Science of the Total Environment 912 (2024) 168926

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Microbial community history and leaf species shape bottom-up effects in a freshwater shredding amphipod

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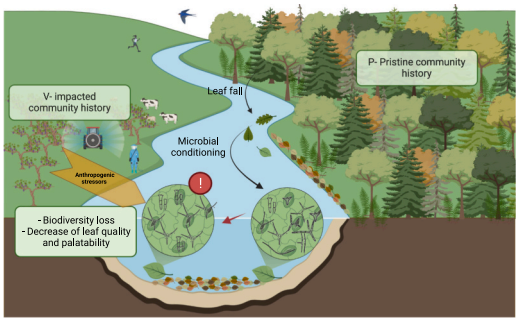
HIGHLIGHTS

- Leaf species and community history shaped the leaf-associated fungal community.
- *Gammarus*' growth, feeding and faeces production were affected by the leaf species.
- Leaf species had a more substantial impact on *Gammarus* relative to the microbial community.
- Sex-specific growth rate in response to the leaf species

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Henner Hollert

Keywords:
Leaf litter breakdown
Shredders
Aquatic fungi
Exposure history
Food quality
Fatty acids

ABSTRACT

Arable land use and the associated application of agrochemicals can affect local freshwater communities with consequences for the entire ecosystem. For instance, the structure and function of leaf-associated microbial communities can be affected by pesticides, such as fungicides. Additionally, the leaf species on which these microbial communities grow reflects another environmental filter for community structure. These factors and their interaction may jointly modify leaves' nutritional quality for higher trophic levels. To test this assumption, we studied the structure of leaf-associated microbial communities with distinct exposure histories (pristine [P] vs vineyard run off [V]) colonising two leaf species (black alder, European beech, and a mixture thereof). By offering these differently colonised leaves as food to males and females of the leaf-shredding amphipod *Gammarus fossarum* (Crustacea; Amphipoda) we assessed for potential bottom-up effects. The growth rate, feeding rate, faeces production and neutral lipid fatty acid profile of the amphipod served as response variable in a 2 × 3 × 2-factorial test design over 21d. A clear separation of community history (P vs V), leaf species and an interaction between the two factors was observed for the leaf-associated aquatic hyphomycete (i.e., fungal) community. Sensitive fungal species were reduced by up to 70 % in the V- compared to P-community. *Gammarus*' growth rate, feeding rate and faeces production were affected by the factor leaf species. Growth was negatively affected when *Gammarus* were fed with beech leaves only, whereas the impact of alder and the mixture of both leaf species was

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https://doi.org/10.1016/j.scitotenv.2023.168926
Received 1 August 2023; Received in revised form 21 November 2023; Accepted 25 November 2023
Available online 27 November 2023

sex-specific. Overall, this study highlights that leaf species identity had a more substantial impact on gammarids relative to the microbial community itself. Furthermore, the sex-specificity of the observed effects (excluding fatty acid profile, which was only measured for male) questions the procedure of earlier studies, that is using either only one sex or not being able to differentiate between males and females. However, these results need additional verification to support a reliable extrapolation.

1. Introduction

The decomposition of allochthonous organic carbon, such as terrestrial leaf litter, is a fundamental ecosystem-level process in streams with forest-dominated catchments (Nelson and Scott, 1962; Minshall, 1967; Fisher and Likens, 1973). After leaching of soluble organic substances, leaf litter is colonised by aquatic microorganisms, such as aquatic hyphomycetes (AH; a polyphyletic group of asexual fungi; Baschien et al., 2006; Ferreira et al., 2016) and bacteria (Gessner et al., 1999). These microorganisms decompose leaf litter by producing exoenzymes responsible for the transformation of complex leaf compounds into more usable and accessible transformation products (Hieber and Gessner, 2002). Moreover, the activity of bacteria and fungi increases the leaves' palatability and nutritional value for leaf-shredding invertebrates, also defined as conditioning. Thereby, microbial conditioning indirectly promotes leaf litter decomposition through the stimulation of shredders' feeding activity (Cummins, 1974; Bärlocher and Kendrick, 1975), which ultimately results in the production of fine particulate organic matter that is an essential resource for collectors and deposit-feeding organisms (Bundschuh and McKie, 2016). Driven by this crucial role in stream food webs, changes in leaf-associated microbial communities can have far-reaching ecological consequences (Gessner et al., 2010).

The structure of leaf-associated microbial communities is shaped by their surrounding environment, including chemicals of anthropogenic origin (Canhoto et al., 2016). A repeated or continuous exposure to anthropogenic chemicals favours the occurrence of tolerant species with consequences for the communities' functioning (Blanck, 2002; Feckler et al., 2018). Indeed, laboratory studies suggest that constant exposure to antimicrobial substances, such as fungicides, can affect leaf palatability (Fernández et al., 2015; Zubrod et al., 2015a) and leaf nutritional quality for shredders (Wallace et al., 2015; Zubrod et al., 2015b; Konschak et al., 2020). It remains, however, unclear whether agricultural field relevant exposure patterns, among others characterised by repeated fungicide exposures (Zubrod et al., 2019), can modify both the leaf-associated microbial community and the nutritional quality of leaves for shredders.

At the same time, the leaf species identity may function as an additional filter for microbial communities due to their unique recalcitrance and nutrient levels (e.g., Cornwell et al., 2008; Hladyz et al., 2009; Swan et al., 2009; Frainer et al., 2016; Grossman et al., 2020; Wang et al., 2020). In fact, most studies assessing impacts of chemicals on leaf-associated microbial communities have been performed with black alder (*Alnus glutinosa* (L.) GAERTN.) leaves, which are characterised by high nitrogen and phosphorous concentrations (Gulis, 2001) combined with a low degree of recalcitrance (Melillo et al., 1982; Malanson, 1993; Gulis, 2001). Consequently, this leaf species likely supports microbial growth and activity through a relatively easy access to nutrients (Gulis, 2001). It may therefore be questioned whether effects of chemicals observed using black alder are transferable to leaf species of a lower quality, characterised by low nutrient concentrations or a high degree of recalcitrance.

To address this knowledge gap, we assessed bottom-up effects on shredders by focusing on leaf-associated microbial communities from distinct streams, one pristine site (P) and one site characterised by repeated fungicide exposure in viticulture (V; Fernández et al., 2015), conditioning two leaf species and their mixture. As leaf species we selected black alder and European beech (*Fagus sylvatica* L.), representing a low and high degree of recalcitrance, respectively (Gulis, 2001; Artigas et al., 2012). Leaf-associated microbial communities were characterised by their exoenzyme activity as a functional endpoint, and AH species composition as well as fungal and bacterial biomasses using species- and group-specific quantitative real-time polymerase chain reaction (qPCR) assays, respectively. Subsequently, those conditioned leaves were offered as food to *Gammarus fossarum* (KOCH) over 21 days. Responses of male and female *Gammarus* were assessed by measuring their growth rate in terms of biomass increase, feeding rate and faeces production, as well as their energy reserves in the form of neutral lipid fatty acid (NLFA) profiles (was only assessed for male individuals). The use of both sexes is motivated by the deviating life history strategies and thus ecological roles in ecosystems (e.g., Pöckl and Humpesch, 1990). Nonetheless, a transferability of results between sexes has been assumed (Naylor et al., 1989; Malbouisson et al., 1995). We hypothesised that i) independent of the exposure history of the microbial community, low quality leaf species (i.e., beech) will be mostly conditioned by AH species that are conjectured as capable of degrading highly recalcitrant material (Baudy et al., 2021). Since published evidence (e.g., Feckler et al., 2018; Bundschuh et al., 2011b) suggests that those species are more tolerant to fungicides (due to the land use around their sampling site), the hypothesised pattern of microbial colonization should be especially pronounced for the pre-disturbed (V) community when compared to the pristine (P) community. At the same time, these more tolerant fungal species that are able to degrade highly recalcitrant material (e.g., Baudy et al., 2021), represent a less nutritional food for shedders (Arsuffi and Suberkropp, 1989; Graça et al., 2001), which will be reflected in a lower food intake, growth rate and altered NLFA profile in both *Gammarus*' sexes. On the other hand, ii) the higher nitrogen concentration and lower recalcitrance of alder leaves will enable AH species with a more limited ligninolytic enzymatic capability to colonise such leaves, compensating for potential differences in palatability of microbial communities from the P- relative to the V-community. Consequently, alder leaves should provide a comparatively high-quality food for *Gammarus* through higher fungal biomass and diversity. Moreover, iii) the mixture of leaf species increases AH diversity because of increasing habitat diversity (Gessner et al., 2010). At the same time, the anticipated lower food quality of beech leaves is compensated by a stimulated feeding on alder leaves, which is reflected by a higher *Gammarus* growth rate. Finally, it was hypothesised that iv) the responses of male and female gammarids to the different food qualities are comparable.

2. Material and methods

2.1. General study design

We used a 2 × 3 × 2-factorial design, where the first factor was the exposure history of the leaf-associated microbial communities sampled from streams dominated either by forest (mainly beech; pristine – P; P-community) or agricultural (vineyard run-off – V, without riparian vegetation; V-community) land use in their catchment, which is supported by earlier publications (Fernández et al., 2015; Schneeweiss et al., 2022). The second factor refers to the leaf species (i.e., alder and beech) and their mixture, colonised by two leaf-associated microbial communities serving as inoculum and the third factor to *Gammarus* sex. The leaf-associated microbial communities were characterised through group- or species-specific qPCR as well as their enzymatic activity. In addition, the conditioned leaf material served as food for *Gammarus*

(males and females) in a 21-day lasting feeding assay ($n = 40$; Fig. 1). The impacts on *Gammarus*' growth rate, absolute feeding rate, faeces production and NLFA profile were assessed.

2.2. Sources and procedures of leaf material and microbial communities

The study was initiated in March 2021 largely following published protocols (Zubrod et al., 2010). Briefly, stream water was collected from a pristine stream (P; Hainbach, Germany, 49° 14′ N, 8° 09′ E) dominated by forest and originated in a nature conservation area (Palatinate Forest Nature Park) and a stream in the agricultural landscape – namely viticulture – with a known history of fungicide exposure as documented elsewhere (V; Modenbach, Germany, 49°25′N, 8°11′E; see more detailed information on chemical characterisation in supplementary information, SI, A.1 Tables S1–S5; Fernández et al., 2015; Schneeweiss et al., 2022; Landesamt für Umwelt, 2016). The temperature of stream water at the time of sampling was between 8.0 and 8.8 °C. The leaves were collected at the time of leaf fall in autumn 2019 close to Landau, Germany (49° 11′ N 8° 7′ E) and stored at −20 °C until use. The conditioning was realised in separate 50-L stainless-steel channels, kept at 20 ± 1 °C in darkness under permanent aeration inducing water movement, for 14 days with a water exchange, freshly collected from the stream, after seven days. Each channel contained, 25 L stream water used to colonise 500 g of unconditioned alder or beech leaves as well as their mixture (250 g of each leaf species). This procedure resulted in six food sources (two inocula crossed with two leaf species and their mixture) provided to the test species *G. fossarum* (20 males and 20 females) as food source over 21 days (Fig. 1). The conditioning was repeated weekly, including stream water collection (i.e.,7d and 14d after the initial colonization), ensuring the provisioning of food with comparable quality over the entire study duration.

2.3. Long-term feeding assay

Coinciding with the first stream water sampling, *G. fossarum* were collected from the Hainbach. In the laboratory, *Gammarus* were passively size separated using sieves with decreasing mesh sizes (Franke, 1997). Adults passing a sieve with a mesh size of 2.0 mm but being retained by 1.3 mm were selected for this experiment. Specimens were subsequently separated by sex, identified by their position in pre-copula pairs (Pascoe et al., 1995; Fielding et al., 2003). *Gammarus* were kept in aerated test medium (SAM—5S; Borgmann, 1996) for 14 days and acclimatized to 20 ± 1 °C in darkness while being fed ad libitum with unconditioned black alder leaves, ensuring *Gammarus* had access to a good quality food source (Bloor, 2011).

During the feeding assay, *Gammarus* were offered six food sources as detailed in Section 2.2. Therefore, eight leaf discs (Ø = 16 mm) were cut from two conditioned leaves, to ensure comparable results on the leaf mixture treatment, including one leaf from each species, and allocated to one replicate, with 40 replicates (20 male plus 20 female gammarids) being prepared for each treatment (Fig. 1). Each replicate consisted of a 250-mL glass beaker and was equipped with a cylindrical mesh cage made from stainless-steel (mesh size: 0.5 mm) containing one *Gammarus* and four leaf discs (two from each leaf). A second, rectangular mesh cage contained the remaining four leaf discs controlling for microbial leaf mass loss. A watch glass separated these two cages preventing adhesion of *Gammarus'* faeces to the leaf discs in the rectangular cage (see Zubrod et al., 2015b; Fig. 1). Replicates were filled with 250 mL test medium (SAM—5S; Borgmann, 1996), which was automatically renewed twice a day. The flowrate was selected to not remobilise the faeces, which was identified during a preliminary experiment. Moreover, every seventh day, remaining leaf discs and faeces were retrieved and gammarids were translocated to a new beaker with fresh medium and fresh leaf discs. The remaining leaf discs from each cage were collected, dried at 60 °C for 24 h and weighed to the nearest 0.01 mg. The old medium was filtered through pre-weighed glass fibre filters (GF/6, Whatman, Dassel, Germany), dried and weighed as detailed above to determine faeces production. At the termination of the experiment (after 21 days), surviving *Gammarus* (mortality did not exceed 5 %) were shock frozen in liquid nitrogen and stored at −80 °C before being freeze-dried and weighed to the nearest 0.01 mg. Those organisms were used to determine growth rates and assess the NLFA profile of five randomly chosen male *Gammarus* per treatment (Section 2.5). The sole focus on male *Gammarus* is motivated by the endeavour to reduce intra-treatment variability (Pascoe et al., 1995; Fielding et al., 2003). Similarly, leaf discs (after 7 days in the test system with *Gammarus*) from the rectangular cage of five randomly chosen replicates were frozen at −20 °C for further analysis. Two of these leaf discs were used to assess microbial community composition (Section 2.4.1) and the remaining two leaf discs served the activity analyses of exoenzymes (Section 2.4.2). Replicates containing dead *Gammarus* (not exceeding 5 %) were excluded from any analyses.

2.4. Characterisation of the leaf-associated microbial communities

2.4.1. Quantitative real-time PCR

DNA was extracted using the FastDNA® Spin Kit for Soil in combination with the FastPrep™-24 5G Instrument (MP Biomedicals, Germany) generally according to the manufacturer's protocol. Fungal and bacterial DNA was quantified following Baudy et al. (2019) and Manerkar et al. (2008) using qPCR reactions. On the species level (10 common and co-occurring AH species; Zubrod et al., 2015a), the amount of DNA was measured as a proxy for fungal biomass based on species-specific TaqMan® qPCR reactions (Applied Biosystems, USA). On the group level, the amount of fungal and bacterial operon copies was measured as a proxy for overall fungal and bacterial biomass via SYBR® Green reactions slightly adapted (Manerkar et al., 2008). PCR reaction mixtures were prepared with 2.8 μL of distilled water, 0.1 μL of forward primer, 0.1 μL of reverse primer, 2 μL DNA extract, and 5 μL of master mix PowerUp™ SYBR® Green, (Applied biosystems). PCR reactions consisted of initial denaturation at 95 °C for 2 min, followed by denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 60 s for 40 cycles. Both types of qPCR reactions were performed on a Mastercycler® ep gradient S (Eppendorf, Germany) using 0.2-mL 8-tube strips covered with clear optical 8-cap strips (Sarstedt AG & Co. KG, Nümbrecht, Germany). More details on the assays and data analyses are provided in the Supplementary Information (A2; Table S6 and S7).

2.4.2. Exoenzyme activity

To quantify hydrolases' and oxidases' activities, we use the method described by DeForest (2009) but modified for leaf litter (see Baudy et al., 2020); detailed information on enzyme names, respective substrates, and targets is provided in the Supplementary Information A.2. Enzymatic activity was expressed as μmol of degraded substrate/mg leaf dry weight/h (DeForest, 2009). Further details on substrate concentrations, plate layout and calculations can be found in Baudy et al. (2020). Additionally, we used enzyme activities to calculate the recalcitrance ratio of the leaf material, after square root transformation to reduce the effect of dominant enzyme activities, as normalised oxidases per total hydrolases activity (Table 2). The higher the ratio oxidase/hydrolase activity, the greater is the relative investment for degradation of recalcitrant carbon (Romero-Olivares et al., 2017).

2.5. Characterisation of Gammarus' physiological fitness

2.5.1. Growth, feeding and egestion rate

The individuals' growth rate was determined by subtracting the average (±sd) dry weight of 20 male (4.89 ± 1.06 mg) plus 20 female (3.00 ± 1.07 mg) lyophilized gammarids collected at the start of the bioassay, from the *Gammarus*' dry weight (after lyophilization) at test termination considering their respective sex, divided by the duration of the experiment (μg biomass gain/d). Although our approach to estimate

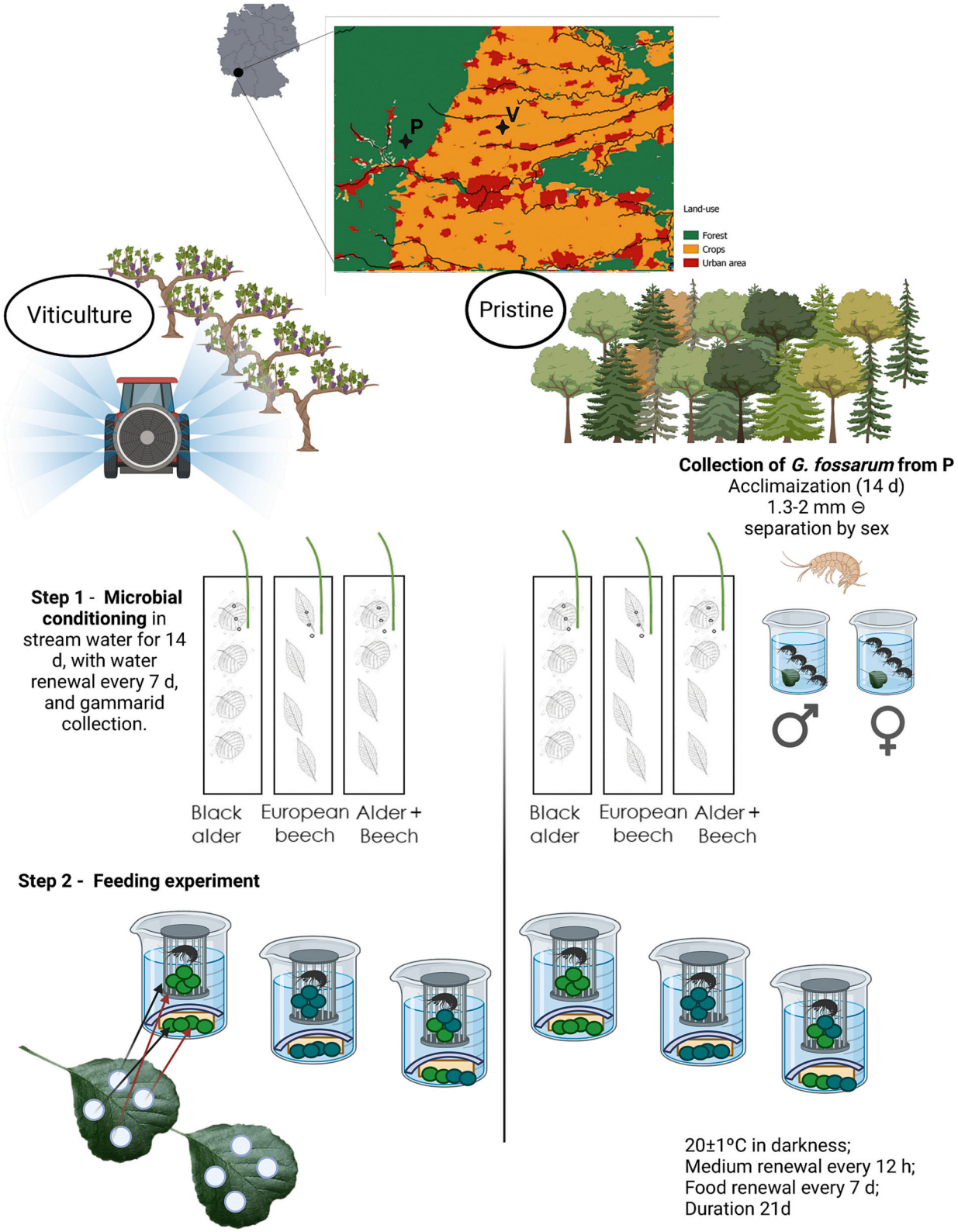

Fig. 1. Schematic overview of the study design. Step 1: Preparation for the feeding experiment: generating inocula and collecting test organisms – sampling stream water and *Gammarus fossarum* from a near-natural stream (pristine, P- community). Simultaneously, a stream surrounded by viticulture (V- community) was sampled. In the laboratory, the stream water was used to microbially colonise alder and beech leaves or a mixture of both in stainless steel channels under continuous aeration (green lines). Gammarids were separated by diameter and sex and kept in aerated medium, while fed with alder leaves ad libitum during acclimatization (14 d). Step 2: 21-d lasting feeding experiment with a 2 × 3-factorial design ($n = 40$). Per replicate 8 discs (Ø = 16 mm) were cut of leaves generated in step 1, here only exemplified for alder treatment. Four leaf discs of each leaf species combination were fed to each gammarid, and another four leaf discs were used to control for leaf mass loss (orange rectangle), separated by a watch glass (grey line).

growth might carry severe uncertainty, alternative approaches, such as the quantification of wet weight before and after the experiment substantially increases stress (unpublished studies point to a substantially higher mortality). The latter will potentially carry severer consequences for the data and conclusions that can be drawn thereof. The individuals collected at the start of the experiment were also used for NLFA profile analysis (see below) to which changes in NLFA profiles of all treatment groups have been related. The consumption of leaf material was calculated using the weight difference between the discs offered as food to the *Gammarus* in the cylindrical cage and those placed in the rectangular cage, divided by the final weight of the respective gammarid and time of the assay (i.e., 21 d; mg consumed leaf material/mg *Gammarus*/d; Zubrod et al., 2011). Faeces production was calculated by subtracting the initial filter dry weight from the final filter dry weight divided by the final weight of the respective gammarid and time between food renewals (mg faeces/mg *Gammarus*/d; Zubrod et al., 2011).

2.5.2. Fatty acid analyses

Five randomly chosen male gammarids from each treatment plus five male individuals collected at the start of the bioassay were lyophilized and weighed to the nearest 0.01 mg for TAG FAs (Triacylglyceride fatty acids i.e., NLFAs) profiling following Bligh and Dyer (1959) and Konschak et al. (2020). We deliberately chose to analyse NLFAs, rather than phospholipid FA, as they are an important energy storage in invertebrates (Azeez et al., 2014) and are more readily affected by changes in the organisms' diet (Iverson, 2012). *Gammarus* were homogenized in a chloroform:methanol:water mixture (1:2:0.8; v:v:v). Subsequently, a TAG with three deuterated 18:0 FAs (Tristearin-D105, Larodan, Solna, Sweden) was added as internal standard, followed by chloroform and water addition to reach a chloroform:methanol:water ratio of 2:2:1.8 (cf. Bligh and Dyer, 1959). The samples were stored overnight at 4 °C. TAGs were separated from glycolipids and phospholipids by solid phase extraction (Chromabond® easy polypropylene columns, Macherey-Nagel, Düren, Germany; conditioned with 4 mL chloroform) and elution with 4 mL chloroform. Afterwards, the solvent was evaporated at 40 °C under a constant stream of nitrogen in a dry heat incubator (VLM Metall- blockthermostate, VLM GmbH, Bielefeld, Germany). TAGs were subsequently solved in 100 μL of dichloromethane and NLFAs were transesterified to fatty acid methyl esters (FAME) using trimethylsulfonium hydroxide (Sigma-Aldrich, St. Louis, US-MO). FAME were analysed via gas chromatography with flame-ionization detection (GC-FID; Trace GC Ultra, Thermo Fisher Scientific, Bremen, Germany) using a Restek FAMEWAX column (30 m × 0.25 mm, 0.25 μm film thickness) and helium (1.4 mL/min) as carrier gas. FAMEs in each sample were determined using the retention times of FAME standards (37-component FAME Mix, Supelco CRM47885) and FAs were quantitatively analysed via external standard calibration (i.e., μg NLFA/mL). NLFA concentrations were corrected using extraction blanks and the recovery rate of the internal standard. The corrected NLFA concentrations were extrapolated to the total sample volume and normalized to *Gammarus*' dry weights (i.e., mg NLFA/g dry sample mass). The results are presented as difference relative to the subsamples of *Gammarus* collected at the start of the experiment.

2.6. Statistics and figures

Visual inspection, Shapiro–Wilk tests and Levene's tests were used to test for normality of the residuals and homoscedasticity of univariate data. When presumptions for parametric testing were met, two-factor or three-factor analyses of variance (ANOVA) were applied depending on the assessed variable (see Tables S8–S10). As the presumptions for parametric testing were violated for data on the number of bacterial operon copies, a two-factor Kruskal-Wallis test, followed by a Bonferroni correction, was used to assess the individual and combined effect of the microbial communities' history and leaf species. Please note that considering the criticism of null-hypothesis significance testing we base our interpretation on both statistical significance and effect sizes (i.e., the difference between treatments (Newman, 2009; Feckler et al., 2018)).

Multivariate data (AH species composition and NLFA profiles) were square root-transformed to reduce the effect of dominant AH species or FAs (Happel et al., 2017). Afterwards, permutational multivariate analyses of variance (PERMANOVA) on transformed data were performed to assess the individual and combined impact of the microbial communities' history and leaf species, applying Bray-Curtis dissimilarities as a distance measure between groups. The assumption of homogeneous within-group dispersion was tested using the "betadisper" function and was fulfilled for all groups. Furthermore, AH species composition was displayed for graphical interpretation via non-metric multidimensional scaling plots using Bray-Curtis dissimilarities (NMDS; Clarke, 1993). Statistics and figures were conducted with R version 4.2.1 for Windows (R Core Team, 2022) as well as the add-on packages "vegan", "ggplot2", "multcomp", "rstatix" and "ggh4x". The graphical abstract was created in Biorender.com. Note that the term "significant(ly)" refers to statistical significance ($p < 0.05$) throughout the study.

3. Results

3.1. Leaf-associated microbial communities

The number of fungal operon copies was lower (up to 40 %) on beech and the mixture of alder and beech compared to alder alone. Although statistically not significant, this impact was more pronounced for the V- relative to the P-community (Tables 1 and S8–S10). Bacterial operon copies were three-fold more abundant on leaves in the mixture conditioned by the P- compared to the V-community (Table 1), but the difference was not statistically significant (Table S8).

The AH community composition assessed through the quantification of DNA of 10 species, showed a difference between treatments. In fact, the factors community history (P vs V; $p = 0.004$), leaf species ($p = 0.001$) and an interaction between leaf species and community history ($p = 0.048$; Fig. 2, Table S10; S12; S13) had a statistically significant impact in the community composition. Some species, such as *Alatospora acuminata* and *Flagellospora curvula*, were present in all treatments but with ~70 % significantly lower abundance on beech leaves conditioned by the V- relative to the P-community was detected, these results suggest a shift in the relative contribution of individual species to the AH community (Tables S12–15).

A distinct pattern of the overall enzymes' activity was found for each of the treatments (Fig. 3) with only one enzyme (namely peroxidase) showing a significant interaction of microbial community history and leaf species ($p = 0.016$; Table S9). Higher ligninolytic activity was found in all treatments conditioned by the V- compared to the P-community. Additionally, beech-associated microbes showed a higher hydrolase activity. On the contrary, alder-associated microbes showed a higher enzyme activity targeting phosphate esters and lignin (see also Table S16; SI A.3). The recalcitrance ratio (Table 2) of alder and beech leaves conditioned by the P-community was about 30 % higher relative to their counterparts conditioned with the V-community. However, the opposite was observed in the mixture of alder and beech leaves, where the recalcitrance ratio of leaves conditioned by the P-community were 25 % lower relative to the V-community. Moreover, alder leaves had overall the highest recalcitrance ratio.

3.2. Gammarus' physiological fitness

Gammarus' growth rate was significantly impacted by the leaf species ($p = 0.001$, Table S9) and showed a significant interaction of leaf species and sex ($p = 0.005$; Table S9). Male gammarids grew faster when fed with alder compared to male gammarids fed with the mixture of alder and beech (up to 60 % depending on the inoculum) and beech leaves only (up to 115 % depending on the inoculum; Fig. 4a). In contrast, the

Table 1
Means (with 95 % confidence intervals; 10^8/mg leaf dw; $n = 3$) fungal and bacterial operon copies of microbial communities colonizing the leaves used as food for *G. fossarum* during the 21-d lasting feeding assay. P: pristine; V: vineyard run-off.

Organism group	Endpoint	Treatment					
		alder-P	alder-V	alder-beech-P	alder-beech-V	beech-P	beech-V
Fungi	Operon copies/mg leaf dw	4.66 ± 3.30	6.78 ± 6.73	5.33 ± 3.6	3.44 ± 3.47	3.76 ± 3.43	3.56 ± 3.2
Bacteria		0.51 ± 0.92	1.72 ± 2.03	1.67 ± 1.22	0.59 ± 0.59	0.71 ± 0.73	0.58 ± 0.56

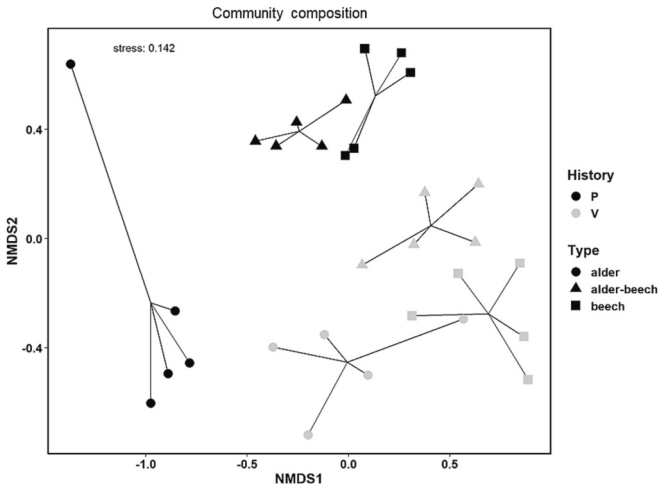

Fig. 2. Non-metric multidimensional scaling (NMDS) plot for leaf-associated aquatic hyphomycete communities. Leaf species are indicated by symbols (alder = circles, beech = squares, the mixture of both = triangles). Colours indicate the source of microbial inocula: pristine stream water (P) = black and vineyard run-off stream water (V) = grey. Spider webs connect the samples of each treatment at their respective group centroid. The stress value is provided as a measure of "goodness-of-fit" for NMDS, with a reasonable fit indicated when below 0.2 (Clarke,1993).

growth rate obtained for female gammarids was in extreme cases 21 times higher when fed with the mixture of alder and beech leaves compared to treatments in which only one of the leaf species was offered – a pattern independent of the inoculum (Fig. 4d). Additionally, a negative average growth rate was obtained for one of the treatments, with the magnitude of the effect in combination with the variation within the data set pointing towards a growth stagnation or a slight loss in weight (Fig. 4a & b). This observation may also be a consequence of a methodological artefact of the method chosen to calculate growth (see Section 2.5.1).

Moreover, the feeding rate of females was slightly (5–30 %) but consistently and significantly higher than that of males ($p = 0.048$; Table S9). *Gammarus'* feeding rate was significantly influenced by the leaf species ($p = 0.014$) and the interaction of community history and leaf species ($p = 0.004$; Table S9) suggesting a substrate-dependent role of the source of the microbial inoculum. Finally, the feeding rate showed a similar pattern among treatments for both sexes while the effect sizes were more pronounced for males (Fig. 4b).

While the feeding rate of female gammarids was higher than that of males, the reverse pattern was observed for the faeces production. Females produced with ~10–20 % significantly less faeces than males

Table 2
Investment in recalcitrant carbon degradation calculated as the ratio of oxidases divided by total hydrolases using square-root transformed data. The lower the ratio the higher the relative investment in recalcitrant carbon degradation (Romero-Olivares et al., 2017). P: pristine; V: vineyard.

Treatment	Total hydrolases	Oxidases	Ratio oxidases/hydrolases
alder-P	191.74	51.79	0.27
alder-V	231.89	45.56	0.20
alder-beech-P	138.92	18.42	0.13
alder-beech-V	310.54	54.86	0.18
beech-P	177.67	25.47	0.14
beech-V	134.07	13.44	0.10

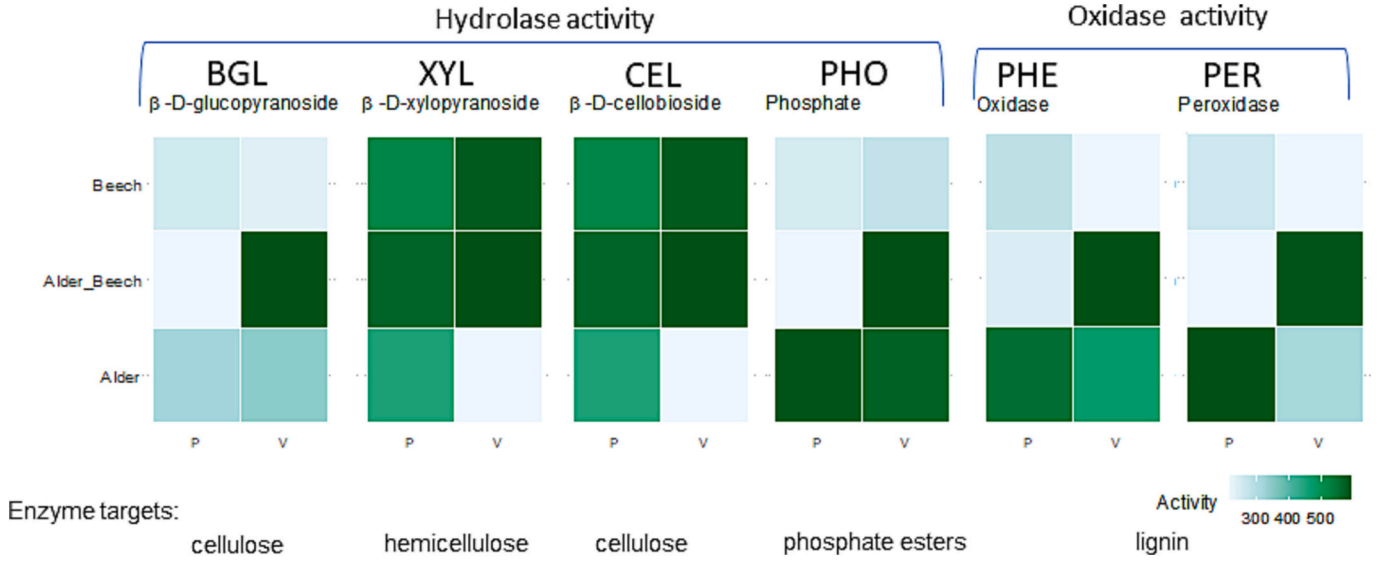

Fig. 3. Heatmaps displaying square root-transformed activities (μmol of degraded substrate/g leaf dry mass/h) of β-1,4-glucosidase (BGL; targeting cellulose), β-1,4-xylosidase (XYL; targeting hemicellulose), cellobiohydrolase (CEL; targeting cellulose), phosphatase (PHO; targeting phosphate esters), phenol oxidase (PHE; targeting lignin) and peroxidaseperoxidase (PER; targeting lignin). Leaf species are shown on the Y-axis, while the community histories are shown on the x-axis (P: pristine; V: vineyard run-off).

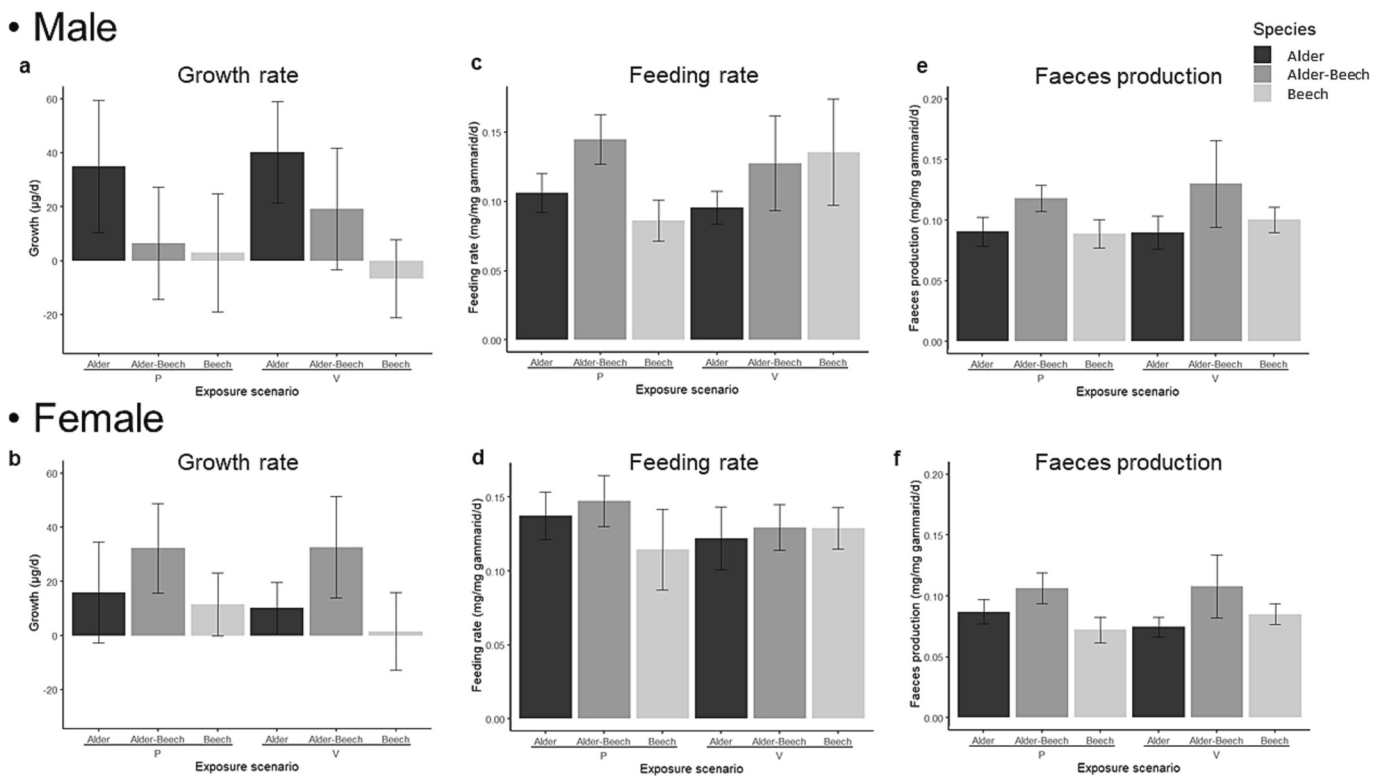

Fig. 4. Mean (± 95 % confidence intervals, $n = 20$) a), b) growth rate as µg biomass gain/day, c), d) feeding rate as mg leaf material/mg gammarid/day, e), f) faeces production as mg faeces/mg gammarid/day of male and female gammarids, respectively, consuming alder (black), beech (light grey) or their mixture (dark grey) colonised by microbes with distinct exposure histories: P pristine; V vineyard.

(Fig. 4c, f; $p = 0.008$; Table S9). Moreover, faeces production was – independent of sex and source of the microbial inoculum – higher when feeding on the mixture of both leaf species (Fig. 4c, f). This observation is supported by a significant effect of the factor leaf species ($p = 0.0001$, Table S9) and may be a consequence of a promoted feeding rate partially observed in those treatments (Fig. 4b, e).

As displayed in Table 3, no significant differences among treatments in the NLFA profiles of male gammarids were found (Table S10). This includes all NLFA groups (saturated FAs, SAFA; monounsaturated FAs, MUFA; polyunsaturated FAs, PUFA) and biologically important FAs and their precursors, such as eicosapentaenoic acid (EPA; C20:5n-3), alpha-linolenic acid (ALA; C18:3n-3), and linoleic acid (LIN; C18:2n-6). Although the overall changes in NLFA profiles among treatments are statistically non-significant, gammarids have partly up to 50 % lower levels of essential FAs and their precursors compared to the experiment initiation (see Table 3 for further details). While these changes suggest implications in the physiology of the organisms, the reliability of the observed trends needs further support by follow-up experiments.

4. Discussion

Gammarus' physiology was partially affected by the tested combinations of leaf species and leaf-associated microbial communities with differing exposure histories. Beech leaves alone resulted, for both sexes and independent of the microbial community, in lower growth rates compared to alder leaves, with effect sizes being more pronounced for the V- than for the P-community, which supports our first hypothesis. In support of our second hypothesis, alder (directly or indirectly) supports *Gammarus'* physiology more efficiently. Moreover, alder seems capable of compensating for the reduced presence of nutritional AH species in the beech-associated microbial community when offered together with beech (see hypothesis (iv)). Additionally, sex played a central role in the responses of *Gammarus* to the different treatments, which contradicts hypothesis (iv). Consequently, extrapolation of responses among sexes is not advisable. However, the partially high variability rendered some of the high effect sizes as statistically insignificant despite its potential biological relevance. Consequently, our strategy to base data interpretation on both statistical significance and effect sizes is further supported

Table 3
Percentage variation to the pre-experimental status of total, saturated (SAFA), monosaturated (MUFA) and polysaturated (PUFA) fatty acid content as well as linoleic acid (LIN; C18:2n-6), alpha-linolenic acid (ALA; C18:3n-3), and eicosapentaenoic acid (EPA; C20:5n-3), that represent FA with biological interest (expressed as %total FA content per mg dry weight) of male *G. fossarum* subjected to different treatments during the 21-d lasting feeding assay. Statistical analyses are displayed in Table 1. P pristine; V vineyard run-off.

% Variation to pre-expriment (%FA/mg gammarid dw)	NFLA	Treatment					
		alder- P	alder-V	alder-beech-P	alder-beech-V	beech-P	beech-V
	TOTAL	−26.97	−11.62	0.12	−14.51	−27.73	−36.74
	SAFA	−23.61	−23.38	−12.39	−25.16	−24.94	−33.21
	MUFA	−20.58	0.29	9.59	2.42	−14.42	−32.67
	PUFA	−33.88	−18.03	−10.52	−28.82	−32.11	−20.27
	C18:2	−29.57	−17.20	4.32	−13.32	−30.99	−37.61
	C18:3	−50.80	−30.36	−24.11	−49.46	−38.64	9.18
	C20:5	−43.72	−35.58	−4.37	−35.68	−39.35	−19.96

(Newman, 2008). Nonetheless, this strategy could introduce some uncertainty to our interpretation and discussion, which requires follow-up initiatives more specifically testing hypotheses that emerge based on the present study.

4.1. Leaf-associated microbial communities

The overall fungal and bacterial biomasses, approximated by operon copies, were statistically insignificantly different among treatments suggesting a limited capacity of these parameters to explain the responses of gammarids' feeding. Although fungi and bacteria's chemical signals are considered attractive to shredders (Large et al., 2005), the role of bacteria in their nutrition remains largely ignored. In contrast, literature suggests a preference of shredders for certain AH species (Arsuffi and Suberkropp, 1984). Indeed, in the present study the AH community composition varied significantly between P- and V-communities and among leaf species. The leaf associated microbial community, in particular AH community, is driving the palatability of leaf litter for shedders. However, no relation between shedders' preference and fungal biomass or enzymatic production could be established (Suberkropp et al., 1983). Instead, shedders' preferences for specific fungal species seems to be a function of the individual AH species traits, such as secondary metabolites (Arsuffi and Suberkropp, 1984), or mycelia's glyceride or FA content (Cargill et al., 1985; Arce Funck et al., 2015). Against this background, species considered more palatable (e.g., *A. acuminata, F. curvula*; (Suberkropp et al., 1983; Arsuffi and Suberkropp, 1989)) had equally high or higher biomasses on leaves conditioned by the P- relative to the V-community, independent on the leaf species. Those AH species are also assumed more nutritional (Rong et al., 1995; Arce Funck et al., 2015) to leaf-shredding organisms such as *Gammarus*. On the other hand, less nutritional AH species (such as *Tetracladium marchalianum or Tricladium angulatum*) were either absent or had a lower biomass on leaves conditioned by the P-community compared to leaves conditioned by the V-community. This pattern is in accordance with several studies (e.g., Bärlocher and Kendrick, 1973; Arsuffi and Suberkropp, 1989; Gonçalves et al., 2014), suggesting that more tolerant species, such as *T. marchalianum* (Maltby et al., 1995), ultimately dominate stressed AH communities (Solé et al., 2008; Bundschuh et al., 2011a). Furthermore, AH species patterns are less consistent among leaf species. *Neonectria lugdunensis* is either clearly dominating on alder conditioned by the P-community or is the second most abundant species when the V-community served as inoculum. This pattern is not confirmed for beech or the mixture of beech and alder. At the same time, *N. lugdunensis* is among the least preferred AH species for detritivores according to Arsuffi and Suberkropp (1989). Consequently, a generalizable pattern of AH community composition among substrates or the origin of the microbial inoculum is not abstractable, particularly as shedders' feeding preference for AH species is variable (e.g., Gonçalves et al., 2014). Moreover, we would like to highlight that laboratory conditions, which may include temperature differences relative to the field (Carl et al., 2022) and the presence of shredders' faeces (Díaz Villanueva et al., 2011), can impact microbial communities. By monitoring the succession of these communities over the study's duration, the magnitude of the effects could be quantified in future studies, further supporting a reasonable interpretation of the results presented here.

4.2. Responses of Gammarus to different food qualities

The fact that different leaf species presented different palatability should have had, according to our hypotheses, an impact on *Gammarus'* physiology. Based on *Gammarus'* growth, both sexes did not perform well when fed with beech only, a potential consequence of its higher recalcitrance and conditioning with less nutritional AH species, such as *N. lugdunensis*. Moreover, males and females showed different general growth patterns: despite the partially high variability within treatments, it may be abstracted that males and females grew faster when feeding on alder and the mixture of both leaf species, respectively, a pattern independent of the leaf-associated microbial community.

This observation of differing preferences may be explained by sex-specific requirements and life history strategies: although literature on this topic is scarce, studies have reported that male *Gammarus* live longer and have larger sizes than females with the aim to increase their competitiveness and support mate-guarding (Pöckl and Humpesch, 1990; Pöckl, 1992; Pöckl et al., 2003), suggesting that males strive for resources optimising their growth. Indeed, males grew faster when their feeding rate was the lowest (i.e., fed with alder) pointing to an efficient use of high-quality leaf litter additionally characterised by an AH community of presumably high nutritional quality. The introduction of beech into the leaf mixture decreases the food quality, as does the presumed nutritional quality of the AH community, leading to a higher feeding rate but lower growth of males. The latter indicates compensatory feeding, a mechanism by which organisms consume higher amounts of low-quality food to meet their nutritional requirements (Rasmussen et al., 2012; Feckler et al., 2015). Although FA profiles did not show significant changes in male gammarids exclusively feeding on beech, highly unsaturated (essential) FAs, such as ALA and EPA, were more strongly reduced compared to the test initiation. This observation was not confirmed when the mixture of both leaf species served as food. Even though data on female gammarids is lacking, this observation supports the assumption that alder may compensate for lower food quality of beech leaves.

The generally lower NLFAs' concentration compared to individuals from the start of the bioassay, points towards the fact that gammarids were fed with lower quality food in the lab compared to the situation in the field, where they are able to supplement their dietary needs with other sources (e.g., algae; Guo et al., 2016, 2018). Earlier studies have shown that laboratory conditions (e.g., changes in temperature, flux, or nutrient availability as for example derived from the amphipod faeces) can change the microbial community compared to field conditions (Carl et al., 2022). These changes in physical and chemical conditions potentially select more tolerant species, with potential implications in food quality as explained in the previous section. These more tolerant fungal species are often less palatable to *Gammarus*, potentially interfering with their feeding and physiology. This calls for further efforts to quantify the impact of such confounding factors, for example through the monitoring of the succession within the microbial community over the study duration. Moreover, the experiment was initiated in March and thus prior to the usual first fungicide application of the growing season. This fact points to the possibility for recolonization of AH from less or even uncontaminated upstream sections influencing the V-community of our study as documented for invertebrates (Orlinskiy et al., 2015). At test initiation we assumed, however, a change in AH communities when sampled from streams in vineyards (i.e., V-community) due to repeated fungicide exposure over the last years or even decades. Consequently, and contrary to our assumption, the impact of fungicide exposure in AH communities may be assumed to be buffered by recolonization over the winter season. Re-running the experiment during or shortly after the main fungicide application period may be recommended to capture a field relevant worst-case scenario.

In contrast to males, females increase their size to enhance fecundity and carry eggs (Pöckl, 1990, 1992), with the latter also affecting their mobility and thus ability to exploit food resources (Lewis and Loch-Mally, 2010). We, consequently, assume females will constantly feed on any leaf species available to survive and wait for better conditions supporting growth, moulting and brood development. Bakkar et al. (2017) supports our assumptions, demonstrating that male and female sesarmid crabs produced faeces with a different chemical signature when feeding on mangrove leaves, suggesting a sex-specific digestive process. Moreover, due to competitive behaviour (e.g., cannibalism as food preference over sex, Ward, 1983; Dick et al., 1990; Ward and Porter, 1993; Dick, 1995; Ironside et al., 2019) and size advantage of males over females, the latter may have evolved to use a mixed quality of

food, which is reflected by the efficient use of recalcitrant leaves in the present study. While this assumption needs further verification also in the field, it points to the fact that an extrapolation – also at the physiological level – from males to females (commonly used in previous studies due to reduced intra-treatment variability; Pascoe et al., 1995; Fielding et al., 2003) is not straightforward and needs particular attention because of their relevance for population development.

Overall, the present study suggests that the leaf species identity, and thus the substrate on which the microbial communities grow, has a larger impact on the physiology of the next trophic level (i.e., the shredders) than the microbial community as such. As this observation is based on a fairly limited number of community history replicates (i.e., one P-community and one V-community), its general applicability needs further scrutiny.

5. Conclusions and future perspectives

The interaction of leaf species and community history shaped the leaf-associated AH community composition. This stirs up a sex-specific change of gammarids' fitness as shown by differences in their growth. Particularly the sex-specific response to the different substrates questions the procedure of earlier studies using either only one sex or not being able to differentiate sex. Consequently, sex-specific responses are not yet properly considered. Moreover, the lack of a clear pattern in energy reserves on males (here the NLFA profile) calls not only for expanding replication but also the use of both sexes in physiological assessment, which is supported by the sex-specific growth pattern in response to the food sources. Thereby, a more comprehensive pattern on potential bottom-up related effects in the wider food web can be developed.

CRediT authorship contribution statement

Sara Gonçalves: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Annika Pollitt:** Investigation, Writing – review & editing. **Sebastian Pietz:** Investigation, Methodology, Writing – review & editing. **Alexander Feckler:** Investigation, Methodology, Writing – review & editing. **Mirco Bundschuh:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors do not see a conflict of interest with the publication of this paper.

Data availability

Data are available from the authors upon reasonable request.

Acknowledgments

We thank V. Gerstle, S. Kolbenschlag, S. Oster and T. Schmitt for the technical support. The project was funded through the German Research Foundation (DFG; project number 411020793).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.168926.

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