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Effects of Drying and Re-Wetting on Litter Decomposition and Nutrient Recycling: A Manipulative Experiment

Beatrice Palmia [1,*], Marco Bartoli [1,2], Alex Laini [1], Rossano Bolpagni [1,3], Claudio Ferrari [1] and Pierluigi Viaroli [1]

1 Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, 43124 Parma, Italy; marco.bartoli@unipr.it (M.B.); alex.laini@unipr.it (A.L.); bolpagni.r@cnr.irea.it (R.B.); claudio.ferrari1@studenti.unipr.it (C.F.); pierluigi.viaroli@unipr.it (P.V.)
2 Marine Research Institute, University of Klaipeda, 92294 Klaipeda, Lithuania
3 Institute for Electromagnetic Sensing of the Environment, National Research Council, 20133 Milan, Italy
* Correspondence: beatrice.palmia@gmail.com

Received: 4 February 2019; Accepted: 3 April 2019; Published: 5 April 2019

Abstract: Climate change and water abstraction may change stream flow from perennial into intermittent lotic systems, modifying their abiotic and biotic benthic environment and impacting ecosystem processes such as nutrient turnover. We conducted a microcosm experiment to investigate the interactive effect of water intermittency, macrofauna and leaf size (*Populus nigra* leaves) on nutrient mineralization and recycling. Leaf disks (1 or 5 cm diameter) were incubated for 40 days with or without the leaf-consumer, *Potamophylax cingulatus* larvae (Trichoptera, Limnephilidae) and with or without an intervening, 10-days simulation of stream drying and subsequent rewetting. Nutrient fluxes, residual leaf biomass and leaf elemental composition were measured to evaluate how intermittency, macrofauna and leaf size affect organic matter mineralization rates and stoichiometry. Results suggest that drying slows decomposition rates, impacting both the microbial and setting to zero macrofauna activities. The presence of macrofauna increases mineralization and nutrient (C, N and P) regeneration rates. Our findings also suggest that leaf disks with higher diameter display higher microbial activity and NH_4^+ regeneration. During the experiment, the C:N:P ratios of residual litter changed, as the leaf material became enriched with N and P. Our study suggests that increasingly frequent dry events might slow mineralization rates and downstream nutrient transport.

Keywords: hydrologic intermittency; macrofauna; leaf size; mineralization; elemental composition; nutrient fluxes

1. Introduction

In many areas of the world, climate change and water abstraction exceeding water availability are expected to change the timing, extent and frequency of drying that will alter the hydrology of many freshwater environments [1]. A rapid decline in flow, with increasing intensity of dry episodes, has been already observed along many rivers [2] and permanent streams are becoming or expected to become intermittent. Shifts from perennial to intermittent flow regimes modify the abiotic characteristics of benthic systems, plant, animal and microbial communities, nutrient concentrations and availability [3,4], food webs [5] and ecological processes [6], resulting in changes of benthic ecosystem functioning.

Headwater stream ecosystems, especially in forested regions, strongly depend on leaf litter inputs as sources of nutrients and energy for downstream sectors [7]. In these systems, upstream organic matter processing is a key ecosystem process [8]. In low order creeks, the efficiency of organic matter breakdown, microbial use, and biogeochemical transformations are highly dependent on

hydrology [9]. Drying profoundly affects leaf-litter decomposition, alters breakdown rates and affects benthic communities and processes [10,11].

Leaf material breakdown has been shown to be much slower in dry than under aquatic conditions, primarily because the absence of water limits the activity of decomposers [10,12]. During dry periods, the organic matter accumulated on riverbed depression or low-energy pools undergoes slow decomposition as the ability of microbes to colonize and initiate the process of litter breakdown is limited by water availability [13].

Another critical aspect of drying concerns the marked changes in community structure and activity of microbial decomposers and invertebrate detritivores. Dry events usually reduce the functional and taxonomic richness of invertebrate communities [14]. This reduction can include the loss of macroinvertebrate shredders, which through their feeding activity, play a substantial role in leaf breakdown and in nutrient cycling within and between ecosystem compartments [15]. Nutrient cycling by shredders may be especially important for the microbial-detritus compartment, stimulating microbial growth on the leaves and organic matter decomposition [16] and can strongly regulate productivity within ecosystems.

Organic matter breakdown may also be influenced by leaf size [17,18]. Leaf size and geometry (e.g., flat, large surfaces) may facilitate the conditioning action that macrofauna produces on leaves and result in higher colonization by a biofilm of bacteria, fungi and algae [17,19]. During skeletonization of large leaves, the lignin to cellulose ratio may increase significantly and influence the residual organic matter breakdown [19]. On the other hand, litter fragmentation produces smaller and smaller debris with C:N ratios significantly lower as compared to those of the original detritus [20]. Increasing nutrient content in smaller debris has been attributed to microbial bioaccumulation due to higher surface-area to volume ratios and thus a greater capacity for microbial nutrient immobilization and adsorption [21]. Microorganisms condition the litter through nutritional enrichment, thereby increasing litter quality and, consequently, the rate of decomposition by invertebrate feeding [16].

Recently, the number of ecological studies on intermittent streams has grown exponentially [22], in part, because of climate-change predictions of stream drying. Several authors have studied leaf breakdown in streams that seasonally dry compared to permanent streams [23], along a natural gradient in immersion and emersion [10], during and after drying [24], in different habitat resulting from flow fragmentation in temporary stream [25] and in stream mesocosms with flow reduction compared to natural flow regime [26]. However, these studies mainly used litter bags method or were focused on consequences to biotic community structure rather than to associated ecosystem processes. Studies based on microcosms generally tested the effects of drying on microbial processes, excluding interactions with other environmental or biological factors [27,28]. Furthermore, while most studies about decomposition in intermittent conditions have been performed in arid or Mediterranean regions [25,29,30], studies on streams in temperate regions, where intermittency is a relatively new phenomenon, are scarce [31,32].

We performed a laboratory experiment using microcosms to test the combined effect of water intermittency, macroinvertebrates shredders and leaf size on organic matter mineralization, nutrient recycling and their ecological stoichiometry. Although these physical, chemical and biological factors are likely to be interacting, they are often not studied in concert. Moreover, we nested experimental approaches in the laboratory to produce a deeper understanding of how processes are influenced by interacting factors. In particular, we nested short-term dark incubations to measure respiration and nutrient regeneration rates of leaf litter within longer-term incubations that measured biomass loss and changes in elemental stoichiometry.

In the context of future climate change and given the importance of both biota and hydrology to biogeochemical cycling in ecosystems, a greater understanding of the effects of hydrological intermittency on the functioning of benthic systems is needed. In this direction, the aim of this work is to better understand how organic matter processing would be affected by the presence of water and to

evaluate the role of benthic communities on leaves decomposition and nutrient recycling in relation to hydrological intermittency.

We hypothesized that drying interrupts biological processes, including microbial and macrofaunal and results in lower leaf breakdown rates. We also hypothesized that shredders hasten organic matter mineralization, microbial activity, and nutrient recycling through direct (e.g., excretion) and indirect ways (litter conditioning). As leaf size may affect breakdown rates we added this factor in our experimental design. We expected faster breakdown for smaller leaves due to higher colonization by microbes and fungi and higher consumption by shredders. The three interplaying factors drying, macrofauna and leaf size likely produce element-specific effects on organic carbon (C), total nitrogen (N) and total phosphorus (P), resulting in different stoichiometry along the decomposition process.

2. Materials and Methods

2.1. Experimental Set-Up

All the material used in this experiment, including water, mineral substrate, macrofauna and leaves, was collected from the Parma stream, an intermittent third-order mountainous stream (44°27′53.3′′ N 10°02′53.9′′ E), located in the Tuscan-Emilian Apennines (Italy). We incubated leaf disks in the presence and absence of larval shredders and under different hydrological regimes (permanent vs intermittent). For each condition, we used leaf disks of different diameter (1 and 5 cm). All microcosms contained 7 ± 0.5 g of sterilized and ignited sand and sifted with a 1-mm mesh size sieve.

The experimental set-up consisted in forty-five PVC microcosms provided with two different lids (Figure 1). We tested nine conditions, each with five replicates: C = Control; L1 = Leaves 1 cm; L1_D = Leaves 1 cm + Drying simulation; L1_M = Leaves 1 cm + Macrofauna; L1_M_D = Leaves 1 cm + Macrofauna + Drying simulation; L5 = Leaves 5 cm; L5_D = Leaves 5 cm + Drying simulation; L5_M = Leaves 5 cm + Macrofauna; L5_M_D = Leaves 5 cm + Macrofauna + Drying simulation.

Microcosms were maintained for forty days in a 200 L incubation tank containing filtered (20 µm plankton net) stream water that was mixed and aerated continuously by aquarium pumps and aerators. Nearly 20% of the tank water was replaced every three days with fresh water from the stream to avoid significant changes in water chemistry. During most of this incubation period, all microcosms were provided with a lid made of a plastic net (mesh size 1-mm) allowing water exchange but not that of macrofauna. The tank was placed outdoor to reproduce light and temperature daily variations and water physical and chemical characteristics were monitored over time to ensure stream water homogeneous conditions.

Macrofauna and leaves were added on 28 April 2018. On 2 May (day 5), 7 May (day 10), and 14 May (day 17) (before drying simulation), and on 30 May (day 33), and 6 June (day 40) (after drying simulation) all microcosms underwent a short (six hours) dark incubation (see detailed methods in next section). The drying simulation was started 22 days after the beginning of the experiment (19 May) (Figure 1). Drying was simulated removing with a syringe most of the water from each microcosm to simulate stream contraction phase, which characterizes a typical intermittent hydrological cycle, and which precedes a complete stream drying. Rapid water removal reflected what happens in permeable riverbeds, where water drains, does not accumulate and does not evaporate slowly. Where present, macrofauna was carefully removed using tweezers and counted whereas leaves, debris (including particulate matter and mineral substrate) were not removed. The removal of macrofauna was decided based on the specific traits of the chosen organism (see the discussion). Microcosms with all their content were maintained outdoor, outside the incubation tank, protected by a net, for ten days. The remaining water evaporated gradually, within the second day from the water removal as suggested by constant weight. After ten days of emersion, microcosms were rewetted by adding a small amount of filtered stream water but not macrofauna. Microcosms were finally re-submersed in the tank, with the net preventing any biomass loss.

At the end of the experiment (day 40), microcosms were retrieved from the incubation tank and transferred to the laboratory. Macrofauna, leaves and debris were carefully separated, removed from microcosms and oven-dried at 30 °C to a constant weight. Thereafter leaves and debris were weighed and powdered for organic carbon, total nitrogen and total phosphorous analyses (see next section). Leaf biomass loss was calculated by the difference between the initial and final dry weights.

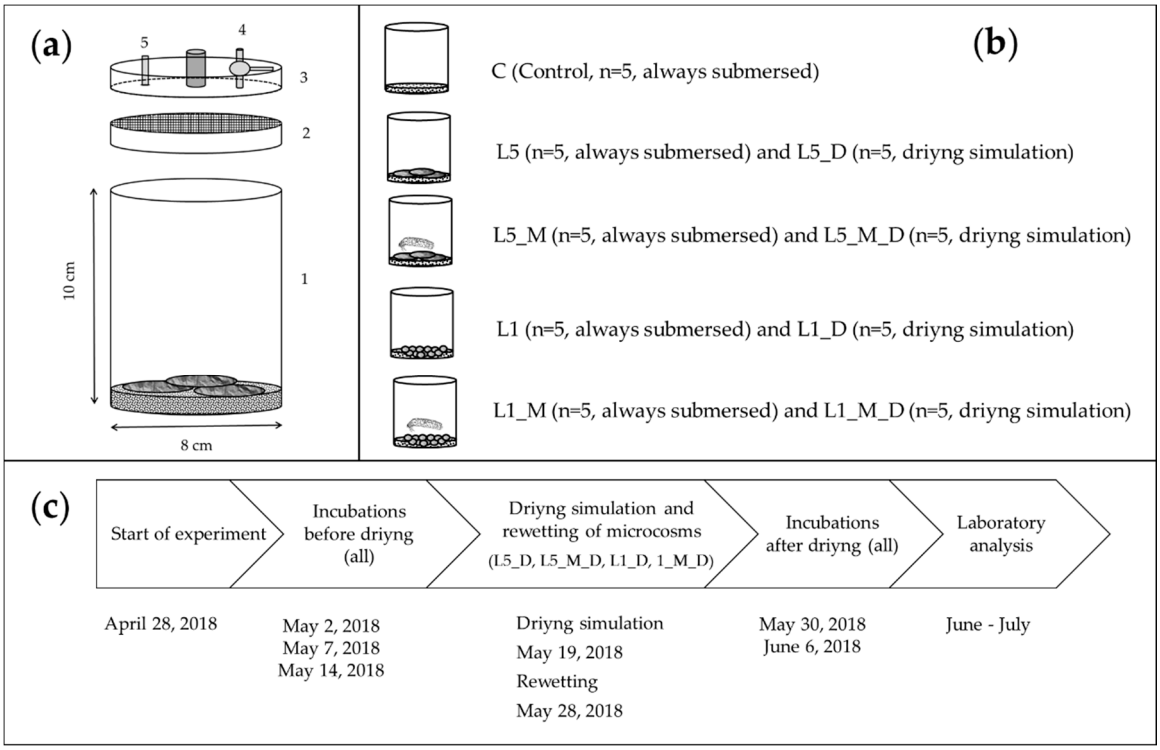

Figure 1. Microcosms set up (**a**), experimental design (**b**) and sequence of actions (**c**). The microcosms used in this experiment were made with opaque PVC (a1). The experimental design consisted of nine treatments, each with five replicates, with various combinations of leaves and macrofauna (b). During maintenance in the tank, microcosms were closed with a lid provided with a net (a2) to allow water exchange between the inner microcosms and the tank. Such lid was replaced with a gas-tight lid (a3) during short-term dark measurements of oxygen and nutrients. The incubation lid was provided with a sampling port (a4) and a compensation valve (a5). Besides control microcosms, incubated to correct fluxes for processes in the water column, we tested four combinations of small and large leaves with or without macrofauna (b). These combinations became eight during the experiment (c) as half of the replicates for each condition underwent a drying simulation.

2.2. Leaves and Shredders

We collected leaves of *Populus nigra*, a common species in the riparian zones of Tuscan-Emilian Apennines that almost exclusively contributes to allochthonous organic matter input at the study site. Leaves were collected in November 2017, just before abscission, were transported to the laboratory and gently rinsed with deionized water. Petioles were removed, leaves were cut into disks of 5 and 1 cm of diameter using a hollow cutter, dried at 25 °C to a constant dry mass and stored at room temperature until needed. Leaves material might have different size and size itself may affect organic matter breakdown rates. This is the reason underlying the choice of two different diameters. Before the start of the experiment, leaves were rehydrated in stream water and then immediately transferred into the microcosms. A total biomass of 0.5 ± 0.002 g of leaves disks of both dimensions was added in each microcosm; to this purpose, three leaves were added to each of the L5, L5_D, L5_M, L5_M_D and 64 leaves were added to each of the L1, L1_D, L1_M and L1_M_D microcosms.

Last instar larvae of *Potamophylax cingulatus* (Trichoptera, Limnephilidae) were collected by hand picking. *Potamophylax cingulatus* is a case-building caddisfly that in the last larval instar construct its cases using entirely mineral particles [33]. The organisms used were at the same developmental stage of the larval forms and similar-sized individuals (0.04 ± 0.003 g_{dw} ind^{-1}) were chosen for the experiment. This species is widely distributed in the study area, where it represents the most dominant shredder taxon participating in the recycling of organic matter and can have strong effects on ecosystem functioning and stream trophic structure.

After sampling, shredders were transported to the laboratory and placed in an aquarium. The day after collection we added 20 individuals in each macrofauna treatment (L5_M, L5_M_D, L1_M, L1_M_D). The number of individuals in each microcosm was chosen on the basis of natural densities found in Parma stream, according to previous studies in the study area (Laini, unpublished).

2.3. Measurement of Benthic Fluxes and Shredders Metabolism

During the 40 days of maintenance in the tank, all microcosms underwent five short-term dark incubations targeting dissolved O_2 and inorganic nutrients regenerated during the mineralization process (NH_4^+, PO_4^{3-}) fluxes. We acknowledge that soluble organic compounds are released as well and might represent important pools of released C, N and P but in this study, we focused only on the inorganic, more reactive fractions. This was also decided in order to discuss the relevance of macrofauna respiration and excretion to measured fluxes. Three incubations were carried out before drying simulation while two were carried out after drying a subsample of the microcosms (L5_D, L5_M_D, L1_D, L1_M_D). The incubations lasted six hours; at the starting water samples were collected in triplicate with 50-mL syringes from the incubation tank and the nets covering each microcosm was replaced with a gas-tight lid. At the end of the incubation, the water samples were collected from each microcosm. An aliquot of the collected water samples was transferred to 12-mL exetainers and poisoned with 100 µL 7 M $ZnCl_2$ to stop microbial activity [34]. Another aliquot was filtered (GF/F Whatman filters) and transferred to 10-mL glass vials for soluble reactive phosphorous determination and to 20-mL scintillation vials for NH_4^+ determination. Dissolved O_2 was measured within two hours by means of polarography with a microelectrode connected to a picoamperometer (Unisense, Denmark). The electrode was calibrated in 100% saturated water at the same incubation temperature and at 0% saturation (N_2 bubbling). Nutrient samples were immediately analyzed with standard spectrophotometric techniques [35,36]. At the end of the incubation, the gas-tight lids were replaced with nets. Fluxes of O_2 and nutrients were calculated according to the equation below.

$$Flux\ x = \frac{\left([x]_f\right) - \left([x]_i\right) \times V}{A \times t} \quad (1)$$

where $[x]_f$ and $[x]_i$, expressed in mM or µM, are the concentrations of the solute x at the end and at the start of the incubation, respectively, V (L) is the volume of the microcosm water phase, A (m^2) is the area of microcosm and t (h) is the incubation time.

Additional individuals of *Potamophylax cingulatus* sampled from the same study stream were also incubated singly and in groups in the dark to analyze O_2 consumption and inorganic nutrient excretion. Individuals were incubated in 50 mL glass vials containing filtered stream water, following a similar procedure as described above.

2.4. Elemental Analysis

C and N content in leaves at the beginning and in residual leaves and debris at the end of the experiment were measured with an EA/NA-1100 CHN elemental analyzer (Thermo Finnigan, Bremen, Germany) coupled with a mass spectrometer. Total phosphorus on the same matrices was determined after ashing at 450 °C, P extraction from ashes with concentrated HCl and spectrophotometry [37]. We analyzed the content of C, N and P in the leaves and in the produced debris and we present data as

a percentage and total content, for budgeting purposes. For the debris, as it was mixed with the mineral particles added to the microcosms, it was not possible to calculate percentages but only total content.

2.5. Data Analyses

We used two-way factorial ANOVA to test the differences among treatments in nutrients fluxes before drying simulation, with leaves dimension and macrofauna as fixed factors. To test differences among treatments in nutrients fluxes after drying simulation, remaining leaf biomass and elemental composition we used three-way ANOVA, with leaves dimension, the presence of macrofauna and drying as fixed factors. The effects of single factors and interactions among treatments were examined. All tests were considered significant if the *p*-value was less than 0.05. Each analysis was performed after assumptions of normality and homoscedasticity were verified. All analyses were performed with statistical software R [38]. Graphs were produced with Sigma Plot 11.0.

3. Results

3.1. Measurements of Shredders Respiration and Excretion Rates

Incubations of macrofauna alone allowed to calculate for organisms with dry weight (g_{dw}) between 0.03 and 0.09 ind^{-1} an average respiration rate of −13.06 ± 0.9 μmol O_2 $g_{dw}{}^{-1}h^{-1}$ (average ± standard error, $n = 9$) and an average $NH_4{}^+$ excretion of 0.75 ± 0.09 μmol $NH_4{}^+$ g_{dw} ind^{-1}. $PO_4{}^{3-}$ excretion was undetectable, due to the difference between initial and final concentrations below the analytical precision of the methods. The literature reports $PO_4{}^{3-}$ excretion rates for the Limnephilidae family averaging 0.2 ± 0.09 μmol $PO_4{}^{3-}$ m^{-2} h^{-1} [39].

3.2. Benthic Respiration and Nutrient Fluxes

We present data for two out of the five incubations: the first, on day 5, and the fourth, on day 33, two days after rewetting. Results from second and third incubation provide rates that lay in between those reported, with consistent outputs of the statistical tests showing the same differences among treatments. Results from fifth incubation suggest very low fluxes likely due to exhaustion of more reactive organic matter pools. Even if they are not shown here, all measured fluxes are reported as Supplementary Materials.

Oxygen respiration and nutrient fluxes in the microcosms varied temporally and among treatments (Figure 2).

At day 5 only four conditions are shown as all microcosms were submerged; at day 33 instead, the four conditions are split in two as half the microcosms underwent 10 days of drying. Microcosms respiration and nutrient fluxes were generally higher at day 5, at the beginning of the experiment. Along the course of the experiment, there was a clear decrease of O_2 consumption rates and nutrient fluxes in all conditions, in particular during the last incubation, in which O_2, $NH_4{}^+$ and $PO_4{}^{3-}$ were very small.

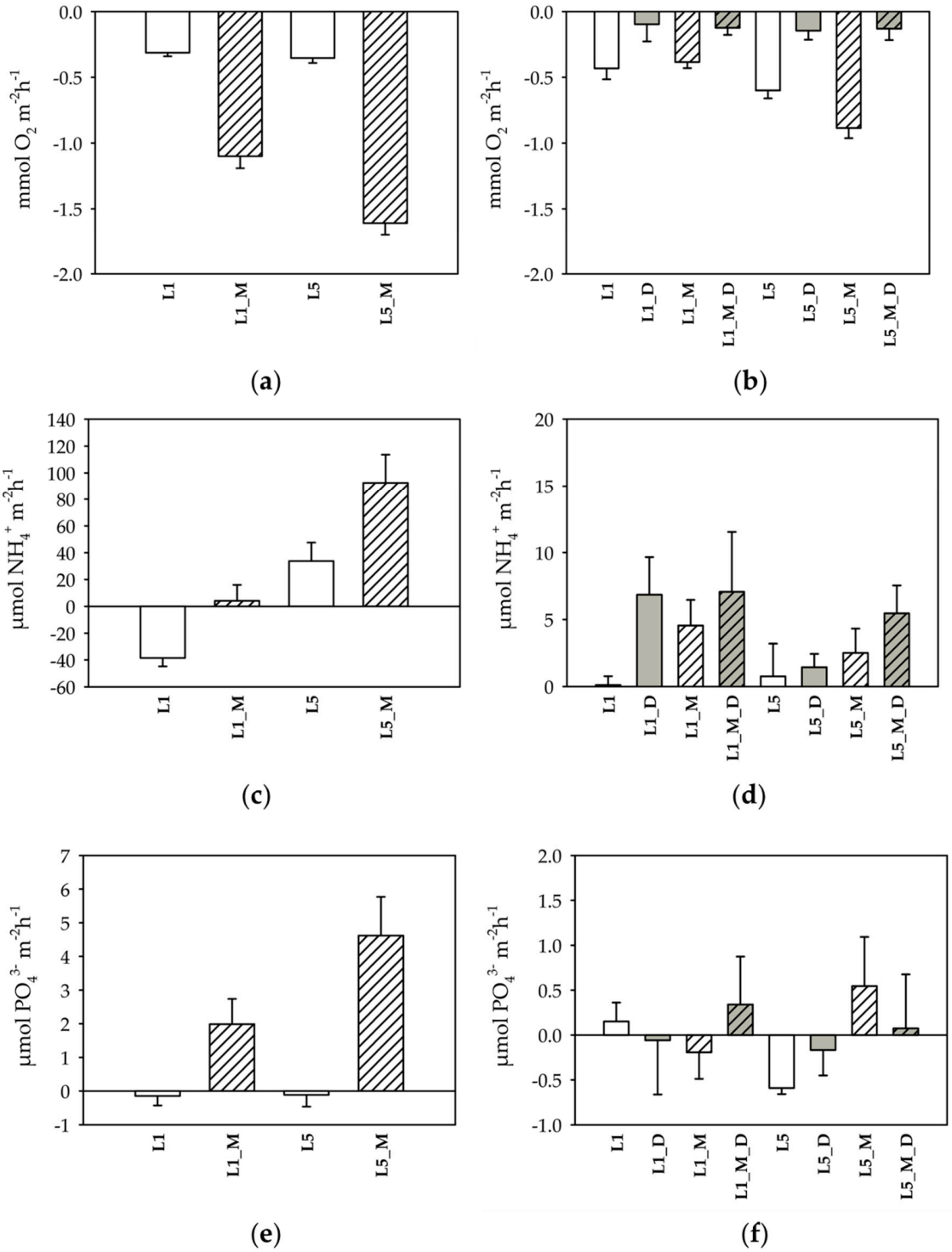

Figure 2. Dark net fluxes of O_2 (**a**,**b**), NH_4^+ (**c**,**d**), PO_4^{3-} (**e**,**f**) at day 5 (**a**,**c**,**e**) and at day 33 (**b**,**d**,**f**). Mean fluxes and standard error (n = 10 at day 5 and n = 5 at day 33) of each condition are reported. All fluxes are expressed in µmol m^{-2} h^{-1} or mmol m^{-2} h^{-1}. White bars represent the permanently submerged condition, grey bars represent dried conditions and hatched bars represent conditions with macrofauna.

At the beginning of the experiment, the conditions with macrofauna displayed significantly higher O_2 consumption rates as compared to microcosms without macrofauna. At day 5, rates varied from a maximum of −1.61 ± 0.09 mmol O_2 m^{-2} h^{-1}, measured in L5_M, to a minimum of −0.31 ± 0.03 mmol O_2 m^{-2} h^{-1}, measured in L1 (Figure 2). At day 33 rates nearly halved and varied between −0.88

± 0.08 (L5_M) and −0.09 ± 0.1 (L1_D). Statistical analyses (Table 1) revealed for day 5 significantly higher O_2 consumption in treatments with macrofauna as compared to treatments without macrofauna ($p < 0.001$; F = 216.5) and in treatments with leaves of 5 cm of diameter as compared to treatments with 1 cm ($p < 0.001$; F = 15.7), however, such differences depended upon the interaction of the two factors ($p < 0.01$; F = 11.4).

Table 1. Summary of results of two-way Analysis of Variance (ANOVA) testing the effects of factors macrofauna and leaf size on benthic respiration (O_2) and nutrient fluxes (NH_4^+, PO_4^{3-}) measured on day 5. Significant values are printed in bold.

	Df	**O_2**		**NH_4^+**		**PO_4^{3-}**	
		***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**
Macrofauna	1	**<0.001**	**216.5**	**<0.001**	**13.2**	**<0.001**	**22.6**
Leaf size	1	**<0.001**	**15.7**	**<0.001**	**33.1**	0.07	3.4
Macrofauna: leaf size	1	**<0.01**	**11.4**	0.6	0.3	0.08	3.2
Residuals	36						

Nutrient fluxes were highly variable ranging from positive to negative values. In the first incubation and in treatments without macrofauna, microbial conditioning of leaves determined negative NH_4^+ and PO_4^{3-} fluxes, likely due to nutrient uptake from the water column. At day 5, NH_4^+ fluxes varied from −38.47 ± 6.3 µmol NH_4^+ m^{-2} h^{-1} measured in L1 to 67.3 ± 16 µmol NH_4^+ m^{-2} h^{-1} measured in L5_M, while PO_4^{3-} fluxes varied from 4.6 ± 1.2 µmol PO_4^{3-} m^{-2} h^{-1} measured in L5_M to -0.14 ± 0.28 µmol PO_4^{3-} m^{-2} h^{-1} measured in L1. At the beginning of the experiment the presence of macrofauna led to significantly higher NH_4^+ and PO_4^{3-} fluxes ($p < 0.001$; F = 13.2 and $p < 0.001$; F = 22.6, respectively). Significantly higher NH_4^+ regeneration was measured in leaves of 5 cm of diameter as compared to leaves of 1 cm of diameter ($p < 0.001$; F = 33.1).

At day 33, two days after rewetting, nutrient fluxes were very low (Figure 2). Treatments subjected to drying displayed significantly lower O_2 demand (Table 2) as compared to treatments permanently submersed ($p < 0.001$; F = 73.9). Leaf size also affected O_2 consumption, with significantly higher O_2 uptake in leaves of 5 cm of diameter at day 33, compared to treatments with leaves of 1 cm of diameter ($p < 0.01$; F = 11.9). However, such difference depended upon the interaction of the two factors ($p < 0.01$; F = 8.6). Drying produced also a significant effect on NH_4^+ regeneration ($p = 0.02$; F = 5.8), whereas the three tested factors did not produce significant effects for PO_4^{3-}.

Table 2. Summary of results of three-way ANOVA testing the effects of factors macrofauna, leaf size and drying on benthic respiration (O_2) and nutrient fluxes (NH_4^+, PO_4^{3-}) measured on day 33. Significant values are printed in bold.

	Df	**O_2**		**NH_4^+**		**PO_4^{3-}**	
		***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**
Macrofauna	1	0.2	1.4	0.3	1.1	0.3	1.3
Leaf size	1	**<0.01**	**11.9**	0.4	0.6	0.8	0.09
Drying	1	**<0.001**	**73.9**	**0.02**	**5.8**	0.8	0.05
Macrofauna: leaf size	1	0.2	1.9	0.8	0.06	0.3	1.1
Macrofauna: drying	1	0.3	1.1	0.6	0.3	0.9	0.02
Drying: leaf size	1	**<0.01**	**8.6**	0.2	1.8	0.8	0.09
Macrofauna: drying: leaf size	1	0.08	3.2	0.6	0.3	0.2	1.8
Residuals	32						

3.3. Residual Biomass and Its Elemental Composition

Assuming random differences in the initial dry weight of the leaf disks and negligible differences among treatments in initial dry weight of leaves, there were large, significant effects of treatments on remaining leaf biomass (Figure 3) and C, N and P content of leaves (Figure 4) at the end of the experiment. The percentage of biomass loss at the end of the experiment ranged from 12.8 to 51.8%, measured in L5_D and in L5_M, respectively. As compared to initial biomass of litter material (110 ± 0.4 g_{dw} m^{-2}, corresponding to 0.5 ± 0.002 g_{dw} per microcosm), the conditions that exhibited lower remaining leaf biomass were those permanently submerged and with macrofauna. For these treatments, the calculated leaf biomass loss was up to four-fold higher compared to conditions undergoing the drying simulation and without macrofauna. Macrofauna and drying significantly affected leaves biomass loss during the experiment (Table 3), with lower remaining leaf biomass in treatments with macrofauna and permanently submersed conditions ($p < 0.001$; F = 32 and $p < 0.001$; F = 13.9, respectively), while the effect of leaf size was very close to the significance level, with lower remaining leaf biomass in larger litter disks ($p = 0.07$).

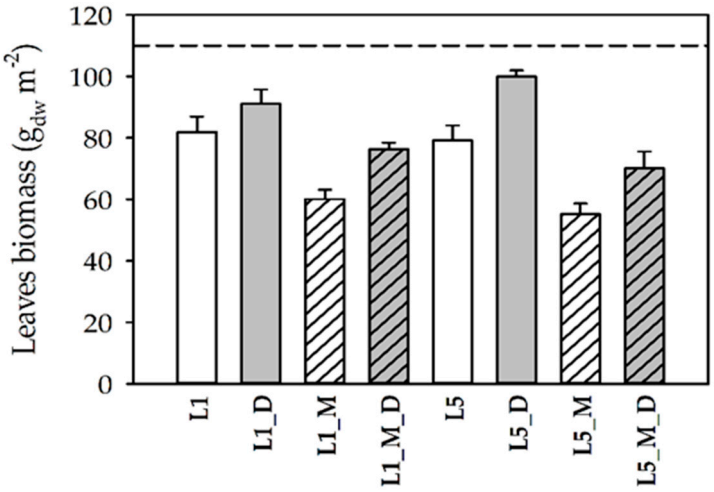

Figure 3. Remaining leaf biomass recovered at the end of the experimental period. Mean ± standard error ($n = 5$) are reported. The horizontal dashed line is the reference value of the average leaf biomass at the beginning of the experiment.

Assuming random differences in the initial elemental composition of the leaf disks and negligible differences among treatments, the leaves elemental composition and stoichiometry changed along the course of the experiment and among treatments. Both percentage and quantity of C in residual leaves decreased during the experiment compared to initial values, while N and P increased, resulting in lower C:N and C:P ratio (Figure 4 and Table 4).

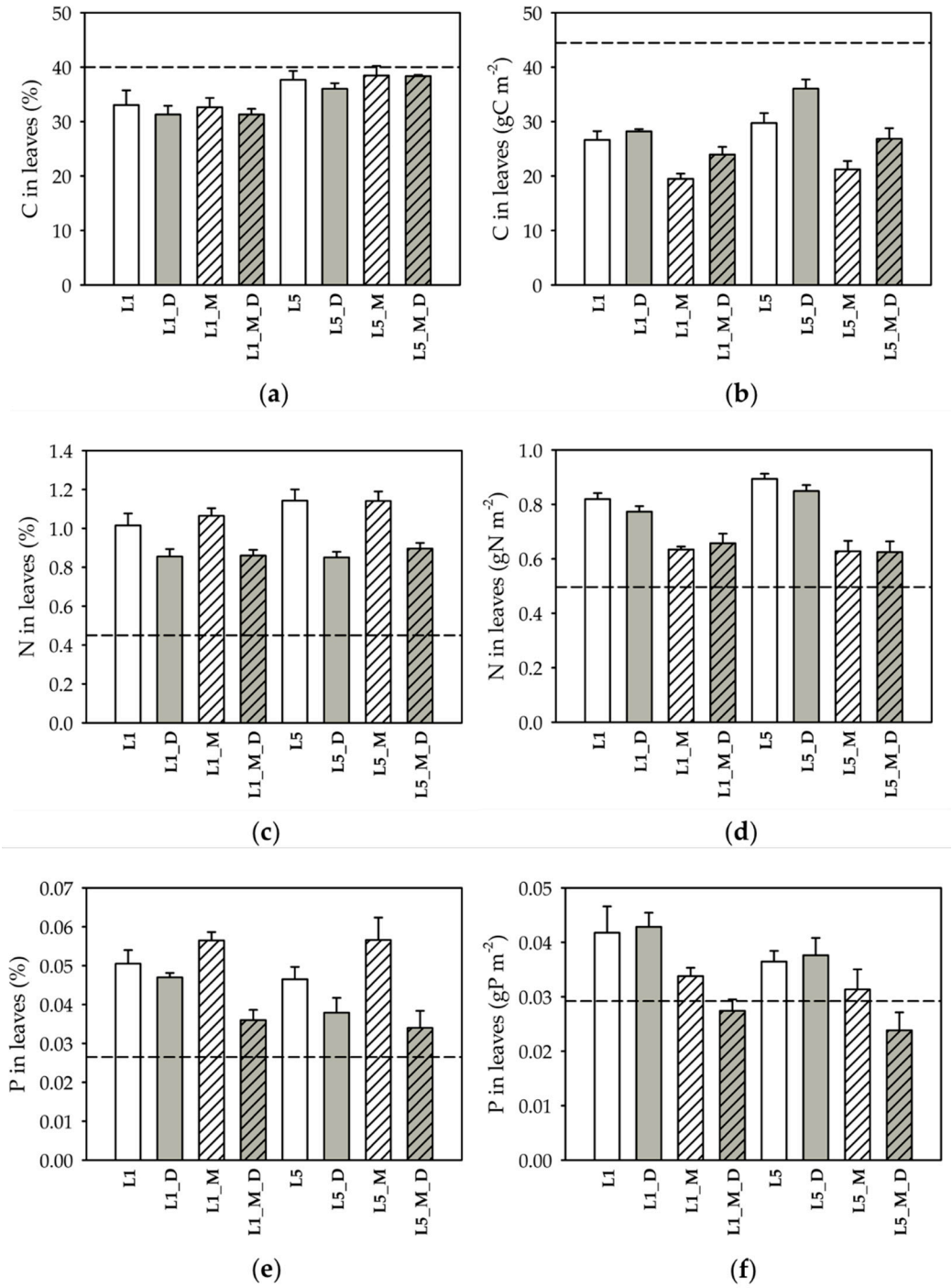

Figure 4. C, N and P percentage (**a**,**c**,**e**) and content (**b**,**d**,**f**) in the residual litter at the end of the experimental period. Mean ± standard error (n = 5) are reported. White bars represent the permanently submerged condition, grey bars represent dried conditions and hatched bars represent conditions with macrofauna. The horizontal dashed line is the reference value of the average C, N, P percentage and content in leaves at the beginning of the experiment.

Table 3. Results of three-way ANOVA testing the effects of the three factors macrofauna, leaf size and drying on residual leaf biomass and its C, N and P percentage and content at the end of the experimental period. Significant values are printed in bold.

Leaves		**Leaves Biomass**		**C (%)**		**C (g m^{-2})**		**N (%)**		**N (g m^{-2})**		**P (%)**		**P (g m^{-2})**	
	Df	***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**
Macrofauna	1	**<0.001**	**32**	0.5	0.4	**<0.001**	**48**	0.4	0.6	**<0.001**	**103**	0.9	0.01	**<0.001**	**24**
Leaf size	1	0.07	3.4	**<0.001**	**24**	**<0.001**	**18.1**	0.06	3.6	0.2	2	0.2	2.2	0.06	3.6
Drying	1	**<0.001**	**13.1**	0.3	1.2	**<0.001**	**13.6**	**<0.001**	**53.4**	0.4	0.8	**<0.001**	**30**	0.2	1.8
Macrofauna : leaf size	1	0.3	1.2	0.4	0.6	0.1	2.2	0.9	0.007	**0.02**	**6**	0.3	1.2	0.6	0.3
Macrofauna: drying	1	0.9	0.005	0.7	0.2	0.6	0.3	1	0.001	0.2	2.1	**0.004**	**9.4**	0.07	3.5
Drying: leaf size	1	0.4	0.7	0.8	0.1	0.1	2.2	0.2	2	0.8	0.1	0.5	0.5	0.9	0.02
Macrofauna: drying: leaf size	1	0.4	0.7	0.8	0.1	0.4	0.7	0.5	0.6	0.7	0.1	0.8	0.09	0.9	0.02
Residuals	32														

Table 4. Molar ratios of C, N and P of leaf biomass (mean ± standard error) at the beginning and at the end of the experimental period.

	C:N	C:P	N:P
Initial litter	104 ± 5	3933 ± 450	38 ± 4
L1	38 ± 2	1721 ± 183	46 ± 6
L1_D	43 ± 1	1729 ± 114	40 ± 2
L1_M	36 ± 2	1497 ± 71	42 ± 2
L1_M_D	43 ± 1	2291 ± 157	54 ± 5
L5	39 ± 2	2124 ± 144	55 ± 3
L5_D	50 ± 3	2566 ± 284	51 ± 3
L5_M	39 ± 2	1826 ± 190	46 ± 4
L5_M_D	50 ± 2	3185 ± 64	64 ± 11

Concerning organic carbon (C), the percentage in leaves at the end of the experiment was lower in all conditions compared to the initial value (40.4% ± 2.56), ranging between 31% and 38%, with a clear tendency of leaves of 1 cm of diameter to have lower C percentage compared to leaves of 5 cm of diameter ($p < 0.001$; F = 24, Table 3). C loss, calculated from percentages and biomass at the end and the beginning of the experiment, was highest in L1_M and L1_M_D conditions (9%), while in the other conditions it was within 2%. Statistical analyses (Table 3) suggest significantly lower C percentage in treatments with leaves of 1 cm of diameter ($p < 0.001$; F = 23.9), while the effect of macrofauna and drying was not significant ($p = 0.55$ and $p = 0.3$, respectively). Compared to C content in leaves at the beginning of the experiment (44.5 ± 2.8 g C m^{-2}), C quantity decreases in all conditions by values between 56% and 19%, with higher calculated C loss measured in L1_M, corresponding to a C quantity of 19.5 ± 0.9 g C m^{-2}. As for biomass, the highest C loss was associated with the presence of macrofauna, with significant lower C content in leaves conditioned by macrofauna ($p < 0.001$; F = 47.85). In addition, both factors drying and leaf size produced a significant effect on C content in residual leaves, with lower values in treatment with drying simulation and with leaves of 1 cm of diameter ($p < 0.001$; F = 18.14 and $p < 0.001$; F = 13.6, respectively).

While C percentages decreased during the experimental period, N percentages in residual biomass almost doubled in all conditions compared to initial values (0.45% ± 0.01), with highest values measured in L5 and L5_M, in which N percentage increased by 2.5-fold (1.14% ± 0.05 in both conditions). Leaves in treatments subjected to drying displayed significantly lower increase in N percentage (0.85% ± 0.03 in L5_D condition), compared to treatment permanently submersed ($p < 0.001$; F = 53.5). Concerning N content in leaves, our results show a general increase in calculated N amount in all conditions compared to initial content (0.5 ± 0.01 g N m^{-2}), varying between 0.6 ± 0.04 and 0.9 ± 0.02 g N m^{-2}, measured in L5_M_D and in L5, respectively. Leaves in treatments without macrofauna accumulated significantly more N compared to leaves subjected to their feeding activity, increasing on average by 40 and 22%, respectively ($p < 0.001$, F = 102.9). However, the effect of macrofauna depended upon the leaf size and was greater for large than for small leaves ($p = 0.02$, F = 6).

Similarly to N, P percentage and quantity in leaves increased during the course of the experiment in most conditions. At the beginning of the experiment, P percentage in leaves averaged 0.026% ± 0.003 (Figure 4e). After forty days, in conditions permanently submerged and with macrofauna (L1_M and L5_M) P percentages exhibited a two-fold increase, while conditions with macrofauna and subjected to drying showed a much smaller increase (L1_M_D and L5_M_D). The factor drying was significant ($p < 0.001$; F = 30.05) and resulted in decreased P percentages, but it depended upon the presence of macrofauna ($p < 0.01$; F = 9.4). Concerning calculated P content, the presence of macrofauna was the only significant factor, without interactions ($p < 0.001$, F = 24), (Table 3). The conditions with macrofauna and undergoing drying simulation (L1_M_D and L5_M_D) were the only with calculated P content lower than the initial value (Figure 4f).

We used the initial nutrient amount in leaves as a reference value for nutrients analyzed in the debris recovered at the end of the experiment (Figure 5). We acknowledge that besides introduced

leaves, additional C, N and P inputs along the course of the experiment may derive from the dissolved inorganic and organic nutrients in the water and from the pellets produced by macrofauna.

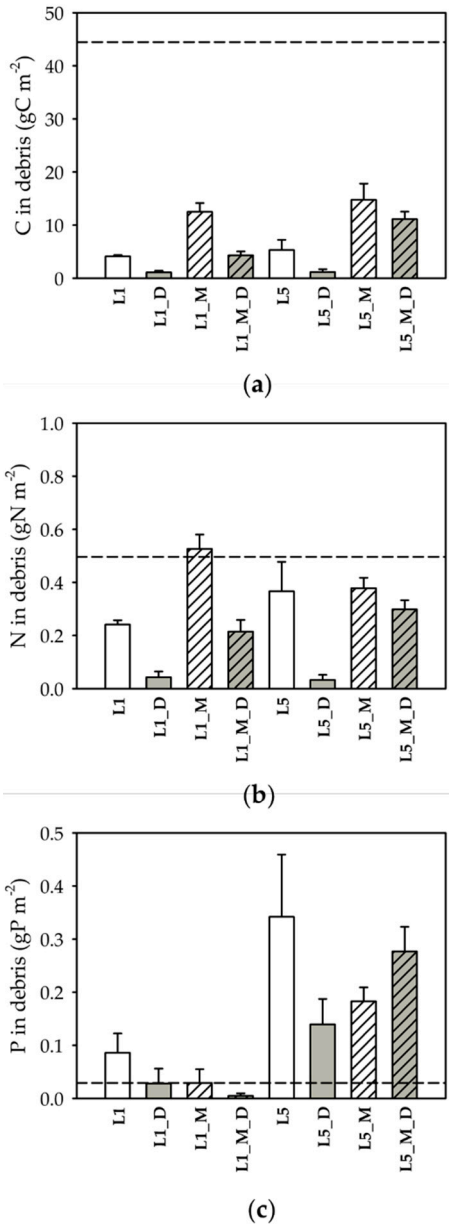

Figure 5. C, N and P content in debris (**a**–**c**) recovered from each microcosm at the end of the experimental period. Mean ± standard error (n = 5) are reported. White bars represent the permanently submerged condition, grey bars represent dried conditions and hatched bars represent conditions with macrofauna. The horizontal dash line is the reference value of C, N, P content in leaves at the beginning of the experiment.

Inorganic nutrient concentrations in the stream water were however very low (<5 µM for NH_4^+ and <1 µM for PO_4^{3-}) whereas the dissolved organic forms were not measured. Produced pellets depended upon introduced leaf material through macrofauna ingestion. We calculated that the quantity of C accumulated in the fine material within the microcosms corresponded to values between 2.5% and 33% of the initial C content in leaves. Results of the effects of the three tested factors on debris elemental composition are reported in Table 5.

Table 5. Results of three-way ANOVA testing the effects of the three factors macrofauna, leaf size and drying on the C, N and P content in the debris recovered from each microcosm at the end of the experimental period. Significant values are printed in bold.

Debris		C (g m^{-2})		N (g m^{-2})		P (g m^{-2})	
	Df	***p*-Value**	**F Value**	***p*-Value**	**F Value**	***p*-Value**	**F Value**
Macrofauna	**1**	**<0.001**	**49.3**	**<0.001**	**26.6**	0.5	0.4
Leaf size	**1**	**0.02**	**5.6**	0.5	0.4	**<0.001**	**39**
Drying	**1**	**<0.001**	**17.2**	**<0.001**	**35.2**	0.09	3
Macrofauna: leaf size	1	0.09	3	0.4	0.9	0.6	0.2
Macrofauna: drying	1	0.3	1.2	0.4	0.8	**0.04**	**4.6**
Drying: leaf size	1	0.09	3	0.8	0.1	0.6	0.3
Macrofauna: drying: leaf size	1	0.2	1.6	**0.03**	**5.5**	0.4	0.8
Residuals	31						

We found significantly higher C amount associated to the debris in treatments with macrofauna (10.7 ± 1.3 g C m^{-2}) compared to conditions without macrofauna (3 ± 0.7 g C m^{-2}) ($p < 0.001$; F = 49.3). In addition, the three-way ANOVA revealed a significant effect of drying and leaves on C amount in residual fine matter. These results show higher C accumulation in residual fine matter of treatments permanently submersed and with leaves with higher diameter ($p = 0.001$; F = 17.2 and $p = 0.02$; F = 5.6, respectively).

Nitrogen enrichment in debris was significantly higher in treatments permanently submerged and with macrofauna ($p < 0.001$; F = 35.2 and $p < 0.001$; F = 26.6, respectively). Treatments undergoing drying simulation and without macrofauna showed the lowest values of N content, while highest values were found in treatments with macrofauna and permanently submerged, with values slightly higher in L1_M conditions compared to initial N content in leaves.

Regarding P, our results show significantly higher content in conditions with leaves with high diameter ($p < 0.001$; F = 38.9), with significant interaction between macrofauna and drying ($p = 0.02$; F = 5.6) and with P amounts exceeding the reference value and 10-fold higher compared to leaves of 1 cm of diameter.

4. Discussion

Studies reporting rates of litter decomposition have recently included drying as a factor due to the increasing frequency of climatic anomalies resulting in temporary periods of water absence. Such periods, despite being short, may produce relevant effects of organic matter conditioning and mineralization, that are consequences of the disappearance of macrofauna and the ecosystem services associated (e.g., nutrient recycling) and of the strong limitation induced by drying on fungal and microbial activity.

During drying in a natural environment several factors interact and affect species and ecosystem processes and our knowledge of combined effects are limited [40]. The experiment described in the present work has two main elements of novelty: it considers simultaneously three interplaying factors: drying, presence of macrofauna and leaf size and it combines a traditional, static approach (remaining leaf biomass evaluation and the analysis of changes in elemental composition) with an approach based on process rates measurement (oxygen and inorganic nutrient fluxes). To the best of our knowledge, such an approach was never used in previously published papers.

Our experiment was conducted in microcosms that inevitably simplify communities and processes occurring in riverine ecosystems, but the controlled conditions in which we operated allowed us to compare rates of oxygen and nutrient exchange along the course of the experiment and to measure after a 40-day period the remaining leaf biomass and the C, N, and P content in the remaining leaf and in debris.

4.1. Submersion, Macrofauna and Large Areas Promote Leaf Litter Decomposition

The mineralization of litter material is a well-studied topic, in particular in perennial rivers as it mobilizes nutrients and represents the engine of downstream processes as pelagic and benthic primary production by phytoplankton, algae and macrophytes [7]. Under submersion conditions, mineralization primarily depends on the activity of macrofauna, and its rate is regulated by organic matter quality, water temperature and nutrient availability [41–43]. Recent intermittent stream studies as that presented here have a different perspective as they focus the effects of river discontinuum on litter mineralization [44,45]. Of special concern for riverine ecology is the understanding of how hydrologic interruptions locally affect river processes and communities and, as a consequence, affect downstream functioning. Drying is expected to be a major concern for low-order and eventually for high order streams due to climate change. Stream intermittency represents a major discontinuum [45] producing poorly studied cascade of consequences for whole riverine ecosystems, in particular on allochthonous organic matter processing [44,46]. This study demonstrates interesting combined effects of a short-term drying event, the presence of macrofauna and leaf size, on leaf litter mineralization and its elemental composition. Results of the statistical analyses suggest that these three factors are sometimes interplaying, as indicated by significant interaction terms (e.g., macrofauna:leaf size for oxygen demand and N content in residual biomass or macrofauna:drying on P percentage in residual biomass). Our results confirm findings from different studies addressing single factors as regulators of decomposition rates [10,17,40]. In particular, highest rates of biomass loss during the nearly 40 days of incubation of the litter material were recorded in microcosms permanently submerged containing leaves with larger diameter and macrofauna. On the contrary, the lowest biomass loss was recorded in dried leaves incubated without macrofauna. Underlying reasons are from one side the larger surface available for macrofauna shredding activity, resulting in leaves fragmentation, the higher release of nutrients and higher colonization and combined activity by fungi and microbes and, at the other extreme, the disappearance of macrofauna and the interruption of the activity of biofilms. Results from this experiments also demonstrate the different paths of leaf-associated C, N and P along the course of the incubation, with the dynamics of the residual matter largely overlapping C content and the dynamics of N and P showing different patterns, ruled by the high C:N and C:P ratios of the original material and the need to import (from the aquatic compartment) additional N and P to allow for microbial decomposition of otherwise recalcitrant material. Here is the key role of macrofauna as a facilitator of microbial activity through nutrient recycling.

Slower decomposition rates are often reported under dry conditions primarily because emersion causes the cessation of water-dependent processes [10,13], with negative impacts on macrofauna community and its stimulatory activity on microbes and changes of litter quality [10,30]. During dry periods abiotic processes such as physical abrasion, and photodegradation or biotic processes as degradation by terrestrial macrofauna [10] may become dominant in driving leaf-litter decomposition [47]. In our study, the most critical aspect of drying regards changes in microbial decomposers and invertebrate detritivores activity, as suggested by low rates of respiration measured after rewetting.

In agreement with previous studies in which negative relationship between litter breakdown and emersion was primarily attributed to negative effects of drying on decomposers communities and to the elimination of drying-sensitive shredders [10,48], our results show a two-fold higher biomass loss in the microcosms with macrofauna. Drying strongly alters growth, activity and functional aspects of decomposers community colonizing and decomposing leaf litter. Such alteration affects organic matter mineralization by temporarily limiting metabolic activity and reducing microbial biomass [11,49,50]. The net effects of drying events likely depend on their duration [50] and severity [49] as macrofauna can only temporary find refuge areas as temporary pools or migrate vertically in the hyporheic zone [14]. Mora-Gómez et al. [11] demonstrated that short term emersion of leaf litter in temperate streams may reduce decomposition rates by 34% 54% and 72% after 7, 14 and 21 days of drying. In the same study, it was demonstrated that fungal and bacterial biomass dynamics changed significantly only after 21 days

of emersion and recovered quickly after rewetting [11,50]. However, only a minor part of the initial live cell biomass was available to immediately start the reactivation of the aquatic microbial food web [28] and some bacterial enzyme activities were significantly reduced after seven days of emersion and may remain affected after rewetting [11].

Results from the present study confirm that even short/term drying events, lasting ten days, are enough to slow biological processes, reducing litter breakdown by nearly 50%. Flux measurements performed after the drying simulation suggest that emersion would not only decelerate decomposition and enlarging the time leaf litter takes to be decomposed in the stream. Emersion also affects microbial assemblages and activity on leaf substrate, reduces the quality of leaf litter as a resource with consequences to detrital food webs [11].

The results we obtained are realistic for areas where drying sets to zero the macrofauna community. *Potamophylax cingulatus* has a univoltine reproductive cycle so, there is one generation per year. If the streambed becomes completely dry, organisms can find refuge in residual pools, die or become adults as a response of environmental stress, and the community temporarily disappears. Data from monitoring macrofauna in intermittent streams suggest that the recovery of community needs at least a few weeks, whereas more time is needed for restoring the initial density of organisms. By removing macrofauna after drying we simulated a situation in which shredders community migrates or emerges as adults, which is realistic for different areas of our study site and shortly after rewetting. We acknowledge that under other environmental settings or with other organisms the effects of drying can be less marked as macrofauna community can restore rapidly. Moreover, at some sites macrofauna can contribute nutrient cycling with its dead biomass, something that was not considered here.

The relevance of macrofauna for leaf breakdown process is well known [18,51] as well as their role in recycling and translocation of nutrients [15]. Nutrient flux measurements, in particular during the early stage of our experiment, suggest that shredders living on leaf litter caused local nutrient enrichment in the sand used as a substrate in our microcosms and within leaf pack in which they feed through excretion and nutrient regeneration, stimulating microbial growth. Nutrient excreted by shredders, especially in oligotrophic conditions, are rapidly immobilized by microbes growing on leaves and decomposing the same resource [16]. As shredders nutrition is highly dependent on leaf-litter microorganisms [51], conditioned leaves become more attractive as a food source to aquatic consumers [52]. This positive effect on fungi and bacteria would imply positive feedback on shredders and in turn on decomposition rates [16].

In addition, leaf size influenced decomposition processes due to litter surface available for microbial colonization, that increases leaf litter palatability to shredders [52]. In our experiment, we used two different leaf sizes and we found higher respiration, nitrogen regeneration and slightly higher values of biomass loss in larger leaves. Our results may be realistic for the litter material we employed and for the macrofauna species we selected but not always. In fact, other studies report higher soluble compounds release and faster degradation in small litter fragments characterized by high surface-area-to-volume ratio, due to higher microbial colonization [17,53]. Overall, results from this study are relevant as under climate change scenario drying events will be more frequent and more prolonged [1,45] and the understanding of their consequences for low order stream metabolism is central.

4.2. What Fluxes Tell Us About Decomposition

Results from single organism incubations were used to assess the share of *P. cingulatus* metabolic activity in the measured benthic oxygen demand and nutrient release, and the percentage of measured flux due to the contribution of macrofauna activity. Oxygen consumption and ammonium excretion by *P. cingulatus* individuals were upscaled and converted to square meter, using the macrofauna densities adopted in our study (4000 ind m^{-2}). Oxygen consumption and ammonium excretion by macrofauna were estimated in -2.2 ± 0.3 mmol O_2 m^{-2} h^{-1} and 127 ± 25 µmol NH_4^+ m^{-2} h^{-1}, respectively, whereas phosphorous excretion, calculated from literature data [39] averaged 34.6 µmol PO_4^{3-} m^{-2}

h^{-1}. Macrofauna-mediated fluxes derived from *P. cingulatus* alone incubations are much larger than those calculated combining measured fluxes in our experiment (e.g., L1_M-L1 oxygen, ammonium or reactive phosphorous fluxes in Figure 2). In particular, they overestimate animal contribution by 216% ± 48, 368% ± 246 and 1018% ± 690.5, respectively. Such overestimation may be due to higher activity of macrofauna when incubated without a substrate: organisms likely do not feel comfortable in a glass bottle with filtered water and move much more than under natural conditions. This was found also in other analogous experiments [54]. Concerning nutrients, we add another explanation. In fact, the degree of overestimation should be similar to that of oxygen, but it's much higher. Our interpretation is that in microcosms with litter and macrofauna, at the beginning of the experiment, a major fraction of the excreted NH_4^+ and PO_4^{3-} is immediately recycled by growing biofilms in the litter biomass and does not accumulate in the water column. Towards the end of the experiment the large difference in the fluxes measured in the conditions with and without macrofauna recorded at day 5 are on the contrary much smaller, likely due to a general decrease of the activity of macrofauna and the microbial communities, following a marked decrease in the quality of residual litter or due to the aging of the experiment. Overall, large differences between calculated and measured fluxes may be due to macrofauna excretion-mediated higher retention of nutrient in the early stage of the decomposition process, to microbial uptake from the water column and biofilms growth, that rapidly immobilized nutrient excreted by macrofauna. If this is true for the early stage of the decomposition process, towards the end of the experiment litter quality is poor and macrofauna and biofilm activity decreases. Such explanation fits with the results of the leaf litter analysis at the end of the experiment, that is highly enriched in N and P and displays a different nutrient stoichiometry at the end of the experiment as compared to the original material [55,56].

Microbial processing of leaf litter may liberate or sequester nutrients depending on characteristics of the microbes and their resources [13]. We used leaves with high C:N and C:P ratios (nearly 105 and 4013, respectively) and microbes may satisfy part of their nutrient demand by removing nutrients from the water column if either N or P is insufficient in the substrate. Our results revealed a general decrease in C:N and C:P molar ratio in leaf litter after forty days of decomposition as a result of N and P increase, coupled with C decrease. Other studies have found this pattern for N and P, while C remained constant [25,57,58]. Permanently submerged conditions and the presence of macrofauna allowed the continuous colonization of leaf litter resulting in higher quality of resources (lower C:N and C:P), compared to drying. Decreased benthic respiration after drying simulation suggests the interruption of biological processes, slowing down leaves biomass loss and affecting leaves elemental composition and nutrient dynamics. On the other hand, measured fluxes after rewetting phase show that rewetting episodes can mobilize labile substances from residual leaves, lysing dead microbial cells and leaching dissolved nutrients and soluble compounds [59]. This could explain higher C:N and C:P in residual biomass subjected to drying, suggesting a possible downstream transport of low-quality organic matter with potential effects on detrital food webs and associated ecosystem processes [60].

5. Conclusions

Drying and the presence of macrofauna were the most important factors determining changes in leaves and debris elemental composition. Leaves permanently submerged and conditioned by macrofauna had lower C and N content, while sand was enriched of both elements. This underlines the importance of water and the role of macrofauna in facilitating nutrient recycling, with impacts on the stream food web and on ecosystem processes across multiple trophic levels. Through feeding activity, macrofauna lead to decreased C, N and P content in leaves, transforming CPOM in FPOM, increasing the organic and nutrient content of the substrate on which they feed. In the natural environment, such nutrient enriched residual litter would be mobilized and transported downstream, becoming available to the stream food web and sustaining secondary consumers. Permanently submerged conditions promote C and N release while drying followed by rewetting leads to higher retention and slow release

of particulate downstream. This may cause a significant alteration of nutrients stoichiometry, with cascading implications for primary producers and water quality.

Under predicted global change scenarios, which are expected to increase the frequency and intensity of drying events, our findings suggest that hydrological intermittence could change streams ecosystem functioning by altering the capacity of benthic fauna to process detritus. In turn, this will impair ecosystem processes and related services.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/11/4/708/s1, Figure S1: Dark net fluxes of O_2, NH_4^+, PO_4^{3-}, at day 10, 17 and 40. Table S1. Summary of results of two-way ANOVA testing the effects of factors macrofauna and leaf size on benthic respiration (O_2) and nutrient fluxes (NH_4^+, PO_4^{3-}) measured on day 10. Table S2. Summary of results of two-way ANOVA testing the effects of factors macrofauna and leaf size on benthic respiration (O_2) and nutrient fluxes (NH_4^+, PO_4^{3-}) measured on day 17. Table S3. Summary of results of three-way ANOVA testing the effects of factors macrofauna, leaf size and drying on benthic respiration (O_2) and nutrient fluxes (NH_4^+, PO_4^{3-}) measured on day 40.

Author Contributions: B.P. carried out the experiment, nutrient analysis and wrote the first draft of the paper. M.B. contributed to data interpretation and to paper writing. A.L. performed the statistical analysis of data and revised the paper draft. R.B. revised the paper draft. C.F. performed DNA sequences analysis of macrofauna. P.V. revised the paper draft.

Funding: This research was funded by NOACQUA project—responses of communities and processes in intermittent rivers—funded by the Italian Ministry of Education and University (PRIN 2015, Prot. 201572HW8F).

Conflicts of Interest: The authors declare no conflict of interest.

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