than in plastics or rocks from the two study sites. However, no interaction between factors substrata and site was observed (Table S2). The algal colonization was lower and slower comparing to that of bacteria and fungi in the substrata tested, with consistent increases after week 2 for sediments and after week 3 for rocks (Figure 1C). No differences in algal biomass were observed between BDBP, LDPE and rocks from the two studied sites (Figure 1C).

4.4. Extracellular enzymatic activities

GLU activity: the activity values increased progressively over the colonization time ($P < 0.05$, Table S2) with a more marked increase during weeks 1 and 2 (Figure 2A). Both the substrata and site factors had significant effects on GLU activity ($P < 0.0005$ for both factors, Table S2). The highest activity rates were measured in leaves, followed by sediments and

finally plastics and rocks (Figure 2A). There was an interactive effect between the factor substrata and site ($P < 0.005$, Table S2), especially for plastics. The activity measured in the BDBP and sediments were similar and significantly higher comparing to that measured in LDPE and rocks from the downstream site. However, these differences were not observed in the upstream site where the activity rates in BDBP and LDPE were similar and even lower comparing to that measured in rocks (Figure 2A). When considering all substrata together, the GLU activity values were higher in the downstream site comparing to the upstream site.

NAG activity: The three tested factors had a significant effect on NAG activity values but no interactions were observed among factors (Table S2). The activity values increased later than those of GLU activity, between weeks 2 and 3, though this was rather variable depending on substrata and sites (Figure 2B). Activity values were higher in substrata from the downstream site than in those from the upstream site. Irrespectively of the study site, NAG activity in leaves and sediment was significantly higher comparing to that measured in plastics and rocks (Figure 2B). When comparing plastics, NAG activity in BDBP was significantly higher comparing to that measured in LDPE and rocks, and this was strongly evident in the downstream site (Figure 2B).

PHO activity: Although PHO activity values increase during the colonization study, the factor time was not statistically significant ($P = 0.238$, Table S2). However, effects were observed from the factors site and substrata ($P < 0.0005$ for both factors, Table S2). Overall, the phosphorus acquisition in the downstream site was higher than in the upstream site (Figure 2C). Moreover, PHO in leaves and sediments was higher than in plastics and rocks. PHO in plastics and sediments were relatively similar, despite an increasing trend of activity in BDBP comparing to LDPE and rocks during the early stages of colonization (weeks 1 to 2, Figure 2C).

4.5. Microbial diversity

The alpha-diversity of prokaryotic communities was influenced by the factor substrata ($P < 0.05$ for Chao1 and $P < 0.0005$ for Shannon) but not by the factor site (Table S2). In contrast, the beta-diversity of prokaryotic communities was much more different between sites (PERMANOVA, $P < 0.005$) than between substrata (Figure 3A). Differences between sites

were explained by higher relative abundance of Firmicutes downstream and Verrucomicrobia upstream (Table S5, Figure 4). Both the structure and composition analyses of communities coincided in grouping separately sediments from rocks (PERMANOVA, $P < 0.05$) since the former had higher species richness and diversity than the latter. The structure and composition of prokaryotic communities in plastics and leaves was relatively similar when comparing substrata for each site separately (PERMANOVA, $P > 0.1$), although strong variance was observed between replicates of BDBP comparing to LDPE and rocks in the downstream site (Figure 3A). Differences among substrata are explained by a higher relative abundance of Firmicutes and Cyanobacteria in sediments and rocks, respectively, compared to leaves and plastics (Table S5, Figure 4).

In the case of eukaryotes, the structure of communities was not affected by the factor substrata but only by the factor site ($P < 0.05$ for the Shannon index, Table S2). Again, consistent differences were observed in the composition of eukaryotic communities between sites (PERMANOVA, $P < 0.005$) rather than between substrata (Figure 3B). Differences between sites were mostly explained by a higher relative abundance of Ascomycota, Euglenozoa and Chlorophyta upstream, while Ciliophora and Rotifera were more abundant downstream (Table S6, Figure 4). The diversity of eukaryotic communities was higher in the upstream site than in the downstream site excepting for leaves, (Table 3), and the composition of communities in sediments was significantly different from that observed in rocks (PERMANOVA, $P < 0.05$, Figure 3B). Leaves and plastics had similar eukaryotic community composition when comparing substrata for each site separately (PERMANOVA, $P > 0.1$), though again a higher variance between replicate samples was observed for BDBP comparing to LDPE and leaves in the downstream site (Figure 3B). Although differences in relative phylum abundance were observed between substrata (Table S6), these were highly variable between sites (Figure 4).

4.6. Relationships between structure and function in microbial communities

Linear correlations between alpha-diversity indices and extracellular enzymatic activities were mostly observed in prokaryotic communities rather than in eukaryotic communities (Table S4). A positive linear correlation was observed between the Shannon and Chao indexes of prokaryotic communities and NAG and PHO activities when considering all substrata and sites together. More precisely, the structure-function correlations were mostly

explained by communities colonizing substrata downstream than by those colonizing upstream (Table S4). Strong positive correlations were observed in prokaryotic communities between i) the Shannon index and NAG and GLU activities and between ii) the Chao 1 index and GLU, NAG and PHO activities of substrata colonized in the downstream site (Table S4). According to correlation plots (Figure 5), the lowest diversity and activity scores were measured in rocks, followed by plastics, and finally, leaves and sediments colonized in the downstream site. No correlations were observed between diversity indices and enzyme activities in prokaryotic communities from the upstream site.

In eukaryotic communities, we only observed a negative correlation between the Shannon index and PHO activity when considering all the substrata and sites together (Table S4). No structure-activity correlations were observed for eukaryotic communities when analyzing study sites separately.

5. Discussion

The objective of this study was to examine the ability of microbial communities to colonise different benthic substrata (including plastics) in two river sites with different level of plastic contamination. In the Artière river (Centre France), no plastics were found in the upstream site while strong plastic contamination was detected in the riverbed of the downstream site. According to the distribution and characteristics of the macroplastics debris found in the downstream site, we suspect their origin from combined sewage overflows release during heavy storm episodes (Horton and Dixon 2017; Kawecki and Nowack 2019). Indeed, the downstream site was located 1.7 km downstream from a WWTP (425000 equivalent inhabitants) collecting waters from the Clermont-Ferrand city. The nature of macroplastic debris found in the downstream section (polystyrene, polypropylene and low- and high-density polyethylene) were quite similar compared to those found in other aquatic systems (Klein, Worch and Knepper 2015; Blettler *et al.* 2019; Kawecki and Nowack 2019). Biodegradable plastic (BDBP) debris were not found in the Artière, and this was probably explained by their lower and more recent industrial production compared to other non-biodegradable plastics (such as polyethylene, polystyrene and/or polypropylene (European Bioplastics 2017)), as well as by their expected lower lifetime in the environment (compostable material, (Song *et al.* 2009)). According to studies quantifying plastic litter

pollution in rivers (*i. e.* 0.5-1.2 items m⁻², Kiessling *et al.* (2019); 5 items m⁻², Blettler *et al.* (2019); 0.6-113 g m⁻², Liro, Mikuš and Wyzga (2022), the downstream site of the Artière was more contaminated by macroplastics (ranging 2 to 29 items m⁻² and 0.1-555 g m⁻²) than other rivers worldwide. However, this result must be taken with caution since protocols used to quantify and identify plastic pollution in rivers are not yet well standardized (*e. g.* Hartmann *et al.* 2019).

The four-week colonization experiment in the Artière river permitted to obtain mature microbial communities in the five substrata analysed, including leaves, sediment, rocks, BDBP and LDPE. The amount of bacteria and algae accumulated in sediments, rocks and leaves in our experiment were in the same range than those measured in other studies in temperate stream ecosystems (Sabater and Romaní 1996; Romaní *et al.* 2004; Artigas *et al.* 2009). Plastics in our experiment reached bacterial densities per surface area (cm²) similar to those measured on polypropylene sheets after four months of experiment in a pre-alpine lake (Hempel, Grossart and Gross 2009). After four weeks of colonization, enzymatic activities (*i. e.* GLU and PHO) measured in sediments and rocks from our experiment were in the same range than those measured in other French rivers (Montuelle *et al.* 2010) but consistently lower comparing to those measured in Mediterranean streams (three to seven times lower comparing to Romaní *et al.* (2004). Such differences between experiments are expected given that enzymatic activity measurements in microbial communities strongly depend on the specific organic matter and nutrient context of each environment (Arnosti 2003). Joly, Mallet and Artigas (2019) measured GLU, NAG and PHO activities and bacterial densities in natural (alder leaves) and standardized organic substrata (wood and cotton strips) colonized for eight weeks in the upstream site of the Artière river (same study site of the present study). This experiment showed that three- to four-week colonization was sufficient to reach a mature microbial community on natural and artificial organic substrata.

The microbial colonization of the two plastic polymers (in terms of microbial densities and enzymatic activities) was closer to that observed on rocks than on sediments and leaves in the two river sites. These differences can be explained by the nature of the substratum where the microbial community develops. For instance, leaves are organic substrata that can provide microbial heterotrophs (such as fungi and bacteria) with carbon and nutrients (Tank and Dodds 2003), whereas sediments are depositional habitats where strong accumulation of organic matter occurs (Romaní *et al.* 2004). When carbon and/or nutrient availability in leaves and sediment is insufficient, microorganisms can also obtain nutrients from the water column (Mulholland *et al.* 1984). Conversely, the functioning of microbial communities on rocks and

plastics rely mostly on nutrients from the water column and/or from the internal recycling of nutrients within the microbial community (*e. g.* Francoeur and Wetzel 2003) since the substrata cannot provide nutrients or does so at very low rates. The above explanation based on nutrient-resources acquisition was not valid for differences observed in microbial communities' composition (beta-diversity) among substrata. After three weeks of colonization, the composition of prokaryotes and eukaryotes communities in plastics (including BDBP and LDPE) was closer to that observed in leaves than on rocks and sediments in the two studied sites. Indeed, higher relative abundance of Firmicutes and Cyanobacteria were observed in sediments and rocks, respectively, compared to leaves and plastics. Another possible explanation for these results may be the different exposure of leaves and plastics to current velocity compared to sediments and rocks. The low weight and strong flexibility of leaves and plastics substrata were probably selecting microbial species more adapted to turbulent flow, whereas rocks and sediments probably selected for microbial species more adapted to intermediate to laminar flow-types (Besemer *et al.* 2007). This is, however, a hypothesis that remains to be tested.

Interestingly, we observed higher GLU and NAG activities and bacterial densities per unit of substratum surface area, in BDBP than in LDPE substrata. More precisely, the activities and bacterial accumulation in BDBP were similar to those measured in sediments, while those measured in LDPE were similar to that measured in rocks. These differences in microbial colonization can be explained by the different nature of plastics. Miao *et al.* (2021) observed variation on carbon utilization capacities between freshwater biofilms on PET and those growing on PVC and glass tiles, indicating the presence of substrate-specific functional diversity. Nauendorf *et al.* (2016) measured five times higher aerobic and eight times higher anaerobic bacterial density in biodegradable plastic bags (> 50% biodegradable polyester and >20 % corn starch) than in polyethylene plastic bags incubated in marine sediments. The BDBP substratum used in our study was composed of 30% potato starch and 70 % biodegradable copolyester, though the supplier does not specify the exact nature of the copolymer (Végéos, France), whereas the LDPE is composed of 100% polyethylene. Up to date, there is no clear or very few evidence of real biodegradation of polyethylene (Ghatge *et al.* 2020), whereas the accessible labile carbon contained in BDBP could explain the stronger GLU and NAG activity measured in this substratum. The higher GLU (hydrolyze 1,4 linked β -D-glucose residues from cellobiose) and NAG (hydrolyze 1,4 linked N-acetyl- β -D-glucosaminide residues in chitooligosaccharides) activities measured in BDBP cannot be related to the use of starch since the cleavage of this polysaccharide is mediated by the action

of α -glucosidases among other enzymes (hydrolyze 1,4 linked α -D-glucose residues from maltose (German, Chacon and Allison 2011). Therefore, the higher rates of GLU and NAG activities could explain the enhanced breakdown of the unknown copolymer used in the BDBP polymer manufacturing.

It is important to highlight that differences between microbial colonization of BDBP and LDPE were only observed in the downstream site of the Artière river. The downstream site had higher water temperature, nutrient concentrations and plastic pollution than the upstream site, certainly due to the natural as well as anthropogenic patterns along upstream-downstream gradients found in urbanized watersheds (Vannote *et al.* 1980; Marti *et al.* 2004). The specific environmental template from the downstream site could explain the strong structure-function relationships observed in prokaryotic communities colonizing the different substrata. Such a difference can result from niche adaptation of prokaryotic communities to colonize in different manner non-biodegradable and biodegradable plastics in a favorable environmental context for microbial development. The more diverse is the prokaryotic community the higher are the enzymatic activity rates permitting to decompose organic matter polymers which agrees with biodiversity-function observations made in the literature (*e. g.* Cardinale *et al.* 2006). In microbial communities, decomposers preferentially decompose labile polymers (*i. e.* cellulose and hemicellulose) when present at any concentration rather than recalcitrant polymers (*i. e.* lignin, chitin), as long as they are within a favorable environment (Ekschmitt *et al.* 2005). However, the decomposition of more recalcitrant polymers can be operated in the presence of labile polymers as the resultant of co-metabolic processes occurring in microbial decomposer communities (Arnosti 2003). The microbial co-metabolism is defined by the transformation of a non-growth substrate in the presence of a substrate necessary for growth (Dalton and Stirling 1982). According to this concept, the higher decomposition activities in BDBP downstream can result from recalcitrant compounds degradation (*i. e.* starch) favored by the high dissolved organic carbon concentrations supplied by the WWTP effluent (boosting GLU activity). Further studies specifically investigating enzyme activities involved in starch decomposition (*i. e.* α -glucosidases, α - β -amylases,) should be conducted on the biodegradable plastic between study sites.

From an ecosystem point of view, upstream plastics are colonized in a similar way than rocks while downstream they are colonized in a similar way than sediment providing higher enzyme degradation capabilities to the river ecosystem and higher heterotrophy. A pattern seems to distinguish the microbial colonization of biodegradable plastic (BDBP), that tends to resemble sediments and leaves, from the microbial colonization of non-biodegradable

plastic (LDPE) that tends to resemble rocks. Accordingly, the presence of biodegradable macroplastics would increase heterotrophy to the river ecosystem more than the presence of non-biodegradable macroplastics. However, the size of these effects at the ecosystem level would depend on the respective availability of the distinct surface substrata in the streambed (*i.e.* natural: rocks, sediment, leaves; and plastics: biodegradable, non-biodegradable). It is important to remark that this conclusion applies to the specific environmental and microbiological characteristics of the Artière's river and may not reproduce in other rivers with different degree of plastic pollution, water physicochemical characteristics, and microbial biodiversity.

6. Acknowledgments

This study was funded by the programm Ecosphère Continentale et Cotière (EC2CO) in the frame of the project DeCoDry. We thank Florent Rossi for his participation in the field samplings.

7. References

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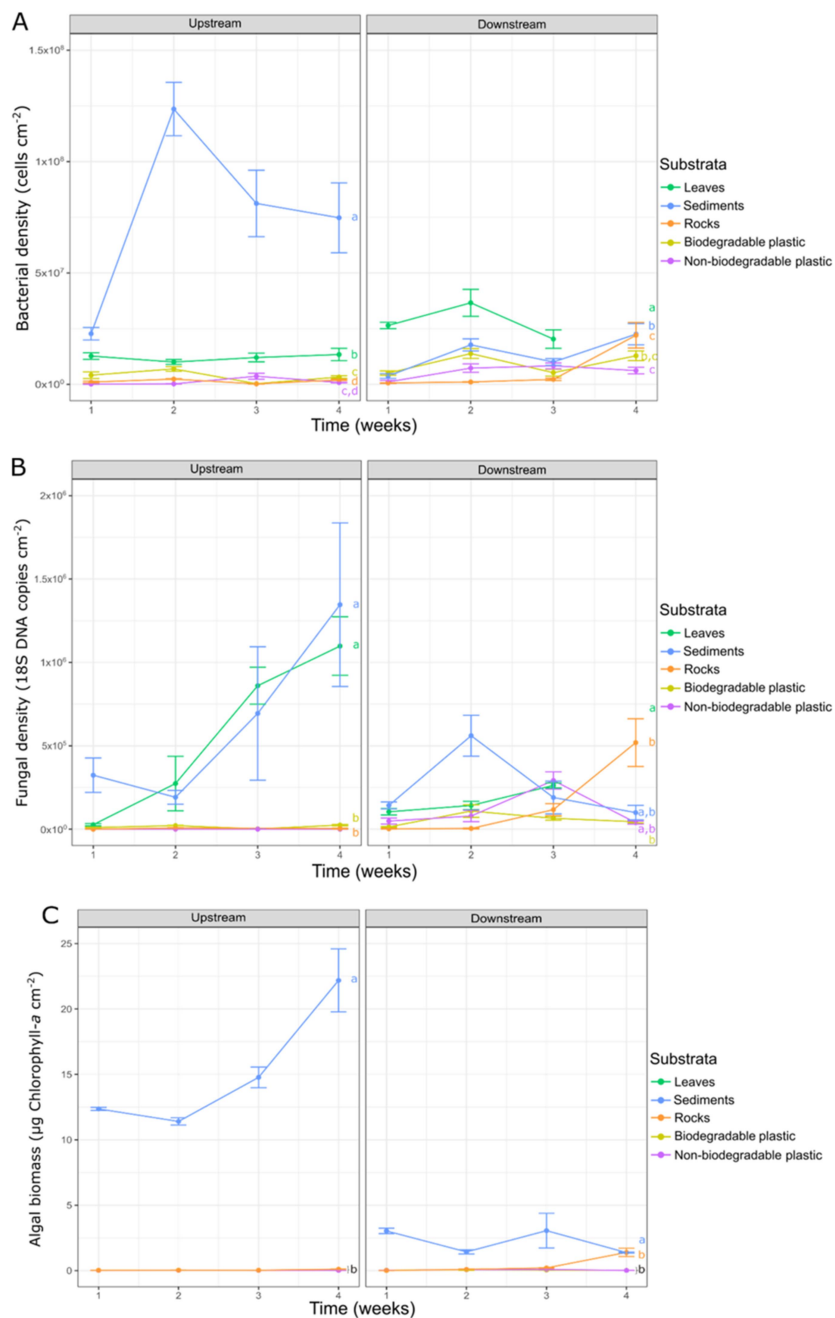


Figure 1: Bacterial density (A), fungal density (B), algal biomass (C) measured in the five substrata colonized in the upstream and downstream sites. Values are means ($n = 5$) \pm SE per substrata, site and sampling time. Statistical differences between substrata were represented by letters ($a > b > c$, Wilcoxon test, $P < 0.05$) and the test has been performed independently for each site.

ORIGINAL MANUSCRIPT

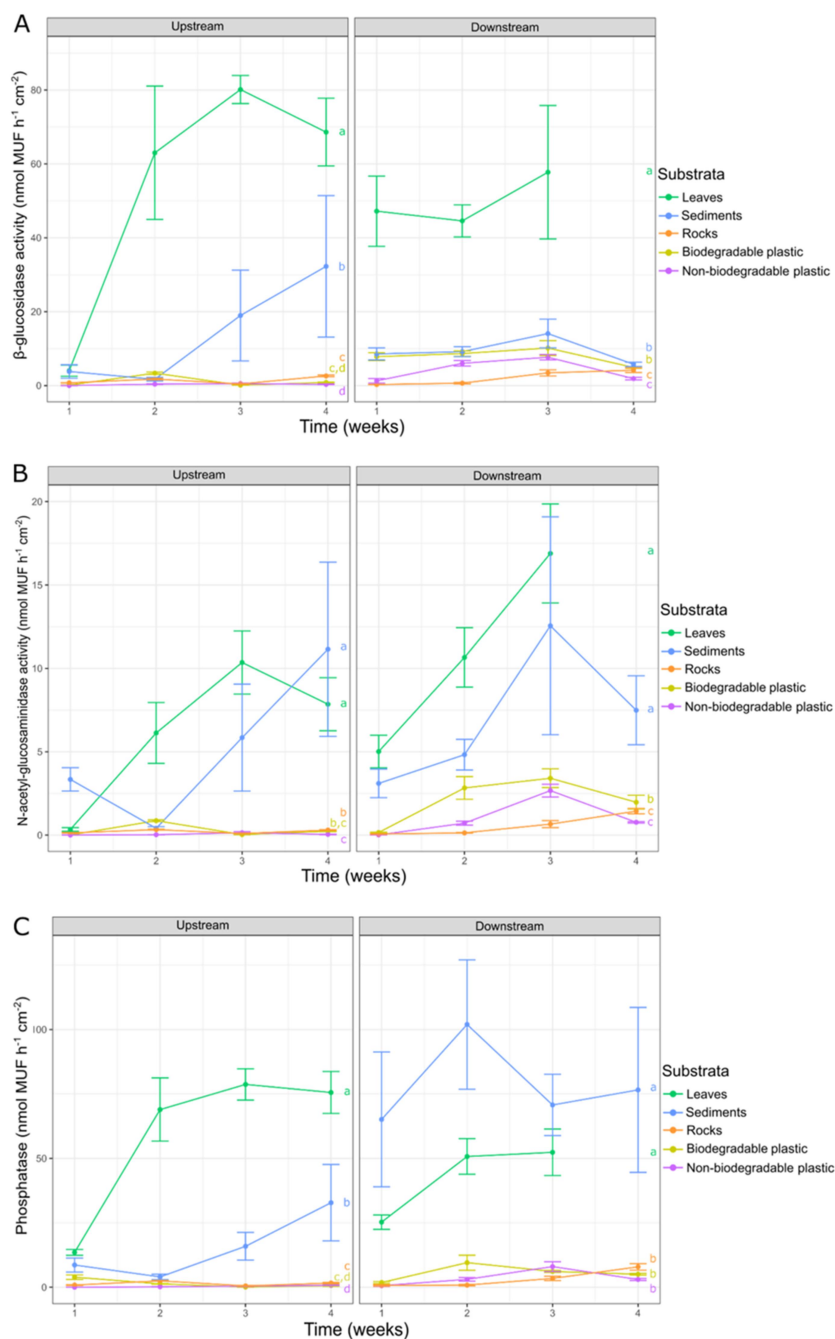


Figure 2: Extracellular enzymatic activities (GLU (A), NAG (B) and PHO (C)) measured in the five substrata placed in the upstream and downstream sites of the Artiere's river. Values are means ($n = 5$) \pm SE per substrata, site and sampling time. Statistical differences between substrata are represented by letters (Wilcoxon test, $a > b > c > d$, $P < 0.05$). The test has been performed independently for each site.

ORIGINAL MANUSCRIPT

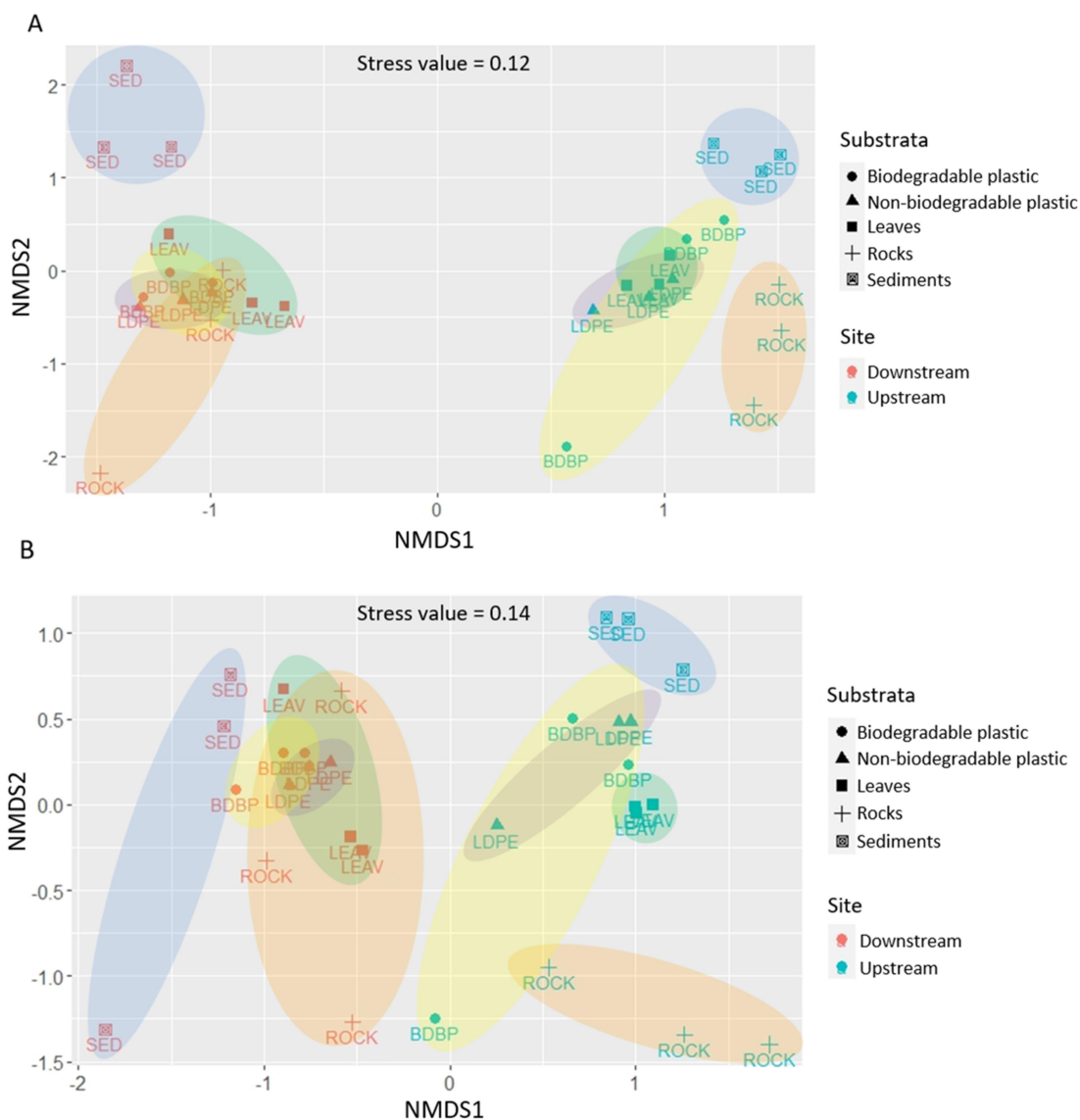


Figure 3. Non-metric multidimensional scaling (NMDS) analysis on prokaryotic (A) and eukaryotic (B) communities at week 3 of colonization of the five substrata placed in upstream and downstream sites of the Artière. Stress values were < 0.2 suggesting that the computation accurately represents the dissimilarity among samples. Ellipse zones are based on the confidence intervals for each substratum and site.

ORIGINAL MANUSCRIPT

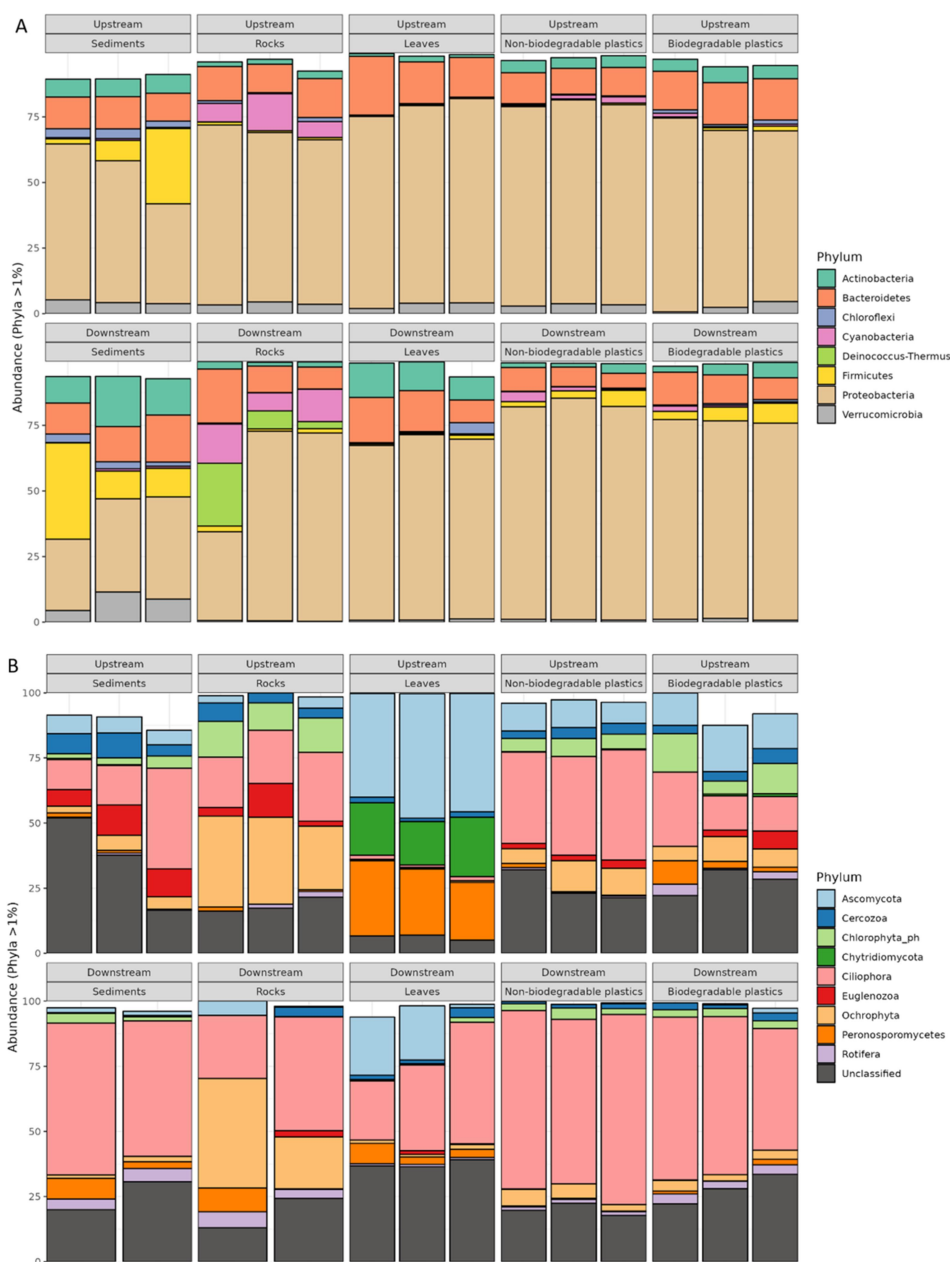


Figure 4: Relative abundance of microbial phyla contributing more than 1 % of total relative abundance of microbial communities at week 3 of the colonization experiment. Bar charts correspond to the percentage of prokaryotic phyla (A) and eukaryotic phyla (B) present in the different samples analysed.

ORIGINAL

SCRIPT

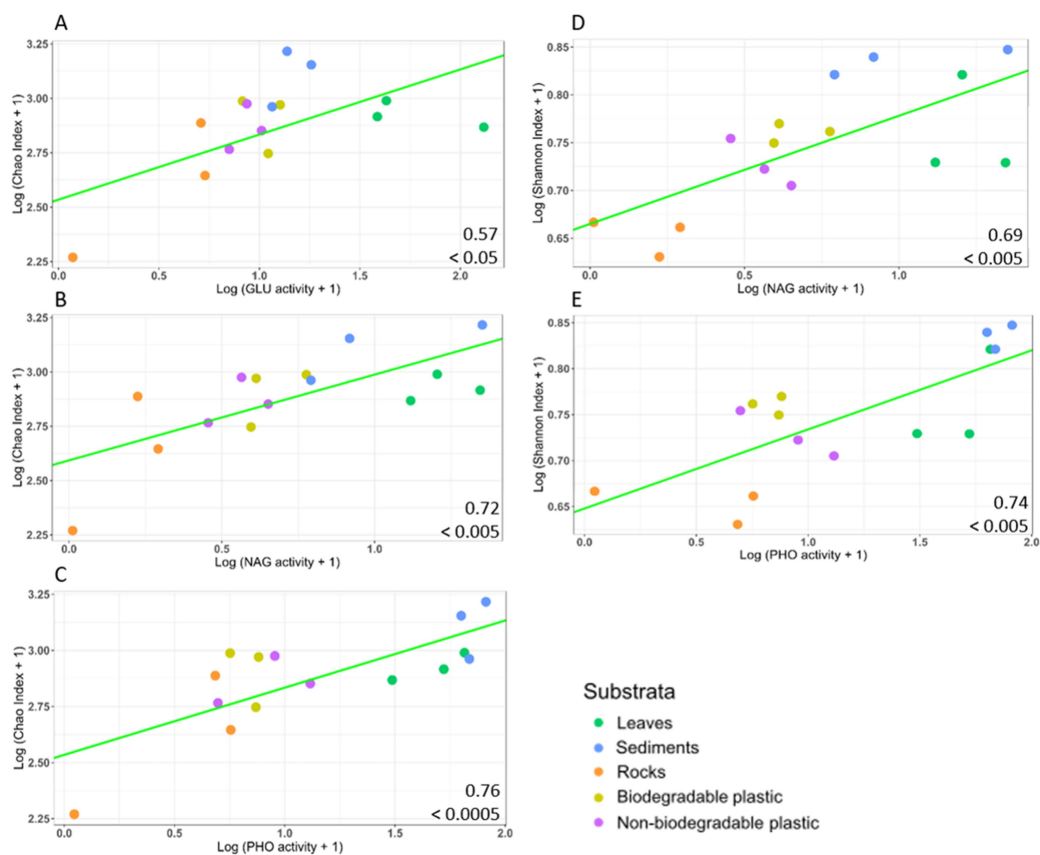


Figure 5. Log relationships between the Chao 1 index of prokaryotic communities and GLU (A), NAG (B) and PHO (C) activities measured at the downstream site as well as between the Shannon index of prokaryotic communities and NAG (D) and PHO (E) activities measured at the downstream site. In each plot we provided the Pearson correlation coefficient and the corresponding *P*-value. The different substrata are represented by different colours.

Table 1: Water physicochemical parameters in the Artière's river, in upstream and downstream sections over the four study weeks (November and December 2020). Average values (N = 4, sampling weeks) \pm SE of pH, conductivity, temperature, concentrations of dissolved oxygen, Dissolved Organic Carbon (DOC), Total Dissolved Carbon (TDC), Total Dissolved Nitrogen (TDN), Soluble Reactive Phosphorus (SRP), Total Dissolved Phosphorus (TDP) are represented. Statistically significant differences between sites were marked by asterisks (*, $P < 0.05$).

	Upstream	Downstream
pH	7.40 \pm 0.17	7.17 \pm 0.01
Conductivity ($\mu\text{S cm}^{-1}$)	222.88 \pm 4.00	723.80 \pm 64.16 *
Temperature ($^{\circ}\text{C}$)	3.98 \pm 0.58	13.56 \pm 0.99 *
Dissolved oxygen (mg L^{-1})	11.54 \pm 0.26 *	7.78 \pm 0.46
DOC (mg L^{-1})	7.00 \pm 0.59	11.88 \pm 1.17 *
TDC (mg L^{-1})	22.83 \pm 1.08	59.09 \pm 3.74 *
TDN (mg L^{-1})	2.70 \pm 0.53	4.29 \pm 0.20
SRP (mg L^{-1})	0.103 \pm 0.06	0.138 \pm 0.07 *
TDP (mg L^{-1})	0.204 \pm 0.05	0.687 \pm 0.10

ORIGINAL UNEDITED MANUSCRIPT

Table 2: Quantification and identification of macroplastics at the downstream site based on three sampling zones: shoreline (at the riverside), intermediate (10 m from the riverside) and distal (20 m from the riverside). The abundance and weight of plastics are represented per unit of surface area. Values are means (n = 3) ± SE. The classification of plastic debris according to the TSG_ML code European Commission (2013) is also provided. The percentages of plastic debris correspond to the total weight collected per sampling zones.

	Shoreline	Intermediate	Distal
Abundance (fragments/m ²)	29.2 ± 2.1	18.8 ± 15.7	2.1 ± 2.1
Weight (g/m ²)	555 ± 552	24.7 ± 14.7	0.1 ± 0.1
Plastic composition (% of weight)			
Bags (G2)	0.02	-	-
Crisps packets/sweets wrappers (G30)	0.40	1.72	-
Fertiliser/animal feed bags (G36)	0.07	-	-
Shotgun cartridges (G70)	-	31.20	-
Plastic construction waste (G85)	-	36.44	-
Other plastic/polystyrene items identifiable (G120)	99.13	-	-
Other plastic/polystyrene items not identifiable (G121)	0.38	19.62	100
Other rubber pieces (G131)	-	11.01	-

Table 3: Richness (Chao 1) and diversity (Shannon) indices for prokaryotic and eukaryotic communities at week 3 of colonization of the five substrata in upstream and downstream sites of the Artière river. Values are means ($n = 3$) \pm SE per substrata and site. Differences among substrata within the same sites are represented by letters ($a > b > c$, $P < 0.05$, Tukey's test).

	Prokaryotes		Eukaryotes		
	Chao 1	Shannon	Chao 1	Shannon	
Up	leaves	877 \pm 94 (a)	4.38 \pm 0.23 (b,c)	113 \pm 3 (a,b)	2.31 \pm 0.04 (a)
	sediment	2106 \pm 484 (a)	6.67 \pm 0.30 (a)	364 \pm 83 (a)	4.75 \pm 0.37 (a)
	BDBP	1110 \pm 407 (a)	5.26 \pm 0.37 (b)	171 \pm 73 (a,b)	4.05 \pm 0.64 (a)
	LDPE	994 \pm 53 (a)	4.37 \pm 0.13 (b,c)	268 \pm 51 (a,b)	4.12 \pm 0.31 (a)
	rocks	785 \pm 136 (a)	4.08 \pm 0.28 (c)	63 \pm 16 (b)	3.30 \pm 0.09 (a)
Down	leaves	861 \pm 68 (a,b)	4.79 \pm 0.42 (a,b)	135 \pm 24 (a)	2.80 \pm 0.48 (a)
	sediment	1311 \pm 217 (a)	5.84 \pm 0.13 (a)	138 \pm 61 (a)	2.20 \pm 0.83 (a)
	BDBP	834 \pm 140 (a,b)	4.79 \pm 0.07 (a,b)	146 \pm 26 (a)	3.10 \pm 0.19 (a)
	LDPE	760 \pm 123 (a,b)	4.39 \pm 0.16 (b,c)	152 \pm 25 (a)	2.95 \pm 0.16 (a)
	rocks	479 \pm 164 (b)	3.72 \pm 0.12 (b,c)	106 \pm 54 (a)	2.87 \pm 0.82 (a)