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Brief Report

Metatranscriptomic outlook on green and brown food webs in acid mine drainage

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Acid mine drainages (AMDs), metal-rich acidic effluents generated by mining activities, are colonized by prokaryotic and eukaryotic microorganisms widely distributed among different phyla. We compared metatranscriptomic data from two sampling stations in the Carnoulès AMD and from a third station in the nearby Amous River, focussing on processes involved in primary production and litter decomposition. A synergistic relationship between the green and brown food webs was favoured in the AMD sediments by the low carbon content and the availability of mineral nutrients: primary production of organic matter would benefit C-limited decomposers whose activity of organic matter mineralization would in turn profit primary producers. This balance could be locally disturbed by heterogeneous factors such as an input of plant debris from the riparian vegetation, strongly boosting the growth of Tremellales which would then outcompete primary producers. In the unpolluted Amous River on the contrary, the competition for limited mineral nutrients was dominated by the green food web, fish and bacterivorous protists having a positive effect on phytoplankton. These results suggest that in addition to direct effects of low pH and metal contamination, trophic conditions like carbon or mineral nutrient limitations also have a strong impact on assembly and activities of AMDs' microbial communities.

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Introduction

Acid mine drainages (AMDs) are generated by the exposition to water and oxygen of sulfide minerals excavated by coal or metal mining activities (Johnson, 2003). Despite their hostility and toxicity (Levings *et al*., 2004; Dsa *et al*., 2008), those metal-rich acidic effluents are colonized by prokaryotic and eukaryotic microbial communities which have been extensively reviewed (Hallberg, 2010; Méndez-García *et al*., 2015; Andres and Bertin, 2016; Chen *et al*., 2016). Although the prokaryotic richness is usually lower in AMDs, observed species are widely distributed among different phyla: Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria, Bacteroidetes, Nitrospirae, sulfur and/or iron-oxidizing archaea and methanogens (Edwards, 2000; Golyshina *et al*., 2000; Baker and Banfield, 2003; Dopson *et al*., 2004; Bertin *et al*., 2011; Volant *et al*., 2012; Mesa *et al*., 2017). The main eukaryotic microorganisms reported in those environments are green algae (i.e. *Chlamydomonas*, *Chlorella*, *Coccomyxa*), protozoa (i.e. Euglenozoa, Amoebozoa, Stramenopiles, Alveolates, Rhizaria) and Fungi (Baker *et al*., 2004, 2009; Hao *et al*., 2010; Amaral-Zettler, 2012; Zirnstein *et al*., 2012; Aguilera, 2013; Aliaga Goltsman *et al*., 2015; Halter *et al*., 2015; Koechler *et al*., 2016; Volant *et al*., 2016; Mesa *et al*., 2017).

Despite a strong focus on metal and sulfur transformations, those studies nonetheless provide some insights on trophic interactions and the role that may be played by the respective microorganisms in the carbon biogeochemical cycle. Primary producers include chemolithoautotrophs oxidizing Fe(II) or sulfur and, in areas exposed to sunlight, photoautotrophs such as Cyanobacteria, Euglena and algae (Baker and Banfield, 2003; Rowe *et al*., 2007; Bertin *et al*., 2011; Halter *et al*., 2015; Koechler *et al*., 2016;

Volant *et al*., 2016; Mesa *et al*., 2017). Heterotrophs feeding on organic compounds may help keep organic levels low by removing lysates and exudates that can be toxic to primary producers (Clark and Norris, 1996; Bacelar-Nicolau and Johnson, 1999; Baker *et al*., 2004; Xiao *et al*., 2013). The AMD prokaryotic communities involved in key biogeochemical transformations may also be affected by the presence of protists able to graze on acidophilic bacteria (McGinness and Johnson, 1992; Johnson and Rang, 1993; Baker *et al*., 2004; Volant *et al*., 2016). Although AMDs are oligotrophic environments (Dodds *et al*., 1998), the abundance of fungi suggests an active decomposition of organic matter (Amils *et al*., 2007; Das *et al*., 2009; Volant *et al*., 2016). The identification of genes coding for a cellulase and an α-amylase in the CARN6 bin of Carnoulès metagenome further suggests that the corresponding strain was able to metabolize complex carbohydrates (Bertin *et al*., 2011). Those complex polysaccharides may originate from components of cell wall and biofilm but also from potential extraneous sources of organic matter like dead leaves, plant debris, decaying wood or bat guano (Bruneel *et al*., 2011; Johnson, 2012; Volant *et al*., 2016). The importance of leaf litter as a source of energy in woodland streams is well known indeed (Fisher and Likens, 1973; Iversen *et al*., 1982; Siefert and Mutz, 2001) and, in the absence of shredding invertebrates, fungi are expected to play a major role as decomposers of complex organic matter (Das *et al*., 2009).

In this study, we present a comparative analysis of metatranscriptomic data from two different sampling stations (Conf and Gal) of the Reigous creek, in the Carnoulès AMD and from a third station (Ams) situated downstream in the nearby Amous River (Fig. 1). Our analyses focus on processes involved in primary production and litter decomposition, aiming at the identification of broad trophic patterns and food web relative activities at each station.

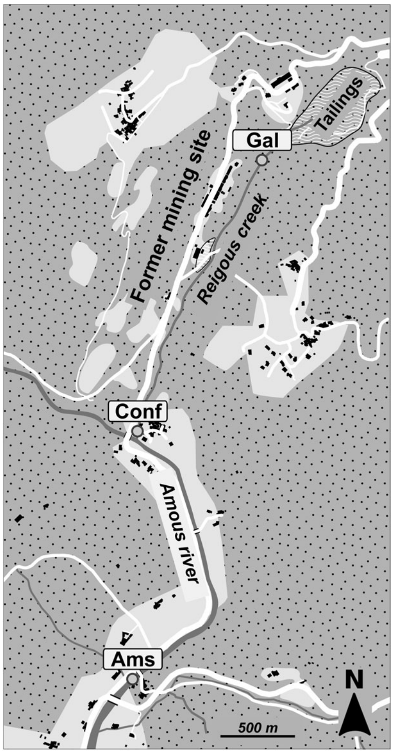

Fig 1. Map of the Carnoulès acid mine drainage area. Tailings that were accumulated during the exploitation of the now abandoned Pb/Zn mine of Carnoulès are drained by the Reigous creek which parallels the former mining site before joining the Amous River in the Fabrègues hamlet. Medium grey-dotted areas represent woodlands and dense vegetation. Light grey areas represent open land. The location of the sampling stations in this study – Gal, Conf and Ams – are represented by a labelled grey disk. The Gal station, situated on the Reigous closest to the tailings stockpiles, is surrounded by woodland. The Conf station is located in open land in the small hamlet of Fabrègues just before the confluence with the Amous River. The Ams station is 1200 m downstream the confluence on the Amous River.

Results and discussion

The first factorial plane of the correspondence analysis (Fig. 2) represented 89.7% of the total variation of the data, indicating that the variation across samples of the relative abundance of orders could be accurately represented on the first two axes. The first axis, accounting for 69.7% of the total variation separated Gal1, Gal2 and Ams1 from all other samples (all Conf samples, Gal3, Ams2 and Ams3) according to the abundance of Tremellales reads. Samples from the contaminated Gal station and from the non-contaminated Amous River (Ams) were found on both sides of the origin, suggesting some heterogeneity of the corresponding factor within the sampling stations independently of their level of contamination. This heterogeneity of Tremellales read abundance across samples from the same station may reflect heterogeneous input of allochtonous organic matter, dead leaves and plant debris, from the riparian vegetation (Fisher and Likens, 1973; Wallace *et al*., 1999; Volant *et al*., 2016). In this study indeed, Tremellales were essentially represented by the *Cryptococcus* genus which includes cellulolytic and ligninolytic primary decomposers dwelling on dead leaves, decaying wood and in freshwater (Nakase *et al*., 1996; Fell *et al*., 2011; Prakash *et al*., 2018). Metatranscript assembly followed by coding sequence (CDS) prediction and annotation allowed the identification of laccase, cellulase, chitinase and α-amylase enzymes belonging to the *Cryptococcus* genus. In addition, the taxonomic classification by Kaiju of reads mapping to the predicted glycoside hydrolase CDSs (Fig. 3) suggested that Tremellales were the main decomposers of lignin, cellulose and other glucans. The degradation of recalcitrant polysaccharides by

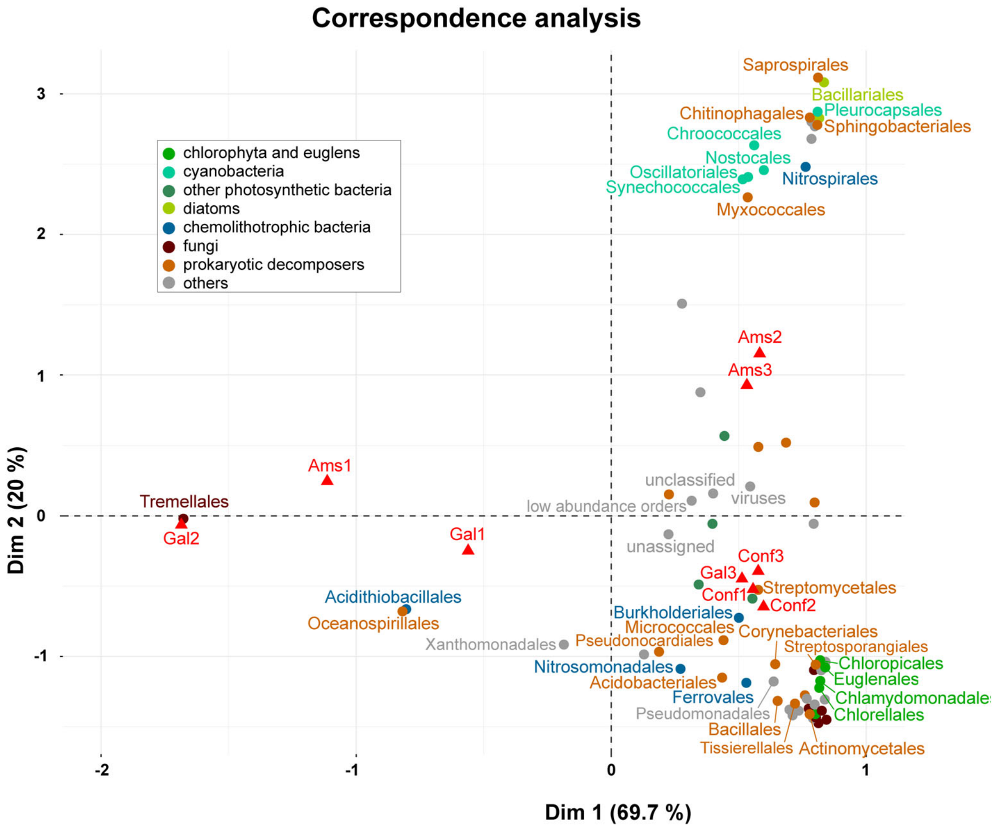

Fig 2. Correspondence analysis of taxonomic classification data by Kaiju (Menzel *et al*., 2016) at the order level, using only orders representing at least 0.1% of all reads in a sample. The first two dimensions account for 89.7% of the total variance in the data. Gal1, Gal2 and Ams1 are related to the saprophytic fungi Tremellales (brown food web) along the first axis. Conf samples, as well as Gal3, are associated with a combination of phyla representative of both green and brown food webs: decomposers (Bacillales, Actinobacteria, etc.) and primary producers such as Chlorophyta and chemolithotrophic bacteria (Burkholderiales, Nitrosomonadales, etc.). The green food web is strongly represented in Ams2 and Ams3 samples by photosynthetic orders of the Cyanobacteria phylum or diatoms.

Tremellales would increase the availability of decaying organic matter as a source of carbon and energy for secondary decomposers and other heterotrophs. Those food chains based on detritus decomposition are denoted by the term ‘brown food web’, in opposition to the ‘green food web’ driven by primary production (Allison, 2006; Butler *et al*., 2008; Zou *et al*., 2016). Thus, in areas receiving plant debris, the microbial communities would not be limited by the amount of organic carbon but by mineral nutrient availability instead, a condition where active cycling of mineral nutrients within the brown food web would favour decomposers and have a limiting effect on primary producers (Zou *et al*., 2016). As a matter of fact, Fig. 3 shows that the expression of genes related to photosynthesis and carbon fixation is lower in samples where the expression of cellulose and lignin degradation genes by Tremellales is higher compared with other samples of the same station (Gal1 and Gal2 vs. Gal3, Ams1 vs. Ams2 and Ams3), suggesting some competition between brown and green food webs in those samples.

The second axis, representing 20.0% of the total variation of data distinguished the non-contaminated samples taken in the Amous River from the contaminated samples of the Reigous creek, reflecting the influence of AMD conditions on Conf and Gal microbial communities in comparison with Ams station. Previous studies have shown that low-pH conditions constitute the main factor affecting diversity of microbial communities in AMDs, although temperature, ionic composition, total organic carbon, and dissolved oxygen may also have a significant influence (Volant *et al*., 2014; Méndez-García *et al*., 2015). In this study, the C:N:P stoichiometry of the Reigous sediments (Table 1) further suggested an absence of deficiency of N and P (Hecky *et al*., 1993). It is thus expected that primary producers were not in competition with decomposers for mineral nutrients, except in Gal1 and Gal2 samples where an input of allochtonous organic matter might have strongly boosted the activity of Tremellales as suggested above. In Gal3 and Conf samples where no expression was observed for lignin degradation genes, suggesting they did not undergo any input

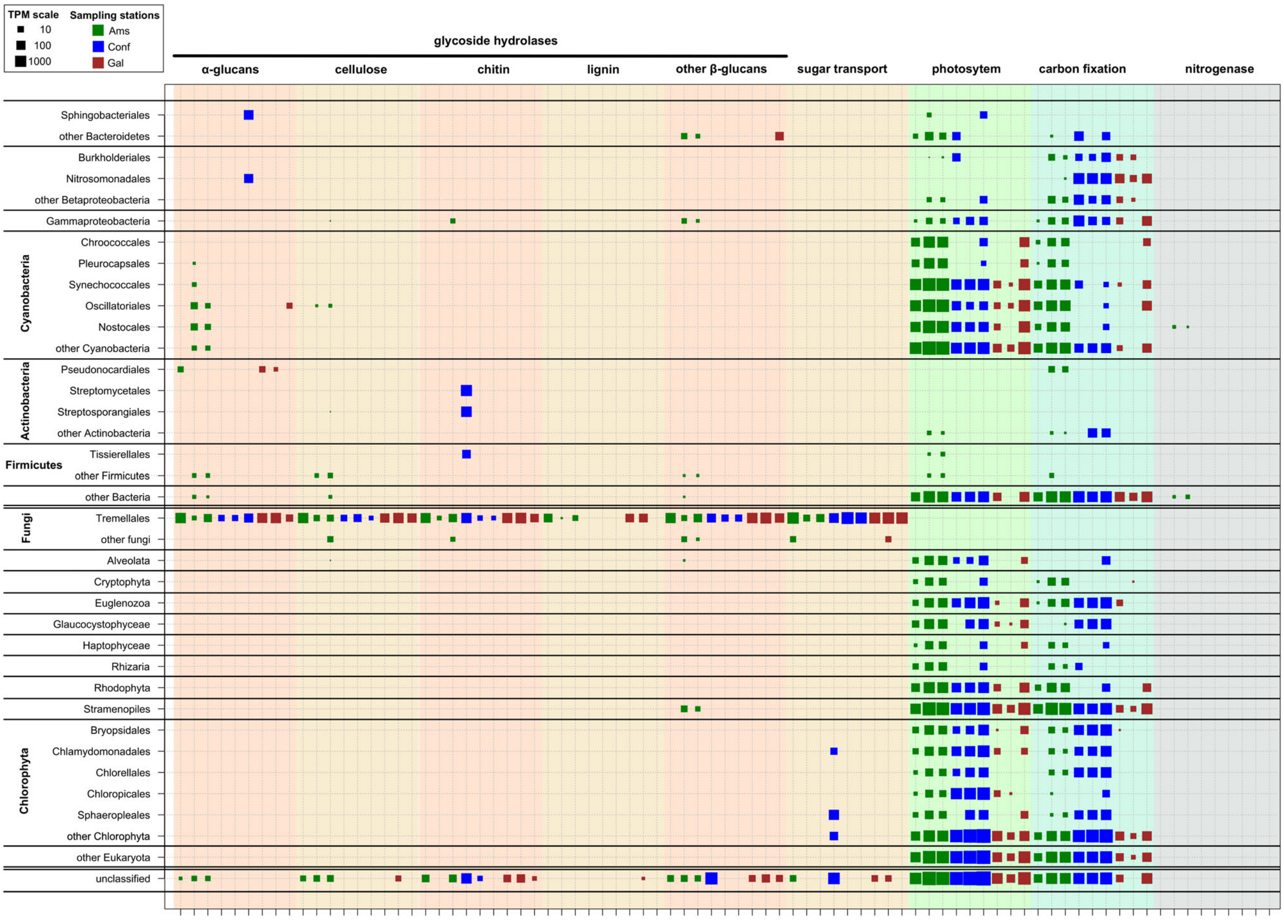

Fig 3. Expression values in transcripts by million (TPM) for functional groups of genes by order in each sample created with the rioja 0.8–5 package in R 3.6.3 (R Core Team, 2020). The colour of squares indicate the sampling station (green = Ams, blue = Conf, brown = Gal) and their size is proportional to the $\log_{10}$ of the corresponding expression value in TPM. The complete figure is available as Supporting Information Fig. S1. After merging using the BBMerge program from the BBTools suite, reads were assembled with rnaSpades 3.13.0 (Bushmanova *et al*., 2019) separately in one batch per sample. CDSs longer than 240 bases were predicted and translated using TransDecoder 5.5.0 (https://github.com/TransDecoder/TransDecoder) and the 128 506 predicted proteins were annotated with Interproscan 5.35–74.0 (Jones *et al*., 2014) and dbCAN2 version 2.0.1 (Zhang *et al*., 2018) with the CaZy database (Lombard *et al*., 2014) as of the 31th of July 2019. The CaZy GH numbers and Interpro entries used to define the functional groups are given in Supporting Information Table S3. Transcript quantification was performed with Salmon 1.1.0 (Patro *et al*., 2017) in quasi mapping mode using quality-filtered read pairs from each replicate of each sample separately. Reads mapping CDSs were kept for each functional group and sample prior to their taxonomic classification by orders with Kaiju. TPM values of each functional group in every sample were computed for each order as: $TPM_{i,j,k} = TPM_{i,j} \times f_{i,j,k}$, where $TPM_{i,j,k}$ is the TPM value for group i, sample j and order k, $TPM_{i,j}$ is the TPM value computed by Salmon for group i and sample j, and $f_{i,j,k}$ is the proportion of reads from sample j mapping to CDSs in group i that were classified as order k by Kaiju.

of plant debris, primary production (photosystems and carbon fixation) is more active indeed than in Gal1 and Gal2 (Fig. 3). The bioavailability of metals and the very low organic carbon content of sediments would favour chemolithotrophs able to use metals for energy production like Burkholderiales, Nitrosomonadales and other *Betaproteobacteria* and *Gammaproteobacteria*. Photosynthetic organisms like Chlorophyta, Stramenopiles, Euglenales and to a lesser extent Cyanobacteria and other photosynthetic bacteria were also active in the Reigous. The primary production of organic matter would benefit C-limited decomposers whose activity of organic matter mineralization would in turn profit primary producers in a synergistic relationship between the green and brown food webs (Daufresne and Loreau, 2001; Zou *et al*., 2016).

The Reigous AMD brown food web was represented not only by fungi but also by orders of the Bacteroidetes, Actinobacteria and Firmicutes phyla. Only the order of Tremellales though was consistently expressing all the genes coding for glucan degradation enzymes examined in this study. Bacterial orders of decomposers showed more specialization towards a few types of glucans only (Fig. 3). Sphingobacterales and Nitrosomonadales in Conf1, Pseudonocardiales in Gal1 and Gal2 were all expressing genes encoding α-glucosidases. However,

Table 1. The main physicochemical parameters (pH, temperature, dissolved oxygen and conductivity) were measured in situ as described in (Resongles et al., 2013). Total dissolved concentrations of Fe, Al, Mn and toxic trace elements (As, Zn, Pb, Cu, Ni, Tl) were determined using ICP-MS after filtration through 0.22 μm Millipore membranes according to routine procedures (Resongles et al., 2013). Analytical error (relative standard deviation) was better than 5%. Sulphate was analysed using nephelometry as described in (Egal et al., 2010). Organic carbon concentration in sediments was determined using dry combustion at the Cirad Laboratory (Montpellier, France).C:N:P stoichiometry of the sediment was determined by IR combustion (carbon), thermal conductivity (nitrogen) and ICP-AES (phosphorus) at the ANALYTICE company (Strasbourg, France).

Sampling stations	pH	T (°C)	DO (mg l^{-1})	Cond (μS cm^{-1})	Fe (mg l^{-1})	SO_4^{2-} (mg l^{-1})	As (μg l^{-1})	Zn (μg l^{-1})	Pb (μg l^{-1})	Al (μg l^{-1})	Mn (μg l^{-1})	Cu (μg l^{-1})	Ni (μg l^{-1})	Tl (μg l^{-1})	OC (% dry weight)	C:N:P[a]
Gal	5.02	12.8	9.7	2370	330	1649	26926	8060	398	9565	4417	248	196	108	0.80	33:9:1
Conf	3.64	9.9	10.1	1360	42	739	1791	8818	556	10833	6672	93	170	22	0.29	45:10:1
Ams	8.18	9.9	11.2	694	4.8	599	26.0	242	0.08	39	362	0.82	9.8	1.38	6.25	208:23:1

DO, dissolved oxygen; Cond, conductivity; OC, organic carbon; T, Temperature.

[a] Organic carbon:nitrogen:phosphorus stoichiometry.

the expression of α-glucosidase genes by Cyanobacteria might be related to the biosynthesis or degradation of their own storage polymer glycogen instead of decomposition (Shinde *et al*., 2020). In addition to Tremellales, chitinase genes were expressed in Conf1 by Streptomycetales, Streptosporangiales and Tissierellales. Since chitin is an essential compound of the fungal cell wall, this observation would suggest that Actinobacteria (Streptomycetales, Streptosporangiales) and Firmicutes were playing an active role as decomposers of dead fungal cells or as predators of fungi (Chet *et al*., 1971; Kumbhar and Watve, 2013; Lacombe-Harvey *et al*., 2018; Starke *et al*., 2020). It is probable as well that the chitin degradation by Tremellales at all stations may correspond to the recycling of dead fungal biomass or to the reorganization of their own cell wall during growth.

Expression of sugar-transport genes could be detected only for fungi except in Conf1 where it was observed also for Sphaeropleales, Chlamydomonadales and other Chlorophyta whose order could not be predicted, suggesting a mixotrophic lifestyle. Sphaeropleales, Chlamydomonadales and other green algae like *Coccomyxa* have been shown indeed to be capable of heterotrophic or mixotrophic growth with a better yield than autotrophy when a source of carbon is available (Laliberté and de la Noüe, 1993; Casal *et al*., 2010; Suzuki *et al*., 2018). Those Chlorophyta may thus have taken advantage of decomposers' activity in Conf1 to enhance their growth and as such would have participated in both green and brown food webs.

The impact of pollution from the Reigous AMD on the Amous River was considerably attenuated at the Ams station, 1.2 km downstream from confluence (Table 1). The C:N:P stoichiometry of the Amous River sediments was quite typical of a primary stream and suggested a moderate limitation of phytoplankton by N and P (Hecky *et al*., 1993). This limitation by mineral nutrients would imply a competition between the green and brown food webs with an outcome depending upon various factors that could not be addressed in this study, like predation rate or nutrient release (Zou *et al*., 2016). Expression of photosystem genes was very active in the Amous River samples for a wide range of microorganisms: Cyanobacteria, Stramenopiles, Rhizaria, Rhodophyta, Chlorophyta, Glaucocystophyceae, Haptophyceae, Euglenales (Fig. 3). Along with the larger proportion of photosystem-related CDSs assembled from Ams samples compared with Conf and Gal (Supporting Information Table S4), this would suggest dominance of the green food web in the Amous River.

Fish, like the common roach or the perch, are known to have a beneficial effect on primary producers, directly by providing mineral nutrients through their excretions and indirectly through predation on grazing microcrustaceans (Carpenter and Kitchell, 1993; Brett and Goldman, 1996; Attayde and Hansson, 2001; Danger *et al*., 2012; Zou *et al*., 2016). Conditions encountered in the Amous River allowed for the presence of fish (Casiot *et al*., 2009) and the green food web may thus benefit from their presence. This sharply contrasted with Gal1 and Gal2 where the brown food web outcompeted the green food web. Fish are absent from Gal station due to the low pH, and the presence of metals like iron, manganese, aluminium and arsenic, zinc, lead, copper. In particular, aluminium concentrations in the Reigous AMD are largely superior to those shown to cause death of fish with a high probability (Witters, 1986; Gagen *et al*., 1993; Baldigo and Murdoch, 1997; Baldigo *et al*., 2020). Bacterivorous protozoa like Ciliophora, Amoebozoa, Choanoflagellida and Kinetoplastida – identified by Kaiju in larger proportions in Ams than in Conf and Gal (Supporting Information Fig. S2) – may also have a positive effect on phytoplankton growth by reducing the amount of mineral nutrients trapped in the brown food web (Caron *et al*., 1988; Ferrier and Rassoulzadegan, 1991; Saleem *et al*., 2016; Zou *et al*., 2016).

Although all photosynthetic phyla were represented in both the Amous River and Reigous AMD, the second dimension of the correspondence analysis clearly indicated that microbial photosynthetic activities were characterized by Cyanobacteria at Ams and Chlorophyta at Conf (Fig. 2). It is probable that this difference could not be strictly attributed to the higher arsenic contamination at Conf since cyanobacterial photosynthetic activity was observed in all Reigous samples. The C:N ratios of 9.0 at Ams and 4.5 at Conf indicated that phytoplankton might be moderately limited by nitrogen in the Amous River but not at Conf (Hecky *et al*., 1993). The expression of nitrogenase *nifH* gene by Nostocales and other bacteria observed at Ams (Fig. 3) further suggested that nitrogen fixation might have contributed to the relative success of Cyanobacteria in the Amous River by allowing them to use dinitrogen, a source of nitrogen that Chlorophyta are unable to tap.

We have performed here a metatranscriptomic analysis on sediments from the Carnoulès AMD and the nearby Amous River, focussing on processes related to primary production, litter decomposition and trophic interactions. Both green and brown food chains comprise several trophic levels from primary producers or decomposers up to top predators. Although nutrient cycling can occur actively within a food chain (Caron *et al*., 1988; Attayde and Hansson, 2001; Leroux and Loreau, 2010; Berdjeb *et al*., 2011), green and brown food webs are coupled by nutrient fluxes (Polis and Strong, 1996; Rooney *et al*., 2006; Boit *et al*., 2012; Cherif and Loreau, 2013; Mougi, 2020). The activity of one food web may generate cascading effects that can influence the activity and production of trophic levels in the other one (Carpenter *et al*., 1985; Carpenter and Kitchell, 1993; Leroux and Loreau, 2010; Zou *et al*., 2016). Bacterivorous protists for instance may reduce the amount of mineral nutrients trapped in the brown food web, making those nutrients more available to phytoplankton (Caron *et al*., 1988; Ferrier and Rassoulzadegan, 1991; Saleem *et al*., 2016; Zou *et al*., 2016). Thus, depending upon resource availability and community structure, the direct and indirect interactions between decomposers and primary producers will result in either synergy or competition (Caron *et al*., 1988; Daufresne and Loreau, 2001; Daufresne *et al*., 2008). Originally developed to describe macroecosystems, these notions have since been extended to microorganisms, which play a major role in detritus decomposition and primary production (Steffan and Dharampal, 2019). In this study, the C-limited Reigous AMD microbial community generally achieved a synergistic relationship between the green and brown food webs. Competition for mineral nutrients would occur though if the resource balance was disturbed by allochtonous input of organic matter, strongly favouring decomposers. In the uncontaminated Amous River, the translocation of organic matter by metazoa would extend the carbon cycle well beyond the microbial community in comparison with the Reigous AMD, with a different outcome to the competition for mineral nutrients in favour of the green food web. In this regard, microbial communities might be significantly affected by their surroundings. Extending the scope of the study beyond the mere sampling points would integrate microorganisms into a more complete network of interdependence within a meta-ecosystemic point of view. Interactions between microorganisms and macroorganisms play an important role in natural ecosystems and the disruption or disturbance of these interactions in anthropized contaminated environments may have dramatic effects on the structure and activity of microbial communities. We thus suggest that the interpretation of metatranscriptomic data should consider information about the surroundings of the studied ecosystem in order to gain a better insight of microbial communities' activity. In our time of global warming and biological diversity crisis, this may be of crucial importance for a better understanding and management of the effects that anthropic perturbations of the Earth microbiota may have, directly or not, on soil fertility and water quality.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

Metatranscriptomic raw data were made available in the SRA database under the bioproject number PRJEB38318. Replicate samples received the accession numbers ERS4560396 (Ams1), ERS4560397 (Ams2), ERS4560398 (Ams3), ERS4560399 (Conf1), ERS4560400 (Conf2), ERS4560401 (Conf3), ERS4560402 (Gal1), ERS4560403 (Gal2) and ERS4560404 (Gal3).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Experimental procedures.

Table S1. Number of reads per sample.

Table S2. Metatranscriptome assembly statistics.

Table S3. List of CaZy and Interpro entries that were predicted in metatranscriptome ORFs to define functional groups.

Table S4: Number annotated CDSs by sampling station.

Fig. S1 Expression values in transcripts by million (TPM) for functional groups of genes by order in each sample created with the rioja 0.8–5 package in R 3.6.3 (R Core Team, 2020). The colour of squares indicate the sampling station (green = Ams, blue = Conf, brown = Gal) and their size is proportional to the $\log_{10}$ of the corresponding expression value in TPM. The CaZy GH numbers and Interpro entries used to define the functional groups are given in Supporting Information Table S3.

Fig. S2 Average proportion of reads classified by Kaiju in the bacterivorous protist taxa Ciliophora, Amoebozoa, Choanoflagellida and Kinetoplastida.