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Effects of sulfonamide antibiotics on aquatic microbial community composition and functions

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HIGHLIGHTS

- Periphyton slowed down microbial leaf-litter decomposition
- Sulfonamide antibiotics did not affect decomposition rates
- Sulfonamides reduced bacterial biomass accrual in leaves
- Sulfonamides stimulated β-glucosidase activity in leaves
- Periphyton did not damp the impact of sulfonamides on decomposers

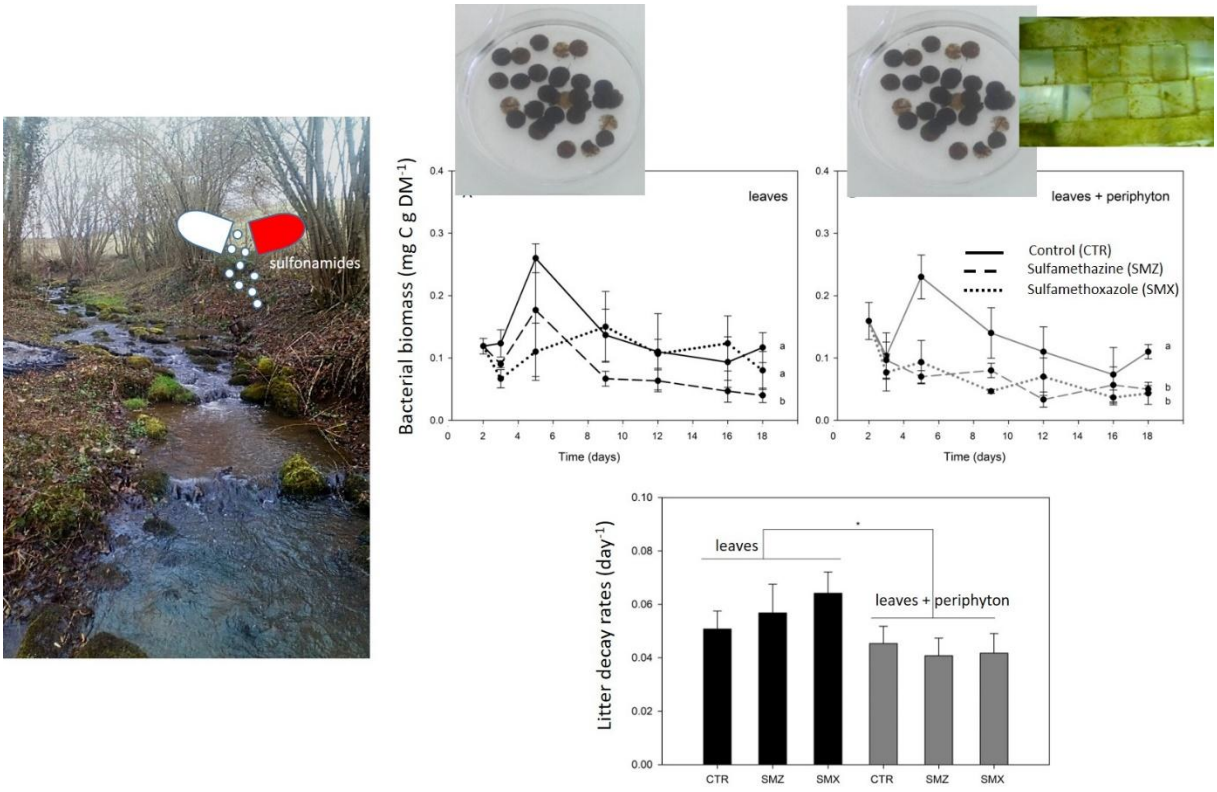
ABSTRACT

Knowledge on interactions among microbial communities colonizing various streambed substrata (e.g. cobbles, sediment, leaf-litter etc.) is essential when investigating the functioning of stream ecosystems. However, these interactions are often forgotten when assessing the responses of aquatic microbial communities to chemical contamination. Using a stream microcosm approach, the respective impact of two sulfonamide antibiotics (sulfamethoxazole and sulfamethazine) on the ability of microbial heterotrophs to decompose alder leaves was investigated in the presence or absence of periphyton. Our hypothesis suggested that sulfonamides would negatively impair microbial litter decomposition and that periphyton could possibly alleviate this effect by stimulating microbial decomposer activity through a priming effect. Results showed that the presence of periphyton enriched water with oxygen and labile dissolved organic carbon forms. However, these labile organic carbon sources did not stimulate leaf-litter decomposition but mostly decoupled microbial decomposer activity from particulate organic matter to dissolved organic matter through negative priming. Also, the two sulfonamide molecules did not affect the leaf-litter decomposition

process but significantly decreased bacterial biomass accrual on leaves. The reduction of bacteria was concomitant with an increase in biomass-specific β-glucosidase activity and this was attributed to a stress response from bacteria to sulfonamides. Further research looking at microbial interactions would provide for better assessment of chemical contamination effects in communities and processes in stream ecosystems.

Keywords: leaf-litter decomposition, sulfamethazine, sulfamethoxazole, periphyton, negative priming, β-glucosidase activity.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Microbial communities are pivotal in stream ecosystem biogeochemical cycles and actively participate in organic matter degradation. The leaf-litter decomposition process is characteristic of forested streams and has been proposed as a tool to assess stream ecosystem integrity (Young *et al.*, 2008; Gessner *et al.*, 2010) though its application is rare. Leaf-litter decomposers are mainly represented by fungi and bacteria, the fungal compartment being predominant and representing on average 96% of the total microbial biomass accrual in leaves (Baldy *et al.*, 2002). However, their relative importance in the degradation process is more balanced. For alder leaves (*Alnus glutinosa*), it has been estimated that fungi contributed to 15% and bacteria to 7% of total leaf mass loss (Hieber and Gessner, 2002), the remaining leaf mass being degraded by the action of macrofauna and/or abiotic processes. Microbial leaf decay is mediated by specific extracellular enzymes excreted by fungi and bacteria (Romaní *et al.*, 2012) and the activity of these enzymes is regulated depending on

the quality and quantity of the dissolved organic matter available in stream water (Sinsabaugh and Follstad Shah, 2012).

Under specific environmental conditions, leaf-litter decomposition in forested streams can be modulated by primary production according to the priming effect, defined as the increase in recalcitrant organic matter mineralization rate in the presence of labile organic matter (Guenet *et al.*, 2010). Some complex macromolecules present in leaves (i. e. phenolics) may be recalcitrant to mineralization because microorganisms are unable to synthesize the enzymes that would degrade them. However, the capabilities of leaf-associated microorganisms to increase the mineralization rate of these complex molecules can be enhanced if extra energy is supplied as labile organic matter (i. e. soluble carbohydrates). Primary producers in streams are mostly accumulated in the periphyton, a type of microbial community colonizing inert substrates and being characterized by a predominant autotrophic metabolism capable of releasing C-labile exudates to stream water (Lock *et al.*, 1984; Romaní & Sabater, 2000). Photosynthetic extracellular release has been estimated at between 0.9 and 48% of primary production, greater release being measured in lentic sections of rivers (e.g. side arms, Sieczko *et al.*, 2015) and under oligotrophic conditions where nutrient-limited algae are forced to increase their release of carbon exudates due to stoichiometric constraints (Ziegler and Lyon 2010). For microbial decomposers, this labile organic carbon supply from periphyton has been shown to stimulate leaf-litter degradation (Daufresne and Loreau, 2001) but the intensity of the priming effect may be modified by shifts in environmental conditions (Guenet *et al.*, 2010). Nutrient availability has been seen to influence the nature of the interactions between decomposers of labile and recalcitrant organic matter (bacteria and fungi, respectively) and their capacities to degrade leaf-litter (Carney *et al.*, 2007). Low nutrient concentrations in stream water have been seen to increase leaf-litter decomposition in the presence of benthic algae but not in their absence, while this priming effect was not observed under high-nutrient conditions (Danger *et al.*, 2013). Also, low quality litter species have been described as less palatable for leaf-litter decomposer which shifts their activity from particulate organic matter (POM) degradation to dissolved organic matter (DOM) degradation (Pastor *et al.*, 2014). Also, a recent study describes the influence of leaf-litter on the primary producer community, i.e. changing microalgae taxonomic composition (Allen *et al.*, 2020). Since considering microbial interactions becomes central when investigating the functioning of stream ecosystems, this study investigates how the priming effect can temper adverse effects generated by antibiotics in leaf-litter decomposer communities for the first time.

Antibiotics are used worldwide for human and animal health. When delivered, antibiotics are far from being completely absorbed by organisms. For antibiotics used in animal husbandry, between 30% and 90% of the parent compound is excreted unmetabolized in animal urine and feces (Sarmah *et al.*, 2006). Due to this incomplete absorption, antibiotics are likely to reach the environment mainly through manure and wastewater (Boxall *et al.*, 2003; Kümmerer, 2009). Sulfonamides (SMs) form a class of antibiotics with a common mode of action. They inhibit multiplication of bacteria by inhibiting folic acid production and therefore the synthesis of purines and DNA (Seydel, 1968). SMs are mainly used for animal health. In France, in 2018, they represented 17.8% of antibiotic sales for veterinary use (Urban *et al.*, 2019). SMs have low sorption properties in soils (Boxall *et al.*, 2002; Thiele-Bruhn *et al.*, 2004) and high water solubilities (Sukul & Spiteller, 2006). Several publications attempted to assess their degradation in the environment. In soils, half-lives range between 10 and 30 days (García-Galán *et al.*, 2008). The degradation of three different SMs was studied in lake water, where half-lives varied between 10.5 and 12.9 days (Zhang *et al.*, 2013). Their high mobility in the environment and their relative persistence are confirmed by many studies highlighting their occurrence in ground and surface waters worldwide (Batt *et al.*, 2006; Peng *et al.*, 2008; Matongo *et al.*, 2015). In a wide study conducted in the USA, Kolpin *et al.* (2002) examined the occurrence of

pharmaceuticals in 139 streams and rivers susceptible to contamination (biased selection). They found that sulfamethazine (SMZ) and sulfamethoxazole (SMX) were detected at the respective frequencies of 4.8% and 12.5% and respective median concentrations of 0.02 µg L^{-1} and 0.15 µg L^{-1} (maximum concentrations: 0.12 µg L^{-1} and 1.9 µg L^{-1} respectively). Sulfonamides have been detected worldwide, their concentrations in surface waters generally varying between a few and several dozen ng L^{-1} (Martin-Laurent *et al.*, 2019).

The impact of SMs was mainly studied on microbial communities from activated sludge. Changes in bacterial community structure were noticed but no significant modification was observed in organic matter microbial degradation (Xia *et al.*, 2012; Collado *et al.*, 2013). However, in soils, at environmentally realistic concentrations, it has been shown that SMs inhibit microbial respiration and possibly reduce specific enzymatic activities like phosphatase or urease (Liu *et al.*, 2009; Gutierrez *et al.*, 2010; Conkle & White, 2012; Roose-Amsaleg & Laverman, 2016). This reduction in microbial activity could negatively impact nutrient renewal and potentially alter biogeochemical cycles in soils. Also, when exposed to SMs, microbial communities in soils show a reduction in the bacteria/fungi biomass ratio (Gutierrez *et al.*, 2010; Pinna *et al.*, 2012). To our knowledge, no studies have attempted to investigate the impact of SMs on natural leaf-litter decomposer communities in streams. Therefore, the objective of this study is to assess the impact of two SM antibiotics (sulfamethazine and sulfamethoxazole) on leaf-litter decomposers, and more precisely, to determine whether the presence of periphyton could modulate the response of litter decomposers to the antibiotics or not. To that end, a microcosm experiment was conducted on previously-colonized alder leaves which were exposed (or not) to an antibiotic in the presence (or in absence) of periphyton. Physicochemical characteristics of water and the biomass and functional responses of leaf-associated microbial communities were monitored over three weeks. Thus, this study aims to answer the following specific questions: i) Does the presence of periphyton increase the activity of leaf-litter decomposers? ii) What are the specific impacts of SMs on the leaf-litter decomposer community? iii) Can the impact of antibiotics on leaf-litter decomposers be alleviated by the presence of periphyton?

Our hypotheses suggest that primary production in periphyton would release labile DOM in water. These labile carbon forms could increase the activity of both labile and recalcitrant organic matter decomposers in leaf-litter through a priming effect. Due to their bacteriostatic activity, SM antibiotics are expected to negatively impact bacterial biomass as well as the decomposition of labile organic matter through decreases in the activity of glucosidases. However, one could expect fungi to take advantage of impaired bacteria by increasing their biomass and their capacity to degrade both labile and complex carbon polymers available in water and of which alder leaves are made up. Finally, the greater availability of labile DOM could enhance the resistance of bacterial populations in leaves to SM antibiotics.

2. MATERIALS AND METHODS

2.1. Microbial communities

Two different substrates were used to grow natural microbial communities: (1) glass slides (7.6 × 2.6 cm) glued onto three independent flagstones to collect periphyton and (2) dried freshly-fallen alder leaves (*Alnus glutinosa* L. (Gaertn.)) divided into three nylon bags of 0.5 mm mesh size to collect leaf-litter decomposer communities. Both the glass slides and the leaves were incubated for one week in spring 2019 in a first-order forested stream (Artière, Centre France; 45° 43' N, 3° 01’ E) before being transferred to microcosms in the laboratory. Aquatic microorganisms can colonize, within a few days,

deciduous leaves submerged in streams (*i. e.* aquatic hyphomycetes; Nikolcheva and Bärlocher, 2004). The 500-meter section in which the microbial communities were grown was characterized by a stream channel 2-4 m wide and 10-20 cm deep. The streambed alternated sand pools and riffles with cobbles of granitic nature and the riparian vegetation was mainly composed of alder and chestnut with a nearly absent shrubby stratum. This section is located within a "zero pesticide area" which means that the use of phytosanitary products is forbidden. Indeed, levels of contamination were low for pesticides and pharmaceuticals at this site (Rossi *et al.*, 2019). More specifically, no SMs were detected in the stream water at this study site.

2.2. Microcosm study

The microcosms used consisted of 20 L glass aquariums (rectangular parallelepiped length 40 cm × width 20 cm × height 25 cm). Previously-colonized alder leaves were cut into 1 cm^2 disks and placed in nylon bags (0.5 mm mesh) before being divided into 18 independent microcosms. Each microcosm contained 10 litter bags and each bag contained 35 leaf disks. Among these 18 microcosms containing litter bags, 9 were filled each with 12 glass slides colonized with periphyton. Since early life forms of macrofauna could be still contained in periphyton (i. e. eggs, larvae) deployed in aquariums, we decided to enclose all leaf disks in fine mesh bags in order to prevent their decomposing activity.

Six different treatments were tested each with three replicates in our experiment (Table 1). Leaves alone (L) and leaves incubated in the presence of periphyton (L+P) exposed to sulfamethazine (SMZ), sulfamethoxazole (SMX) or without antibiotic (CTR). A nominal concentration of 5 µg L^{-1} in water was chosen for the antibiotics. These nominal concentrations were based on SMZ and SMX concentrations detected in agricultural and urban streams worldwide, which can reach a few µg L^{-1} during acute exposure scenarios (Matongo *et al.*, 2015, Peng *et al.*, 2008).

ANTIBIOTICS / BIOFILMS	CTR	SMZ 5 µg L^{-1}	SMX 5 µg L^{-1}
L	a	b	c
L + P	d	e	f

Table 1. Experimental design. Six treatments were tested with leaves alone a) without contamination, b) with SMZ in water at 5 µg L^{-1}, c) with SMX in water at 5 µg L^{-1}, or with leaves and periphyton d) without contamination, e) with SMZ in water at 5 µg L^{-1}, f) with SMX in water at 5 µg L^{-1}.

The water in the microcosms consisted in 12 L of dechlorinated tap water plus 3 L of natural stream water taken fresh from the study site in the Artière. The tap water was filtrated using a BRITA PURITY C Quell ST (Brita, Germany). Every week, the water was fully replaced in all the microcosms to ensure supply of nutrients and microbial species succession during leaves degradation. Antibiotics were reintroduced at their nominal concentration (5 µg L^{-1}) into each microcosm at the same time the water was replaced. Flow velocity was set at 6.6 L min^{-1} using NEWA Jet NJ1200 submersible pumps (Newa, Italy) and the temperature maintained at 20 °C, whereas the light regime followed the

natural light (1054 ± 122 Lux) dark (0 Lux) cycles. The experiment was conducted for two weeks with, in total, seven sampling days (d): d0, d1, d3, d7, d10, d14, and d16.

2.3. Physical and chemical characteristics of water and leaves

pH (Accumet AE150, Fisher Scientific, USA), water temperature (ProODO, YSI, USA) and light (Testo 545, Testo AG, Germany) were measured (Table S1) on each day of sampling. Dissolved oxygen concentration in the water was measured each hour between 9:00 a.m. to 6:00 p.m. in each of the 18 microcosms over four sampling days (d1, d7, d10, and d14) with a Pro ODO (YSI, USA) optical oximeter. The average of the dissolved oxygen measures between 12:00 p.m. and 4:00 p.m. (maximum irradiation, Young and Huryn 1999) in each microcosm was used to calculate the differences between L and L+P exposure treatments (Table 2).

Dissolved nutrient concentrations in the water were assessed on filtered (0.45 µm, cellulose acetate, Dutscher) samples, in order to remove the particulate fraction. Dissolved organic carbon (DOC) was determined by the combustion catalytic oxidation method and total dissolved nitrogen (TN) by chemiluminescence (TOC VCPN Analyzer, Shimadzu, Japan). Total dissolved phosphorus (TP) concentration was measured by the molybdenum-blue colorimetric method (Murphy & Riley, 1962) after basic digestion of water samples (Grasshoff *et al.*, 1983).

The $SUVA_{254}$ index was used to assess the quality of dissolved organic matter (DOM). This index is defined as the ratio between the absorption of a water sample at 254 nm and its corresponding concentration of dissolved organic carbon (Weishaar *et al.*, 2003). $SUVA_{254}$ is positively correlated with the aromaticity of dissolved organic matter. A high $SUVA_{254}$ may indicate the predominance of terrestrially-derived DOM that is commonly more recalcitrant than autochthonous-derived DOM (more labile). Measurements were performed on filtered water samples (0.45 µm) with a UV spectrophotometer (Ultrospec 2000, Pharmacia Biotech, United-Kingdom) and combined with the DOC results described above.

For antibiotic concentrations in water, samples (100 mL) were taken from microcosms thirty minutes before and after each water renewal. After adjusting the pH to 4 and adding an internal standard (50 µL of a 500 µg L^{-1} SMX-d_4 solution), samples were concentrated by a factor 200 on Oasis HLB-200mg cartridges (Waters™) according to the manufacturer's recommendations (elution solvent: methanol). The antibiotic concentrations were determined by LC/ESI-MS on a Thermo Scientific UHPLC Ultimate 3000 RSLC coupled with an Orbitrap Q-Exactive analyzer. The analyses were carried out in positive mode. The UHPLC was equipped with a Luna Omega Polar C18 column; 100 x 2.1 mm; 1.6 µm (Phenomenex) at 30 °C with acetonitrile gradient + 0.1% formic acid (Solvent A) and water + 0.1 % formic acid (Solvent B): 0–2.5 min: 30–64.5 % A (linear); 2.5– 2.6 min: 64.5-99 % A (linear); 2.6-5 min: 99 % A; 5–5.1 min: 99-30 % A; 5.1-8 min: 30 % A. Flow: 0.45 mL min^{-1}. For the mass spectrometer, gaseous N_2 was used as nebulizer gas (50 L h^{-1}). The spray voltage was 3.0 kV.

Nutrient content in leaves (including its associated decomposers community) was determined at each sampling time and treatment in triplicate. Three leaf disks were mixed with 1.3 mL of deionized water and placed in previously-rinsed (deionized water × 2) Lysing Matrix E tubes (MP Biomedicals, USA). Samples were then homogenized with a Precellys 24 tissue homogenizer (three times for 30s, Bertin Technologies, France). After centrifugation of the homogenate (30s at 17000 *g*), the supernatant was collected and separated into two different aliquots. The first aliquot (diluted 1:50) was used to determine total carbon and nitrogen content following the same method described above for water (TOC VCPN Analyzer, Shimadzu, Japan). Total phosphorus content was

assessed using the second aliquot (diluted 1:16) following the molybdenum-blue colorimetric method previously described. Eight blanks containing only deionized water were run to assess carbon, nitrogen and phosphorus contaminations in Lysing Matrix E tubes. Results were expressed as C:nutrient molar ratios.

2.4. Microbial biomass and diversity in leaves

Fungal biomass was estimated through ergosterol concentration determination. Extraction and determination of ergosterol concentration were performed on fourteen alder leaf-disks per sample following the protocol described by Artigas *et al.* (2012). Finally, ergosterol concentrations were converted into fungal carbon using conversion factors described by Gessner and Chauvet (1993) and Baldy *et al.* (1995). The ergosterol results were therefore expressed in mg fungal carbon per g leaf dry mass $(DM)^{-1}$.

Bacterial biomass was assessed through bacterial density analyses using flow cytometry (Borrel *et al.*, 2012). At each sampling time, one leaf disk per replicate was placed in sterile Ringer's solution and immediately fixed with paraformaldehyde (2% final concentration). Before cytometric counts, samples were incubated with sodium pyrophosphate (10 mM final concentration) and sonicated twice 30 s + 30 s to improve cell disaggregation (Artigas *et al.*, 2008). Later, the bacterial suspension was centrifuged for one minute at 800*g* in order to pellet large debris and the supernatant was diluted 100-fold in TE buffer (10 mM Tris, 1 mM EDTA). Finally, bacterial cells were stained with SYBR GREEN (1X, Molecular Probes, USA) and counted with a BD FACSCalibur (15 mW at 488 nm, Becton Dickinson, USA). Bacterial density results were converted into mg of bacterial carbon per g of DM using conversion factors described in Theil-Nielsen & Sondergaard (1998) and Bratbak & Dundas (1984).

Fungal and bacterial species' richness was analyzed by Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis. DNA was extracted from leaves at days 0, 1, 3, 7, and 14 using the FastDNA 2 SPIN Kit for soil (MP Biomedicals, Santa Ana, California, USA) following the manufacturer's instructions. Extracted DNA was amplified targeting the regions i) Internal Transcribed Spacer (ITS) of the fungal rRNA and ii) the V6-V8 regions of bacterial 16S rDNA according to the primers and conditions described in Crouzet *et al.* (2010). DGGE was run in a TIGEK 2401-220 device (CBS Scientific, San Diego, North Carolina, USA) in 1X TAE buffer for 16 h, at a temperature of 59 °C and a voltage of 90 V. Denaturing gradient urea conditions were 20-50% for fungi and 35-55% for bacteria. At the end of the electrophoresis, gels were stained in 1X TAE buffer containing 1/20000 dilution of Gel star (Lonza, Rockland, USA) and digitized using a BioSpectrum AC Imaging System (UVP, Upland, California, US). DGGE band (operational taxonomical unit, OTU) profiles were analyzed using Gelcompar II software (Applied Maths, Belgium) in order to obtain the fungal and bacterial OTU richness.

2.5. Microbial degrading-activity in leaves

At each sampling date, five leaf disks per experimental treatment were dried at 37 °C for 72h and weighed with an analytical balance with 0.1 mg sensitivity (Precisa 80A 200M, Precisa, Switzerland). The amount of mass loss over time in these disks was used to determine microbial decay rates in alder leaves.

A total of five different extracellular enzymatic activities were assessed in leaf-associated microbial communities using fluorogenic or chromogenic substrate analogs (Sigma-Aldrich, USA).β-glucosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91) and phosphatase (EC 3.1.3.1–2) enzymatic activities were measured using methylumbelliferyl (MUF) substrates (Sigma).Leucine-aminopeptidase

(EC 3.4.11.1) enzymatic activity was determined using L-leucine-4-methyl-7-coumarinylamide, a substrate labelled with aminomethyl coumarin (AMC). Finally, phenol oxidase (EC 1.10.3) enzymatic activity was assessed using 3,4-dihydroxy-L-phenylalanine (L-DOPA).

All enzymatic activity bioassays consisted in one leaf disk immerged in 1 mL of Ringer's media supplemented with the corresponding substrate analog concentration expected to saturate enzymatic activity (0.3 mM for β-glucosidase, phosphatase, leucine-aminopeptidase and 1.5 mM for cellobiohydrolase, phenol oxidase) (Artigas *et al.*, 2008 and 2012). Samples were incubated for one hour at 20 °C in the dark under agitation. After incubation, the enzymatic reaction was stopped by adding glycine buffer (pH 10.4) and substrate turnover was determined fluorometrically (360/460 nm excitation/emission) for MUF and AMC substrates with a Fluoroskan (Thermo Fisher Scientific, USA). For phenol oxidase, the activity was determined spectrophotometrically at 460 nm (Ultrospec 2000, Pharmacia Biotech, United-Kingdom). After enzymatic activity bioassays, disks were dried (37 °C for 72h) and weighed to correct enzymatic activities by the corresponding DM and further corrected by the microbial biomass (fungal + bacterial) accumulated in the disk.

Dose-response experiments on β-glucosidase activity in alder leaves were run afterwards in order to assess the interaction between the increasing concentrations of the two antibiotics (SMZ and SMX) and that of glucose in the media. We selected glucose to assess to which extent β-glucosidase activity rates decreased when increasing the concentration of an end product of cellobiose hydrolysis (Desphande and Eriksson, 1988) in the presence of SMZ or SMX. Incubations were performed in triplicate per each glucose and antibiotic concentration tested and run for 72h at the same temperature and light conditions described for the microcosm experiment. After 72h, β-glucosidase activity was determined and corrected by the corresponding bacterial cell density present in the leaf disk (as described above). Dose-response curves were plotted for each of the antibiotic concentrations tested and after expressing β-glucosidase activity values as the % response against the treatment without glucose.

2.6. Data analyses

Differences in water (dissolved oxygen and organic carbon concentrations, $SUVA_{254}$, C:N and C:P molar ratios) and leaf-litter (biomass of fungi and bacteria, C:N and C:P molar ratios) variables among experimental treatments were assessed by two-way repeated measures ANOVA. Between treatments, effects were tested for the following factors: incubation (I; including L and L+P levels), the antibiotic treatment (A; including CTR, SMZ, SMX levels) as well as their interaction (I × A). Within treatments, effects were tested for the factor Time and the interaction between Time and the factors described above (I, A, and I × A).

Microbial decay rates of alder leaves were determined fitting an exponential decay model on disk mass loss over the time of the experiment and using the following equation: $M_t = M_0 e^{-kt}$, where M_0 is the initial DM (g), M_t is the DM (g) at time t and k is the decay rate coefficient (d^{-1}) (Petersen & Cummins, 1974).

Accumulated enzymatic activity (AEA) was calculated for β-glucosidase and phenol oxidase activities (both responding to I and A factors) by means of areas under the time curve calculations in each microcosm using Origin 9.1 software (OriginLab, USA). To assess the efficiency of these enzymes in degrading alder leaves, turnover activities were calculated according to the method described by Mora-Gómez *et al.* (2016) with few modifications. Briefly, Turnover Activities (TA), i.e. TA = 1/k, where k is the slope of ln ash free dry mass (AFDM) remaining regressed to accumulated enzyme activities (AEA) (Simon *et al.*, 2009). However, leaf mass loss was not studied in terms of ash-free-dry-mass (AFDM) but in terms of dry mass (DM) in our experiment. Higher TA values would mean low

enzyme efficiencies for alder leave decomposition while lower TA indicates the opposite. TA was expressed as μmol of enzyme produced to decompose one gram of alder litter DM.

Integrated variables on microbial communities' degrading activity (leaf-litter decay rates, enzyme activity AUC or TA) were tested for differences among I, A, and I × A factors using two-way ANOVA. Post-hoc Tukey's test was used to determine differences among levels within the factor A (CTR, SMZ, SMX) as well as on dose-response curves between antibiotics and glucose.

All ANOVA tests were run on response variables having equality of variances and normality. The different statistical tests were run with SPSS Statistics Ver. 17.0 (IBM, USA).

3. RESULTS

3.1. Impact of periphyton on leaf-litter decomposers

Microcosms containing leaf-litter and periphyton had greater average dissolved oxygen concentrations in water than microcosms containing leaf-litter alone ($P < 0.005$, Table 2 and S2). The moderate increase in dissolved oxygen concentrations was accompanied by slightly higher dissolved organic carbon (DOC) concentrations and lower DOM aromaticity indices ($P < 0.05$ and $P < 0.005$ for DOC and $SUVA_{254}$, respectively; Tables 2 and S2) compared to L treatment, suggesting the presence of photosynthetic extracellular release in L+P treatment. Carbon: nutrient molar ratios in water (C:N and C:P) were in line with the enhanced C production by the periphyton since C:P and C:N were greater in L+P than in L (Table 2), although results were only statistically significant for the C:P ratio ($P < 0.05$ and $P = 0.098$, respectively; Table S2). Average water C:P ratio (days 1 to 16) was 383.9 ± 9.8 in L and 614.6 ± 54.5 in L+P. Water N:P molar ratio was also higher in L+P treatments (days 7, 14 and 16, Table 2) indicating greater availability of dissolved N over P molecules for leaf-litter decomposers.

The microbial decomposition of alder leaves was lower in L+P (0.042 ± 0.004 day^{-1}) compared to L (0.057 ± 0.005 day^{-1}) treatments (Figure 1). In average, the microbial decomposition of leaves was reduced by 25% in L+P compared to L treatments.

Table 2. Comparison of water characteristics (including dissolved oxygen (DO) and organic carbon concentrations, $SUVA_{254}$ index and C:N and C:P molar ratios) between aquariums with leaves alone (L) and in the presence of periphyton (L+P). $Oxygen_{12\text{-}16}$ values correspond to the average of hourly dissolved oxygen measures taken in L and L+P microcosms (including controls and contaminated microcosms) between 12h and 16h. Values are means (n=9, including CTR, SMZ and SMX treatments) and standard errors (*in italic*) in L and L+P aquariums at days 1, 7, 10 and 14 of the experiment. Asterisks represent significant differences between L and L+P (Tukey's test, $P < 0.05$). n.a. means not analyzed.

	Day 1		Day 3		Day 7		Day 10		Day 14		Day 16	
	L	L+P	L	L+P	L	L+P	L	L+P	L	L+P	L	L+P
DO ($mg\ L^{-1}$)	8.21 *0.01*	8.34 *0.02*	n.a.	n.a.	8.13 *0.01*	8.29* *0.01*	8.18 *0.01*	8.50* *0.05*	8.23 *0.01*	8.71* *0.08*	n.a.	n.a.
DOC ($mg\ L^{-1}$)	0.33 *0.06*	0.63 *0.15*	0.44 *0.10*	1.08* *0.13*	0.69 *0.06*	0.93* *0.12*	0.48 *0.09*	1.64* *0.43*	0.66 *0.06*	1.21* *0.16*	0.55 *0.03*	1.68* *1*
$SUVA_{254}$ ($L\ mg^{-1}\ m^{-1}$)	14.74 *2.57*	10.24 *1.85*	14.03 *4.67*	4.22* *0.74*	8.67 *1.43*	6.01* *0.64*	8.84 *2.25*	3.96* *0.62*	8.14 *1.10*	5.19* *0.54*	11.37 *1.90*	8.04* *1.91*
$C:N_{water}$	16.07 *0.14*	16.36 *0.92*	15.45 *0.17*	15.89 *0.64*	14.34 *0.13*	14.25 *0.49*	14.32 *0.70*	17.07 *1.67*	14.96 *0.11*	21.66 *3.25*	16.88 *0.16*	23.25* *3.87*
$C:P_{water}$	370.5 *11.4*	433.7 *61.1*	380.5 *15.1*	427.2 *45.9*	464.4 *26.8*	564.3* *42.6*	373.2 *30.2*	438.3 *29.2*	338.5 *12.9*	954.3* *222.7*	383.3 *26.5*	869.7* *156.7*
$N:P_{water}$	26.92 *0.88*	30.11 *2.09*	28.69 *0.96*	30.90 *2.21*	37.63 *1.86*	46.04* *3.01*	30.93 *2.68*	30.88 *2.20*	26.43 *1.09*	42.60* *5.05*	26.51 *1.86*	43.66* *5.12*

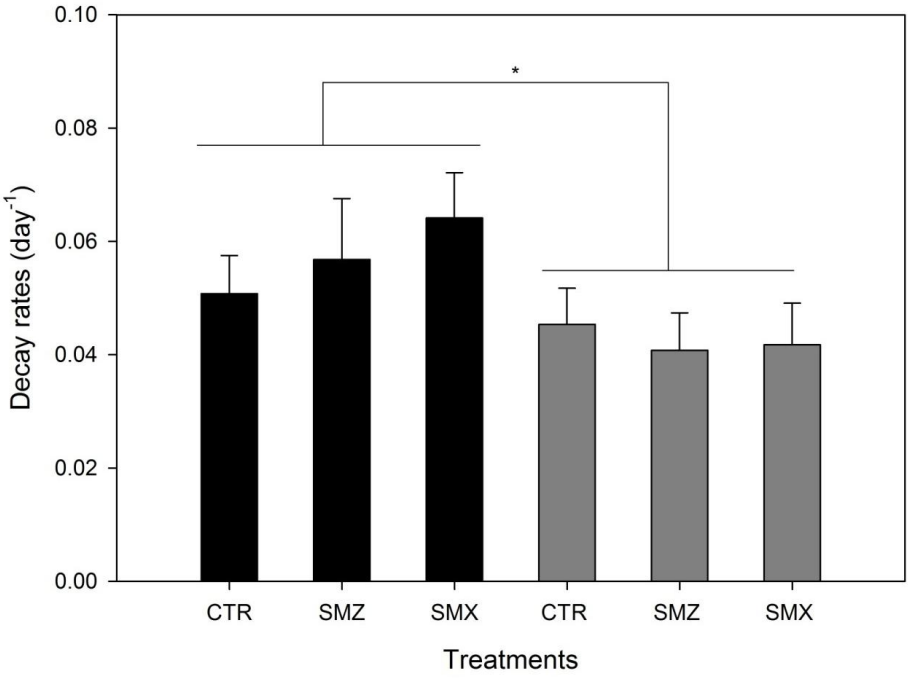


Figure 1. Decomposition rates of alder leaves during the microcosm experiment. Control (CTR), sulfamethazine (SMZ) and sulfamethoxazole (SMX) treatments for leaves exposed alone (black bars) or in the presence of periphyton (grey bars). Values correspond to means (n=3) and standard errors. Significant differences between tested treatments are represented by an asterisk (ANOVA test, $P < 0.05$).

The extra labile C available in L+P treatments was not allocated to the growth of leaf-litter decomposers. Fungal biomass accrual values in leaves were similar between L (29.1 ± 1.2 mg fungal C g DM^{-1}, average days 1 to 16 including control, SMZ and SMX treatments) and L+P (30.7 ± 1.2 mg fungal C g DM^{-1}) treatments ($P = 0.181$, Figures 2C and 2D) and that of bacteria were moderately higher in L (0.109 ± 0.011 mg bacterial C g DM^{-1}) than in L+P (0.084 ± 0.008 mg bacterial C g DM^{-1}) ($P < 0.05$, Figures 2A and 2B). Among the five different extracellular enzymatic activities measured (β-glucosidase, cellobiohydrolase, phenol oxidase, alkaline phosphatase and leucine-aminopeptidase), only the phenol oxidase activity calculated as the Area Under the time Curve (AUC) was influenced by the treatment (Table S2, Figure S1). Phenol oxidase was higher in L treatment (617 ± 68 mmolDIQC g microbial C^{-1}) than in L+P treatment (480 ± 30 mmolDIQC g microbial C^{-1}) evidencing faster breakdown of recalcitrant polymers per unit of microbial biomass in L, since phenol oxidase is responsible for the degradation of lignin polymers from leaves.

The average species richness of fungi (15.2 ± 0.6 OTUs) and bacteria (17.2 ± 0.5 OTUs) in leaves were relatively similar when considering together all treatments and times (Figure 3). However, the species richness of bacteria tended to be greater in L+P (16.0 ± 0.6 OTUs, average day 3 to 16) than in L (12.3 ± 0.4 OTUs) from day 3 and until the end of experiment. During the same time period, fungal species richness remained similar between L+P (17.5 ± 0.6 OTUs) and L (17.0 ± 0.6 OTUs) treatments.

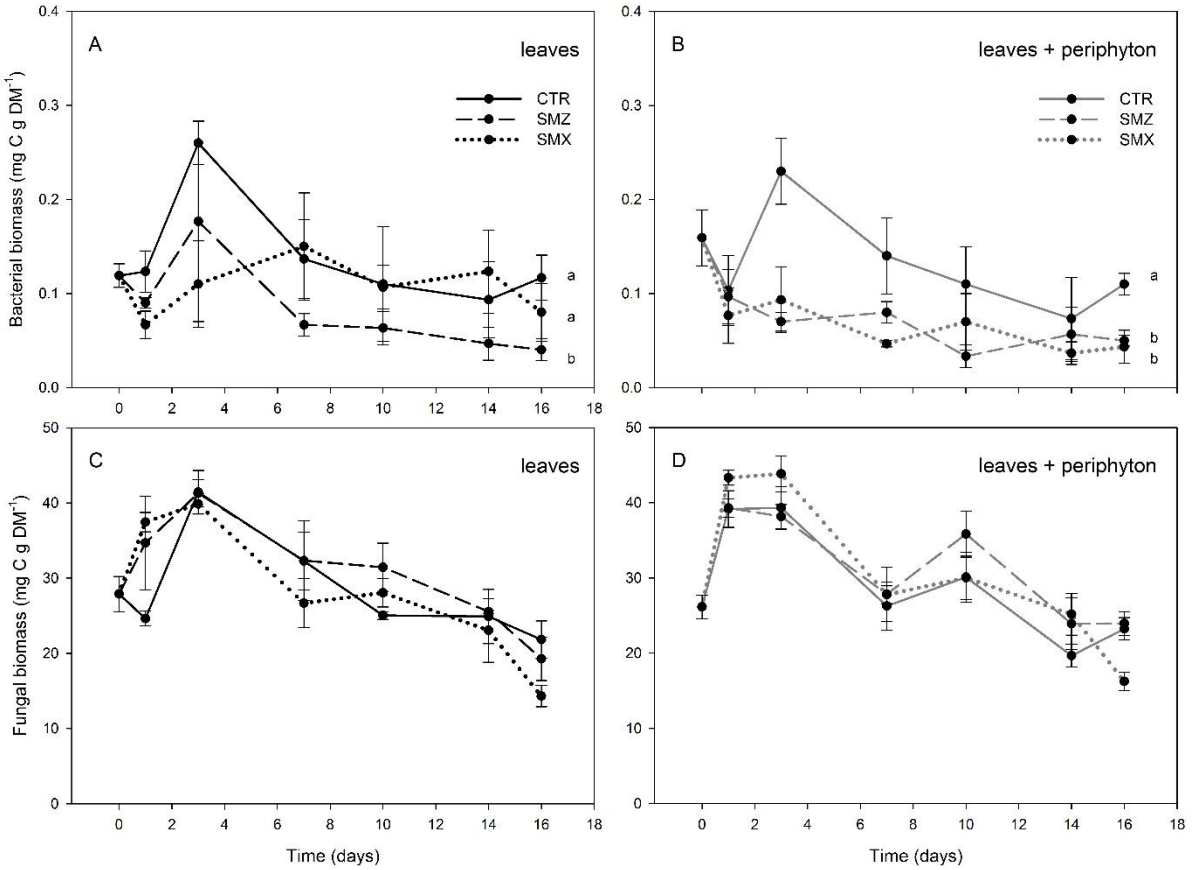


Figure 2. Bacterial (plots A, B) and fungal (plots C, D) biomass accrual in alder leaves during the 16-day experiment. The three experimental treatments (control, SMZ, SMX) are represented for leaves incubated alone (left plots) and in the presence of periphyton (right plots). Values are means (n=3) and standard errors for each sampling time, treatment and exposure condition. Letters indicate statistically significant differences between treatments including the whole sampling dates (a>b, Tukey's test, $P < 0.05$).

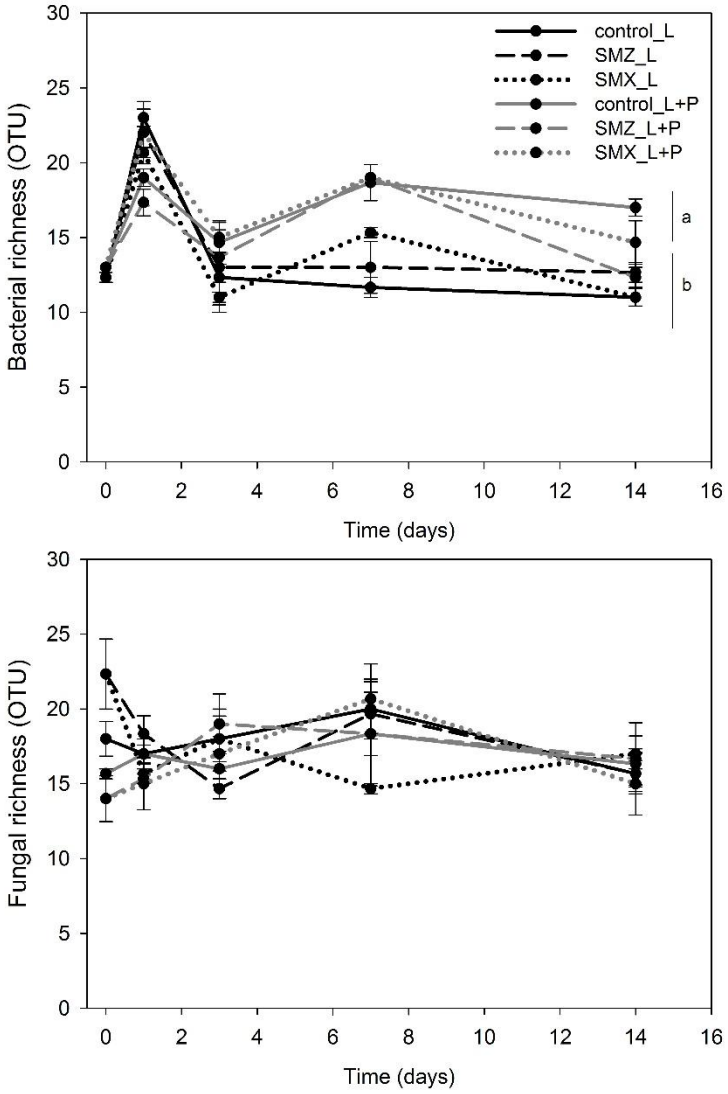


Figure 3. Band richness (as operational taxonomic unit, OTU) in leaf-associated bacterial and fungal communities during the experiment is represented. Controls, SMZ and SMX treatments are represented for L (black-colored line) and L+P (grey-colored line) treatments. Values are means (n=3) and standard errors per treatment and sampling time. Statistical differences between treatments are marked by letters (a > b, $P< 0.001$, repeated measures ANOVA test).

3.2. Impact of antibiotics on leaf-litter decomposers

Average SMZ and SMX concentrations in water from microcosms measured 30 minutes after weekly contamination at days 1 and 7 (5.1 ± 0.2 and 4.6 ± 0.2 μg L^{-1}, respectively) were relatively close to the expected nominal concentration of 5 μg L^{-1}. Chemical monitoring in water from microcosms revealed different dissipation rates between SMZ and SMX. Dissipation percentages were calculated during the second week of the experiment (between days 7 and 14) and resulted in almost double the percentage in SMZ (53.8 ± 6.4 %) compared to SMX (34.8 ± 8.0 %) treatment (Table S3). The greater dissipation of SMZ coincided with the detection of the metabolite, 2-amino-4,6-dimethylpyrimidine

(ADMP). The lower dissipation percentage of SMX was the consequence of the low dissipation values obtained in the treatment with leaves incubated in the presence of periphyton (15.44 ± 1.36 %) compared to those incubated alone (54.19 ± 3.22 %).

Alder leaf-litter decomposition rates were neither affected by the exposure to antibiotics (P = 0.798) nor by the interaction between antibiotics and the presence of periphyton (P = 0.562). Breakdown rates in L and L+P averaged 0.045 ± 0.004 day^{-1} in control and 0.049 ± 0.004 day^{-1} in antibiotic treatments (Figure 1). However, the antibiotics significantly reduced bacterial biomass accrual in leaves between 3 and 7 days of exposure and onwards compared to controls (P < 0.005, Figure 2A and B). While the effect of SMZ and SMX was similar in bacterial communities from L+P treatment (Figure 2B), their effects varied in communities from the L treatment (Figure 2A). In the L+P treatment, bacterial biomass accrual in leaves was 2.5 times lower in both SMZ and SMX treatments (0.058 ± 0.006 mg bacterial C g DM^{-1}) compared to the control (0.143 ± 0.02; average days 1 to 16, Figure 2B). In the L treatment, bacterial biomass accrual in leaves was reduced 2-fold by SMZ (0.079 ± 0.023 mg bacterial C g DM^{-1}, average days 1 to 16) whereas only a transitory reduction (2.5-fold) was seen between days 1 to 7 for SMX (0.066 ± 0.034) comparing to the control (0.150 ± 0.022, Figure 2A). Fungal biomass accumulation in alder leaves followed a similar pattern than that observed for bacterial biomass (Time P < 0.0001, Figures 2C and 2D), though they were neither impacted by antibiotics (P = 0.329) nor by the interaction between antibiotics and periphyton (P = 0.747, Table S2).

Among the five extracellular enzymatic activities measured in alder leaves (β-glucosidase, cellobiohydrolase, phenol oxidase, alkaline phosphatase and leucine-aminopeptidase), only β-glucosidase activity was affected by antibiotics (P < 0.005, Table S2, Figure 4). Activity expressed both over time (Figure S1) or integrated as the AUC (Figure 4) showed higher scores in the antibiotic treatments than in the controls (Tukey's test, P < 0.05, Figure 4). However, these higher activity scores did not suggest more efficient decomposition of alder leaves since turnover activity (TA = 1/k) calculations were higher on average in the antibiotic treatments (111.4 ± 18.8 mmol MUF gDM^{-1}) than in the controls (81.0 ± 11.2 mmol MUF gDM^{-1}).

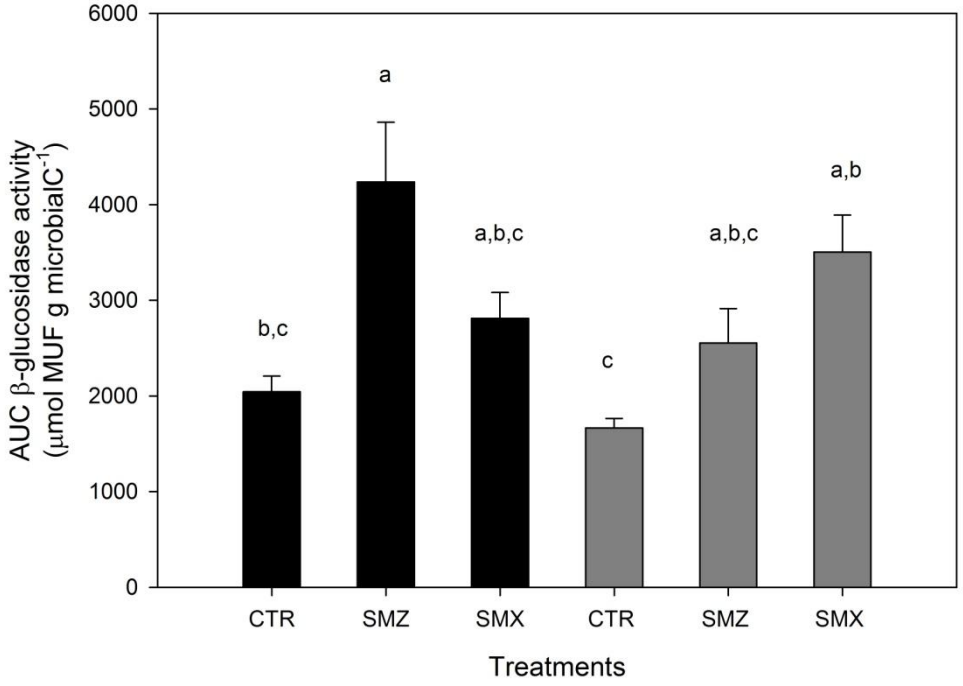


Figure 4. Area under the curve (AUC) of biomass-specific β-glucosidase activity in alder leaves incubated alone (black bars) or in the presence (grey bars) of periphyton subjected to control, SMZ and SMX treatments. Values are means and standard errors of triplicate AUC measures per experimental treatment. Differences between treatments are marked by letters (a>b>c, Tukey's test, $P < 0.05$).

3.3. Interaction between periphyton and antibiotics on leaf-litter decomposers

The only interaction between exposure to periphyton (I) and antibiotic (A) treatments was observed for β-glucosidase activity (I x A interaction, $P < 0.05$, Table S2). The highest activity values were recorded in the presence of SMZ in the L treatment and in the presence of SMX in the L+P treatment (Tukey's test, $P < 0.05$; Figures 4 and S1).

Dose-response curves for β-glucosidase revealed different activity responses to glucose concentrations depending on the concentration of SMZ ($P < 0.001$) and SMX ($P < 0.001$) tested. While increase in glucose concentration tends to repress progressively β-glucosidase activity at SMZ and SMX concentrations ranging from 0.05 to 50 μg L^{-1}, these relationships are the opposite for antibiotic concentrations ranging from 500 to 5000 μg L^{-1}. Dose-response results confirm that at antibiotic concentrations ≥ 500 μg L^{-1}, and despite the increase in glucose concentration in the media, leaf-litter decomposers continue to increase β-glucosidase activity. This response was more marked for SMX than for SMZ (Figure 5), which contrasted with the results obtained in Figure 4 where SMZ had a stronger effect on β-glucosidase than SMX.

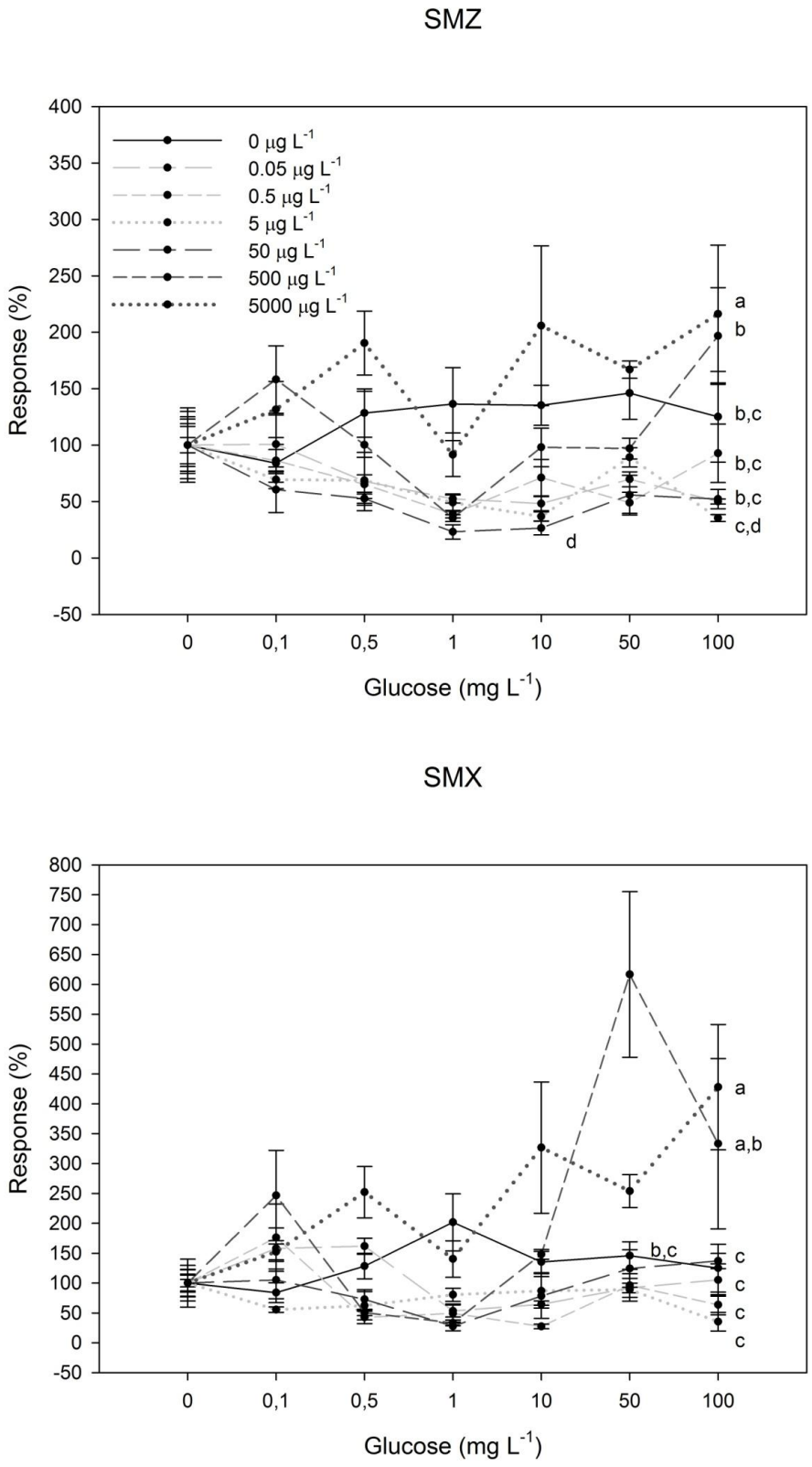


Figure 5. Dose-response curves for biomass-specific β-glucosidase activity depending on antibiotic concentration (from 0 to 5000 μg L^{-1} of SMZ and SMX) and glucose concentration (from 0 to 100 mg L^{-1}). Activity response after 72h incubation is expressed as the % response against treatment without glucose. Values correspond to means (n=3) and standard errors of the activity response variable. Differences between treatments are marked by letters (a>b>c>d, Tukey's test, $P < 0.05$).

4. DISCUSSION

4.1. Periphyton effect on leaf-litter decomposers

Photosynthetic extracellular release in our microcosm experiment was moderate (2.3 times more DOC in L+P than in L) and this can be explained by the high dissolved nutrient conditions (averaging 1.94 ± 0.03 mg N L^{-1} and 0.16 ± 0.01 mg P L^{-1} in this study for all treatments and sampling times) which are proved to repress labile carbon excretion from algae (Daufresne and Loreau, 2001; Danger *et al.*, 2013). Photosynthetic extracellular release is essentially favored by nutrient-limited algae subjected to stoichiometric constraints (Ziegler and Lyon, 2010). Algal-mediated priming intensities tend to be negative under low dissolved N:P and positive under high dissolved N:P conditions, exhibiting an apparent switchpoint between molar N:P of 16 and 64 (Halvorson *et al.*, 2019). Considering that N :P molar ratios in the water from L+P microcosms (including control, SMZ and SMX treatments) averaged 37.4 ± 1.7, conditions reached in our study were at the switchpoint between the positive and the negative algal-mediated priming.

The decomposition rates of alder leaves measured in this study (from 0.018 to 0.072 day^{-1}) were higher compared to those measured in field studies in temperate streams (0.0166, Gonçalves et al., 2006; 0.0169 +/- 0.0027, Ferreira *et al.*, 2012; 0.0193 +/- 0.0052, Bruder *et al.*, 2014). These differences in alder decay rates can be explained by the relatively high and constant temperature conditions setup in our experiment (19 ºC 24h/24h) comparing to those observed in the literature. These higher temperatures lead to an increase in microbial processing of alder leaves.

In the presence of periphyton (L+P treatment), the microbial decomposition of alder leaves was reduced by 25% compared to treatments without periphyton (L treatment), which contradicts our initial hypothesis of positive priming effect on leaf-litter decomposition in the presence of periphyton (Guenet *et al.*, 2010, Hotchkiss *et al.*, 2014, Danger *et al.*, 2013). Some authors describe this negative priming as leaf-litter decomposers exposed to high dissolved N and P likely to allocate labile algal C towards growth, respiration or reproduction instead of degradative enzymes and decomposition (Halvorson *et al.*, 2018, Catalán *et al.*, 2015). This is probably due to a preferential substrate use (Guenet *et al.*, 2010). However, decomposer responses in our study are contradictory to the negative priming observations made in the literature. For instance, Halvorson *et al.* (2018) observed that the presence of active periphyton increased bacterial abundance in leaves of *Liriodendron tulipifera* and *Quercus nigra* species by 141% and 733%, respectively. Moreover, active periphytic algae could potentially enrich the microbial biomass associated with leaves in N and P (Danger *et al.*, 2013, Halvorson *et al.*, 2016) thus modifying their corresponding C:N and C:P molar ratios. In our study, the periphyton neither stimulated bacterial abundance nor decreased C:nutrient molar ratios in leaves. An explanation for such inconsistencies is the duration of the experiments. Whereas the bacterial biomass and C:P ratio responses to the presence of algae were observed after 30 days and 42 days exposure in previous studies (Danger *et al.*, 2013; Halvorson *et al.*, 2018), the responses in our experiment appeared much earlier between 7 and 16 days of incubation. This faster response could be linked to the fast decaying character of alder leaves (N-rich species, Chauvet 1987), thought this cannot be confirmed since the same litter species were used to prove algal priming in the study of Danger *et al*. (2013). Another possible explanation is that litter decomposers might compete with algae for dissolved inorganic nitrogen in the L+P treatment. The N supply may be insufficient to support heterotrophic investment in N-costly degradative enzymes, thus limiting degradation of the litter (Jabiol *et al.*, 2018; Halvorson *et al.* 2019) and biomass growth. Finally, our study revealed an

increase in the extent of bacterial species richness in leaves accompanied by periphyton, suggesting an increase in the accommodation capacity of the community, whereas the presence of periphyton did not modify fungal species richness.

4.2. Antiobiotics effect on leaf-litter decomposers

Sulfonamides reduced bacterial biomass accrual in leaves but did not affect litter decomposition rates. It is recognized that sulfonamides (including SMZ and SMX molecules) exert a bacteriostatic effect characterized by the inhibition of folic acid synthesis, and therefore amino acid synthesis, in both Gram-negative and Gram-positive bacteria (Petri *et al.*, 2001). This bacteriostatic effect can explain the lower biomass accumulation in leaves subjected to SMZ and SMX. However, the effect of the two sulfonamides varied between L+P and L. The recovery of bacterial biomass in L exposed to SMX could be attributed to adaptation and resistance processes setup by microorganisms as observed in other studies (Thiele-Bruhn and Beck, 2005; Liu *et al.*, 2009). The greater dissipation of SMX in L (41.4 ± 4.8 %) compared to L+P (20.5 ± 13.8%) and the transitory increase in bacterial OTU richness in L compared to L+P (at day 7) could confirm the better adaptation of the former decomposer community to SMX. The dissipation percentages in our study were in the same range as those obtained in the study by Collado *et al.* (2013) at 50 μg SMX L^{-1} in a lab scale sequencing batch reactor bacterial community seeded with activated sludge. Concerning SMZ dissipation, Topp *et al.* (2013) reported the isolation of *Microbacterium* sp. C448, a bacterium able to mineralize the benzylic portion of SMZ and to excrete stoichiometric amounts of ADMP. The ability of the white-rot fungus *Trametes versicolor* to degrade SMZ has also been reported through the action of laccase-type enzymes (García-Galán *et al*., 2011). A similar SMZ-degradation mechanism occurring in the leaf-litter decomposer community cannot be ruled out. The differences observed in sulfonamides-dissipation percentages between treatments can be explained by a range of biotic and abiotic factors that would need to be investigated in further experiments focusing on sulfonamide biodegradation.

As expected, the mode of action of the two antibiotics tested did not alter fungal biomass accrual in leaves. Potential indirect effects of antibiotics enhancing fungal biomass accrual in leaves due to reduced competition with bacteria for organic matter use (*e. g*. Romani *et al.*, 2006) were hypothesized, but not observed in this experiment.

Sulfonamides increased β-glucosidase activity in leaves. β-glucosidase enzyme degrades cellobiose and other relatively small oligomers containing β-d-glucose linkages by splitting off the terminal β-d-glucose residue (Desphande and Eriksson, 1988). Then, the β-glucosidase increase was perhaps linked to the use of an external C-supply in the antibiotic treatments of both L and L+P. This external C-source can be linked to C-oligomers release from bacterial cells impaired by the antibiotics and later taken up by fungi. This enzymatic response resulting from fungal-bacterial interactions has also been suggested in other studies (Cycón *et al.*, 2006, Artigas *et al.*, 2012). However, this external C-supply was not noticeable on the C:N and C:P molar ratios of water or biofilms in antibiotic treatments compared to controls (Table S2). Furthermore, the increase in β-glucosidase could also be associated with a general stress response from the decomposer community to resist antibiotics. Although the molecular mechanism controlling this enzymatic response would deserve further research, one could hypothesize that β-glucosidase activity will increase the glucose available for the metabolism of microbial decomposers which is probably impaired by the presence of antibiotics.

4.3. Interaction between periphyton and antibiotics

The presence of periphyton did not alleviate the impact of antibiotics on leaf-litter decomposers (*i. e*. bacterial biomass) which contradicts our hypothesis. Only β-glucosidase activity in leaves was

affected by the interaction between periphyton and antibiotics. The highest activity values were recorded in the presence of SMZ in the L treatment and in the presence of SMX in the L+P treatment. These differences are probably explained by the different concentration of antibiotics and C-sources tested in both experiments. The in-depth study on β-glucosidase response to antibiotics and glucose (dose-response curves) revealed that sulfonamide concentrations ≥ 500 μg L^{-1} stimulated β-glucosidase even when increasing the concentration of the end-product (glucose) of β-glucosidase enzymatic reaction. One could expect an increase in bacterial biomass in leaves concomitant with the increase in glucose concentration in the media, thus leading to enhanced β-glucosidase activity values as observed in other studies (e.g. Ylla *et al.*, 2009). Unexpectedly, neither antibiotic nor glucose adjustment significantly modified bacterial biomass accrual in alder leaves during the 72h experiment (data not shown). After correction of β-glucosidase by the corresponding bacterial cell density present in the leaf disk, we can conclude that at high sulfonamide concentrations (≥ 500 μg L^{-1}), SMX produced greater stress than SMZ on the metabolism of litter-decomposer communities.

5. CONCLUSIONS

Photosynthetic extracellular release from the periphyton neither accelerated microbial decomposition of alder leaves nor buffered the impact of sulfonamides on the bacterial decomposer community, and this can be explained by the relatively high N:P ratio in stream water. Further experiments mimicking positive algal-mediated priming (low water N:P ratios) should be conducted in order to assess whether the effect of sulfonamides is alleviated on the bacterial decomposer community. The concentration of antibiotics tested in this experiment (5 μg L^{-1}) does not represent an ecotoxicological risk for the microbial litter decomposition process itself but consistently impaired the bacterial community which sustains higher trophic levels in the aquatic food web. Special attention should be paid to β-glucosidase activity response to sulfonamides and its potential impact in the stability of dissolved organic matter recycling in stream ecosystems.

6. ACKNOWLEDGMENTS

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Table S1. Average water temperature, pH and light measured in L and L+P microcosms at the different sampling times (n=108).

Temperature (°C)	**22.2** ± 0.66
pH	**8.14** ± 0.19
Light (Lux)	**1054** ± 121.9

1

Table S2. Statistical analyses performed on water and microbial descriptors measured during the microcosm experiment.

Two-way ANOVA

Descriptor	Factor	*F*-value	*P*-value
Decayrates	**I**	$5.3_{1, 12}$	**< 0.05**
	A	$0.2_{1, 12}$	0.798
	I x A	$0.6_{1, 12}$	0.562
β-glucosidaseactivity (AUC)	**I**	$2.4_{1, 12}$	0.146
	A	$10.7_{2, 12}$	**< 0.005**
	I x A	$5.5_{2, 12}$	**< 0.05**
Phenol oxidase activity (AUC)	**I**	$3.5_{1, 12}$	0.085
	A	$0.3_{2, 12}$	0.745
	Ix A	$1.9_{2,12}$	0.185
TA β-glucosidase	**I**	$1.3_{1, 12}$	0.269
	A	$1.9_{2, 12}$	0.197
	Ix A	$0.1_{2, 12}$	0.955

Repeated-measures ANOVA

Descriptor	Within-treatments effects			Between-treatmentseffects		
Water	**Factor**	***F*-value**	***P*-value**	**Factor**	***F*-value**	***P*-value**
Dissolvedoxygen	**T**	$6.5_{1, 13}$	**< 0.05**	**I**	$15.3_{1, 12}$	**< 0.005**
	T x I	$3.0_{1, 13}$	0.101	**A**	$0.4_{2, 12}$	0.692
	T x A	$0.6_{2, 13}$	0.558	**I x A**	$1.0_{2, 12}$	0.393
	T x I x A	$1.0_{2, 13}$	0.371			
DOC	**T**	$1.3_{1, 13}$	0.271	**I**	$5.2_{1, 12}$	**< 0.05**
	T x I	$1.0_{1, 13}$	0.346	**A**	$0.3_{2, 12}$	0.752
	T x A	$1.4_{2, 13}$	0.286	**I x A**	$1.3_{2, 12}$	0.577
	T x I x A	$1.3_{2, 13}$	0.307			
$SUVA_{254}$	**T**	$3.3_{2, 21}$	0.062	**I**	$21.1_{1, 12}$	**< 0.005**
	T x I	$1.1_{2,21}$	0.349	**A**	$2.9_{2, 12}$	0.095
	T x A	$1.5_{4, 21}$	0.243	**I x A**	$1.5_{2,12}$	0.253
	T x I x A	$1.8_{4, 21}$	0.162			
C:N water	**T**	$4.2_{2, 19}$	**< 0.05**	**I**	$3.2_{1, 12}$	0.098
	T x I	$2.3_{2, 19}$	0.135	**A**	$0.2_{2, 12}$	0.810
	T x A	$0.5_{3, 19}$	0.719	**I x A**	$0.3_{2, 12}$	0.759
	T x I x A	$0.8_{3, 19}$	0.494			
C:P water	**T**	$4.5_{1, 15}$	**< 0.05**	**I**	$6.5_{1, 12}$	**< 0.05**
	T x I	$5.5_{1, 15}$	**< 0.05**	**A**	$0.1_{2, 12}$	0.974
	T x A	$0.3_{3, 15}$	0.782	**I x A**	$0.1_{2, 12}$	0.941
	T x I x A	$0.4_{3, 15}$	0.754			
N:P water	**T**	$5.4_{3, 32}$	**< 0.005**	**I**	$13.4_{1, 11}$	**< 0.005**
	T x I	$3.5_{3,32}$	**< 0.05**	**A**	$0.2_{2, 11}$	0.857
	T x A	$0.8_{6, 32}$	0.541	**I x A**	$0.4_{2, 11}$	0.679
	T x I x A	$0.6_{6, 32}$	0.699			
Leaves						
Bacterialbiomass	**T**	$4.9_{3, 36}$	**< 0.01**	**I**	$4.8_{1, 12}$	**< 0.05**
	T x I	$0.4_{3, 36}$	0.779	**A**	$11.8_{2, 12}$	**< 0.005**
	T x A	$1.25_{6, 36}$	0.302	**I x A**	$0.8_{2.12}$	0.464
	T x I x A	$0.6_{6, 36}$	0.690			
Fungal biomass	**T**	$52.6_{3, 36}$	**< 0.001**	**I**	$2.0_{1, 12}$	0.181

	T x I	$3.9_{3, 36}$	**< 0.05**	**A**	$1.2_{2, 12}$	0.329
	T x A	$2.4_{6, 36}$	**< 0.05**	**I x A**	$0.3_{2.12}$	0.747
	T x I x A	$1.0_{6, 36}$	0.424			
C:N leaves	**T**	$0.9_{1, 15}$	0.371	**I**	$2.0_{1, 12}$	0.181
	T x I	$1.4_{1, 15}$	0.269	**A**	$0.8_{2,12}$	0.488
	T x A	$1.3_{2, 15}$	0.298	**I x A**	$1.4_{2, 12}$	0.284
	T x I x A	$1.4_{2, 15}$	0.279			
C :P leaves	**T**	$10.2_{3, 31}$	**< 0.001**	**I**	$0.2_{1, 12}$	0.664
	T x I	$2.7_{3, 31}$	0.072	**A**	$1.5_{2, 12}$	0.262
	T x A	$1.6_{5, 31}$	0.181	**I x A**	$2.5_{2, 12}$	0.125
	T x I x A	$1.8_{5, 31}$	0.140			

Table S3. Antibiotic concentrations (mean and standard errors) after 7 and 14 days of experiment.

Day	I	A	Concentration ($\mu g\ L^{-1}$)
7	L	SMZ	4.86 (0.38)
	L+P	SMZ	5.73 (0.08)
	L	SMX	5.91 (0.14)
	L+P	SMX	4.57 (0.04)
14	L	SMZ	2.20 (0.21)
	L+P	SMZ	2.65 (0.04)
	L	SMX	2.70 (0.09)
	L+P	SMX	3.86 (0.04)

Figure S1. Biomass-specific activity of β-glucosidase and phenol oxidase enzymes measured in L (left-side plots) and L+P (right-side plots) treatments subjected to controls without antibiotics (solid line), SMZ (dashed-line) and SMX (dotted line) treatments. Values are means (n=3) and standard errors per each treatment at each sampling time. Differences between experimental treatments are marked by letters (a > b, $P < 0.05$, Tukey's test)

