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THE ROLE OF MICROORGANISMS IN THE DIET OF *VERGER* CF. *LIMNOPHILUS* (TRICHOPTERA: LIMNEPHILIDAE) LARVAE IN A PATAGONIAN ANDEAN TEMPORARY POND

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Abstract: The importance of fungi and bacteria attached to leaf litter in the diet and growth of shredders in flowing waters is well-documented. This study focuses on the role of microorganisms colonizing submerged leaf litter in the diet and growth of *Verger* cf. *limnophilus* (Trichoptera: Limnephilidae) larvae in a Patagonian Andean temporary pond (Fantasma pond, 41°07′S, 71°27′W). First, the feeding habits were analyzed through an experiment that compared consumption of CPOM and FPOM. Once we determined that *V.* cf. *limnophilus* consumed CPOM, we performed an experiment to compare consumption and growth rates of larvae fed on non-autoclaved and autoclaved decaying leaves. Algae was the most abundant group to colonize leaf surface, comprising 74% of total biovolume. Consumption of non-autoclaved leaves was fourfold that of autoclaved treatments, which produced negative insect growth rates. Although *V.* cf. *limnophilus* processed leaves by shredding, microorganisms living on the leaf litter were found to be an important food resource. As microbial biomass represents a small percentage of the ingested food (0.22 %), *V.* cf. *limnophilus* appears to process relatively large quantities of detritus to obtain sufficient resources for growth (100 mg leaves to grow 3 mg).

Key Words: shredders, microorganisms, temporary pond, organic matter cycling, algae

INTRODUCTION

Temporary ponds, like many other wetlands, are detritus-based systems where benthic heterotrophs dominate metabolism (Wetzel 2001). Processing of microbially colonized leaf litter is mainly by aquatic detritivore insects (Merritt et al. 1984). The processing of coarse particulate organic matter (CPOM) by macroinvertebrates has been studied mainly in streams (Arsuffi and Suberkropp 1985, Jacobsen and Sand-Jensen 1994, Albariño and Balseiro 1998, Dobson 1999, Hein et al. 2003), whereas in temporary ponds, it has received little attention (Oertli 1993, Oertli and Lachavanne 1995). In these ecosystems, the marginal vegetation growing adjacent to or within the pond influences the loading of CPOM. Submerged vegetation can be not only a food source for macroinvertebrates but also can provide surfaces to live on, material for building cases, and sites for egg-laying (Campeau et al. 1994, Oertli and Lachavanne 1995).

In temporary ponds, detritivores such as limnephilid caddisflies are among the first animals to colonize when the pond re-floods (Ward 1992). These larvae are typically shredders (Lamberti and Moore 1984) and play an important role in the organic matter cycling because they transform coarse particles to fine particles, accelerating decomposition (Graça 2001). In flowing waters, the role of fungi in the diet of macroinvertebrate shredders is well-documented (Bärlocher 1980, 1985, Arsuffi and Suberkropp 1985, 1986, Graça et al. 1993). The trophic significance of bacteria was also reported (Meyer 1994, Hall et al. 1996, Hall and Meyer 1998). However, the role of other microorganisms attached on submerged leaf litter as complementary food for shredders remains almost unexplored.

In this study, we aimed to assess the role of microorganism assemblages on leaf litter in the consumption and growth of the trichopteran *Verger* cf. *limnophilus* (Schmid). First, we determined its feeding habits, and then we compared the consumption and growth of *V.* cf. *limnophilus* larvae fed on natural and sterilized leaf litter of *Potentilla anserina* Linée (Rosaceae) through a laboratory experiment.

MATERIAL AND METHODS

Study Area

Fantasma pond is a temporary, fishless pond located at 41°07′S, 71°27′W, 780 m a.s.l. in Patagonia, Argentina. The pond is fed by precipitation and runoff.

Annual flooding period generally lasts 8 to 10 months, from April to January (autumn to early summer). Maximum water levels occur in June or July, when the surface area is 1 ha (170 m length and 80 m wide approximately) and maximum depth is ca. 2 m. Water temperature varies from 20° C in November to 0° C in July and August, when ice may cover the surface (Vega 1995). The bottom of the pond is covered by *Potentilla anserina* Linée, which is widely distributed in wet soils in Patagonia (Correa 1984). This plant grows during the dry season and remains submerged during the flooding period, becoming an important source of decaying organic matter. Riparian vegetation is scarce, so other inputs of allochthonous POM to the pond are negligible.

The relative abundance of *V.* cf. *limnophilus* to total macroinvertebrate abundance in Fantasma pond was assessed through field sampling carried out on June 27, 2002. Five 1-m^2 wooden frames were placed where the pond depth was 50 cm, and the whole volume was sampled with a D-frame aquatic net (200-μm pore size). Samples were fixed in the field with 10% formalin. In the laboratory, the macroinvertebrates were identified and counted under a stereomicroscope. They were then dried and weighed to estimate dry biomass.

Verger cf. *limnophilus* Feeding Habit

In order to determine the diet of *V.* cf. *limnophilus*, we carried out an experiment and observed feeding behavior. The experiment compared consumption of CPOM and FPOM under three treatments: treatment C offered leaves of *P. anserina* (CPOM), treatment F offered fine particulate organic matter (FPOM), and treatment C+F offered both items. The larvae and the organic matter (coarse and fine particulate) were collected on June 29, 2002 near the shore at a water depth of 50 cm. Larvae were acclimatized in the laboratory for one week at ambient pond temperature (6°C) and photoperiod of 12L:12D hours. They were supplied with detritus from the pond.

FPOM was obtained by filtering pond detritus through a 1000-μm pore-size mesh (which retained CPOM), and FPOM was retained with a 100-μm pore-size mesh. *Potentilla anserina* leaves and FPOM were weighed after being at room temperature for 24 h to obtain initial fresh mass (FM). Initial dry mass (DM) was estimated from a fresh mass-dry mass regression obtained by drying leaf litter (n = 17) and similar amounts of detritus (n = 20) at 80° C for 48 h (DM), which were then weighed. Treatment C contained 33.1 ± 0.5 mg CPOM (DM, mean ± SE), treatment F 31.0 ± 5.6 mg FPOM (DM), and treatment C+F 32.4 ± 1.4 mg CPOM and 30.2 ± 4.6 mg FPOM (DM). Organic matter was introduced in the containers 48 h before the beginning of the experiment.

The experiment consisted of five replicates with one larva each. The larvae were placed in plastic containers of 6.5-cm diameter with 250 ml of filtered pond water (Whatman GF/C filters). The experiment was run at 6° C and photoperiod of 12L:12D. After seven days of experimentation, larvae were picked out of the containers, and the remaining organic matter (CPOM and FPOM) was collected. Leaves were taken manually, while FPOM was obtained by filtering the water through a 100-μm pore-size mesh. The filters were then dried to obtain final dry masses of FPOM. Consumption rate was estimated as the difference between initial and final dry masses of CPOM and FPOM divided by time of exposure.

The observations of feeding behavior were carried out under a stereomicroscope at 25×. Three larvae were placed in Petri dishes with detritus (CPOM and FPOM) and observed for 15 min. This procedure was repeated 10 times. Additionally, several individuals were placed for one week in a 30-L glass container with pond water and detritus (CPOM and FPOM) to observe their behavior.

Experimental Design

Once the feeding habit of *V.* cf. *limnophilus* had been determined, we compared consumption and growth of larvae fed on natural and sterilized leaves of *P. anserina* through a second experiment. The experiment consisted of two treatments with five replicates each. In treatment A, the CPOM added to the experimental units was previously autoclaved (1.5 atm, 20 min), and in treatment N-A, the CPOM was added non-autoclaved. Controls without larvae were run for both treatments in five replicates. Five larvae were kept without food to compare the growth of fed larvae with starved ones.

The larvae and the senescent leaves were collected on July 7, 2002 near the shore at a water depth of 50 cm. Larvae were acclimatized as in the previous experiment. Leaves were weighed after being at room temperature for 24 h to obtain initial fresh mass (FM). Initial dry mass (DM) was estimated from the fresh mass-dry mass regression obtained in the previous experiment.

Larval initial biomass was obtained after gently placing the larvae on a drying paper with their organic cases (total wet biomass, TWB), and they were weighed with a precision of 0.01 mg. Initial dry mass (B_i) was estimated from the TWB through a regression (TWB-B_i). With this purpose, a set of 91 larvae were weighed alive, as in the experiment, and killed with hot water. Afterwards, they were separated from their

cases and dried at 80° C for 48 h to obtain insect dry mass.

$$B_i = 0.012 + 0.059\ TWB\ (R^2 = 0.873).$$

Estimated larval initial dry mass (B_i) was 2.46 ± 0.25 mg (mean ± SE). We measured head width (1.21 ± 0.04 mm) from living larvae of the experiments to ensure that all of them belonged to the same instar.

The experiment was carried out in the containers described above, with filtered pond water, one larva in each container, and at the same temperature and photoperiod as in the previous experiment. The amount of CPOM placed in each container was calculated to ensure that no treatment lacked CPOM during the 24 days, according to the results obtained for the first experiment (76.0 ± 5.7 mg DM). This CPOM was placed in the containers for 48 h before the addition of the larvae, which had been starved for 24 h.

The experiment was finished one month later on August 7. During the experiment, no larvae died or molted. At the end of the experiment, larvae were collected and killed with hot water to obtain final dry mass (B_f). The remaining CPOM was also collected and dried to obtain final CPOM dry mass (M_f). Feces and little fragments of leaves were collected by filtering the water through pre-weighed Whatman GF/C filters, which were then dried at 80° C for 48 h and weighed.

In order to evaluate if sterilization decreased organic matter in leaves, 10 autoclaved leaves and 10 non-autoclaved leaves were dried, weighed (DW), and then combusted at 550 °C for 1 h to obtain ash-free dry mass (AFDM by subtraction).

Analysis of Larval Consumption and Growth

The rate of mass loss of CPOM not due to consumption was calculated in the controls as $R = (M_i - M_f)/M_i$, where M_i represents initial dry mass and M_f final dry mass. Daily net consumption (C) was calculated as the difference between final and corrected initial mass ($M_i^* = M_i \times R$) divided total time (24 days). Then, daily consumption rate (CR) was calculated as

$$CR\ (d^{-1}) = \frac{(\ln M_i^* - \ln M_f)}{t}$$

Daily larval growth (G) was calculated as the difference between the estimated initial dry biomass (B_i) and final dry biomass (B_f) divided by time (24 days). Then, daily growth rate (GR) was calculated as

$$GR\ (d^{-1}) = \frac{(\ln B_f - \ln B_i)}{t}$$

Efficiency of conversion of ingested food (ECI) was calculated following Arsuffi and Suberkropp (1986) as

$$ECI = \frac{G}{C} \times 100$$

The daily fecal production (Fc) was estimated as the difference between dry mass of FPOM obtained in treatments and FPOM obtained in the controls, divided by time. This value would be overestimated, since there were little fragments of leaves as a result of the shredding activity that we were not able to separate from feces. The percentage of feces in relation to ingested food (Fc%) was calculated as

$$Fc\% = \frac{Fc}{C} \times 100$$

Differences between consumption of CPOM and FPOM in the previous experiment and consumption and growth in the treatments Autoclaved and Non-autoclaved were compared with *t*-tests (Sokal and Rohlf 1981).

Microorganism Quantification

To analyze microorganism communities on non-autoclaved leaves, 20 leaves collected on July 7 from Fantasma pond were weighed after being at room temperature (20° C) for 24 h. Ten were autoclaved, and all 20 leaves were then preserved in 4% formalin, in the dark, and at 5°C for further inspection under the microscope. Autoclaved leaves were examined to determine the efficiency of sterilization.

Each leaf was introduced in 5 ml distilled water, and microorganisms were removed by adding 2 ml buffer pyrophosphate 0.02 M (final concentration 2 mM) and sonicating for 2 min (Grossart and Ploug 2000). This technique dislodged material from the surface of the leaves; thus, fungal hyphae growing inside leaf tissues may have been underestimated.

Slides for bacteria counting were prepared by filtering 1 ml of the suspension after being stained for 1 min with 2 % (V/V) DAPI (4,6-diamidino-2-phenylindole), through black polycarbonate filters (Poretics, 0.2 μm pore size) (Porter and Feig 1980). Counting was carried out at 1000× magnification with an Olympus BX50 epifluorescence microscope. Ten images for each leaf were processed with an image analysis system (Image ProPlus; Media Cybernetics, Silver Spring, MD, USA), and bacterial biovolume was then calculated by approximating shapes to an ellipsoid. Carbon content was estimated following Fisher and Push (2001).

Counting of the other microorganisms (algae, fungi, ciliates, flagellates, testate amoebae, and nematodes) was performed in 10-ml Utermöhl chambers under an inverted microscope at 400×. Algae were identified to genus level and grouped in class levels. Biovolume for

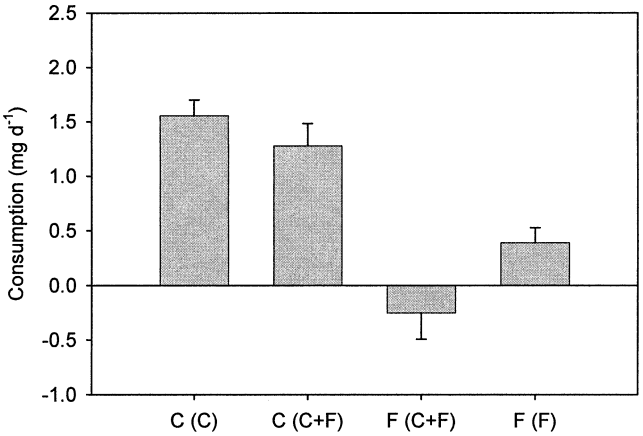

Figure 1. Net consumption of coarse (C) and fine (F) particulate organic matter in treatments with coarse particulate organic matter (C), fine particulate organic matter (F), and both items together (C+F). (Error bars are standard error, n = 5).

each taxon was calculated with best fitting geometric models (Wetzel and Likens 1991, Hillebrand et al. 1999). Ciliates, colorless flagellates, and testate amoebae were identified to genus level whenever possible following Patterson (1998), otherwise to class level. Biovolume was estimated using the measured lengths and widths and common geometric equations. To estimate fungal biovolume, hyphal length and width were measured and biovolume was calculated as the volume of a cylinder. Carbon content was estimated for each group following Menden-Deuer and Lessard (2000).

Leaves were scanned to measure their surface area with an image analysis system. Microorganism biovolumes were expressed per unit leaf area. The leaves were then dried and weighed, combusted, and reweighed to obtain AFDM by subtraction. Carbon content was estimated as the 50% AFDM (Goluecke 1991). We calculated the percentage of microorganism biomass (as estimated carbon content) in relation to leaf biomass (also as estimated carbon content).

RESULTS

V. cf. *limnophilus* dominated macroinvertebrate fauna in Fantasma pond, accounting for 42% of the total density and 48 % of the total macroinvertebrate biomass. Other members of the macroinvertebrate fauna were Chironomidae pupae (38% of the total density) and larvae (3%), Dytiscidae larvae (10%) and adults (0.6%), Scirtidae larvae (4%), Oligochaeta (1%), and Amphipoda (*Hyallela* sp.) (0.3%).

The comparison between the consumption of CPOM vs. FPOM (Figure 1) showed that the larvae consumed significantly less FPOM (0.62 ± 0.20 mg d^{-1} DM, mean ± SE) than CPOM (1.56 0.15 mg d^{-1}) (t-test, $P<0.001$). This result was also obtained when both items were offered together (Figure 1). Consumption of CPOM was not different between C and C+F treatments (t-test, $P>0.05$). In contrast, consumption of FPOM was significantly greater in F than in C+F treatment (*t*-test, $P<0.05$). The negative consumption in treatment C+F could have been a result of an increase in FPOM derived from CPOM.

In addition, microscopic observations showed that *V.* cf. *limnophilus* cut the leaves with their conspicuous mandibles. They were never observed handling fine detritus. Larvae behavior observed in the 30-L glass container showed that *V.* cf. *limnophilus* prefers to feed on leaves and to perch on stems.

Algae, bacteria, fungi, ciliates, flagellates, testate amoebae, and nematodes constituted microorganism assemblage on the leaves. They represented 0.22% of the leaves biomass. Leaves weighed 14.67 ± 0.77 μg AFDM mm^{-2}; hence, estimated carbon content was 7.33 μg mm^{-2}, from which 0.016 μg mm^{-2} was microorganism carbon content. Algae dominated the assemblage (74% of total biovolume) (Table 1). The best represented algae were Xanthophyceae (mostly *Ophiocytium parvulum* (Perty) Braun), Dinophyceae (*Peridinium* sp), and Bacillariophyceae (Table 2). Bacteria accounted for 15% of total biovolume, and ciliates, which consisted of small scuticociliates and peritrichs

Table 1. Microorganism assemblage on *Potentilla anserina* leaves (n = 10, mean ± SE).

	Biovolume (10^3 μm^3 mm^{-2})	Percentage (%)
Algae	145.62 ± 39.54	74.20
Bacteria	29.09 ± 3.11	14.82
Ciliates	12.21 ± 9.09	6.22
Testate amoebas	5.63 ± 1.56	2.87
Fungi	3.17 ± 1.96	1.62
Flagellates	0.31 ± 0.18	0.16
Nematodes	0.22 ± 0.07	0.11
Total	196.25 ± 12.09	100.00

Table 2. Biovolume and density of the different classes of algae present in *Potentilla anserina*'s leaves in Fantasma pond (n = 10, mean ± SE).

	Total biovolume (10^3 μm^3 mm^{-2})	Density (ind. mm^{-2})
Xanthophyceae	72.26 ± 7.31	11.84 ± 1.46
Dinophyceae	20.74 ± 7.60	0.32 ± 0.08
Bacillariophyceae	26.67 ± 15.01	9.93 ± 5.00
Chlorophyceae	15.48 ± 9.36	0.90 ± 0.60
Euglenophyceae	0.46 ± 0.25	0.08 ± 0.05

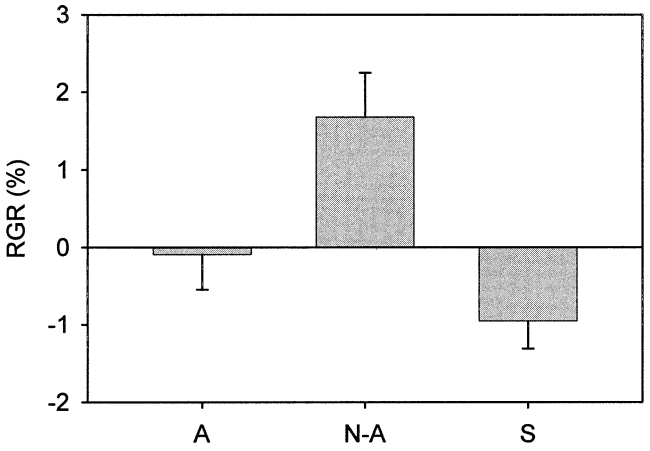

Figure 2. Specific growth rate (%) for *Verger* cf. *limnophilus* larvae fed on autoclaved (A) and non-autoclaved (N-A) *Potentilla anserina* leaves and for starved (S) larvae (Error bars are standard error, n = 5).

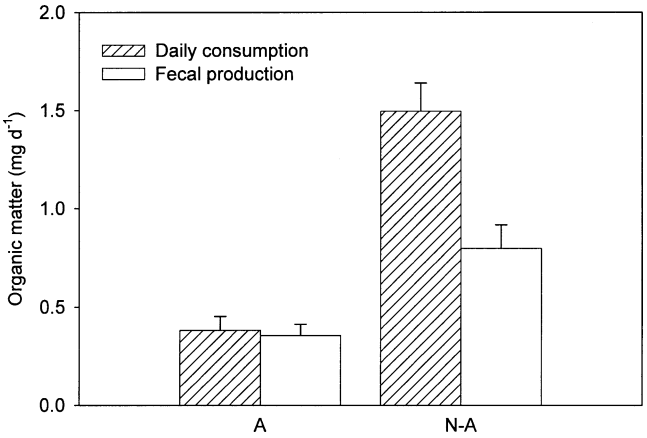

Figure 3. Larval consumption of leaves and stems of *Potentilla anserina* and fecal production in autoclaved (A) and non-autoclaved (N-A) treatments (Error bars are standard error, n = 5).

(*Vorticella* spp.), constituted 6%. Testate amoebae, particularly the genus *Trinema*, accounted for 3%, and Fungi (hyphae and conidia) growing outside of leaves reached only 1.6%. Less abundant groups were colorless flagellates (0.2%) and nematodes (0.1% of total biovolume). Leaf AFDM was not significantly reduced in sterilized CPOM (autoclaved leaves = 72.9 ± 4.4%, non-autoclaved leaves = 71.2 ± 4.7%, t-test, $P > 0.05$).

Consumption and Growth

The analysis of *V.* cf. *limnophilus* larvae, fed on natural and sterilized decaying leaf litter, indicated that each larva consumed 1.49 ± 0.14 mg d^{-1} DM (mean ± SE) of non-autoclaved CPOM and 0.38 ± 0.07 mg d^{-1} of autoclaved CPOM. The specific consumption rate (CR) was higher for non-autoclaved leaves (0.029 ± 0.004 d^{-1}) than for autoclaved leaves (0.006 ± 0.001 d^{-1}) (t-test, $P < 0.05$).

Growth was greater for larvae fed on non-autoclaved leaves (t-test, $P < 0.05$) (Figure 2). Larvae biomass of treatment N-A increased 0.048 ± 0.017 mg d^{-1} (GR = 0.013 ± 0.004 d^{-1}), while larvae biomass of treatment A decreased (G = −0.011 ± 0.006 mg d^{-1}, GR = −0.002 ± 0.003 d^{-1}). Efficiency of conversion of ingested food was calculated only for treatment N-A (ECI = 3.01 ± 0.79 %) since growth was negative in treatment A. None of the larvae died during the experiment, not even those of starved treatments, which declined at G = −0.017 ± 0.004 mg d^{-1} (GR = −0.016 ± 0.005 d^{-1}).

Fecal production in treatment N-A (0.80 ± 0.12 mg d^{-1}) was significantly greater than in treatment A (0.35 ± 0.06 mg d^{-1}) (t-test, $P < 0.05$) (Figure 3). However, the percentage of feces in relation to ingested food was greater in treatment A (94.17%) than in treatment N-A (52.25%) (t-test, $P < 0.05$).

Ingested food (C) is allocated for growth (G), egestion (E), respiration (R), and excretion (Ex) (C = G + E + R + Ex). So, for *V.* cf. *limnophilus* larvae in treatment N-A, 1.49 mg d^{-1} = 0.048 mg d^{-1} + 0.80 mg d^{-1} + R + Ex, then Respiration and Excretion should account for 0.6 mg d^{-1}.

DISCUSSION

We found that *Verger* cf. *limnophilus* larvae ingested and assimilated CPOM and a mixture of CPOM and FPOM at higher rates than FPOM alone. In addition, microscopic observations showed how these larvae cut leaf litter with their mandibles, which indicates that this species belongs to the shredder feeding guild (Merritt and Cummins 1996).

Caddisfly larvae ingested naturally colonized CPOM four-fold faster than autoclaved CPOM. This result corroborates that shredders prefer conditioned (i.e., colonized with microorganisms) over unconditioned leaves (Bärlocher 1980, 1985, Arsuffi and Suberkropp 1985, 1986, Graça et al. 1993). The lower consumption of the autoclaved leaves may be attributed to two possible reasons: the reduction of microorganisms and/or a decline in edibility as a consequence of the alteration of leaf physical/chemical structure due to autoclaving. A reduction in palatability is a common consequence of the absence of microorganisms. Graça et al. (1993) indicated that the palatability of leaf-fungus complex could be determined by factors like differential ability of fungi to eliminate plant allelochemicals, fungal synthesis of micronutrients, production of mycotoxins, and the ability of detritivores to use fun-

gal enzymes to enhance leaf digestion. These factors may also be attributed to other members of the biofilm that colonize submerged leaves.

In addition to fungi, submerged plants provide a well-illuminated substrate for epiphytic algae (Cattaneo and Kalff 1980, Cattaneo et al. 1998) and bacteria. In our study, algae and bacteria represented 74% and 15% of the total microorganism biovolume, respectively. Microscopic algae and bacteria have large protein contents (up to 54 % of their dry weight in algae and 67 % in bacteria) and are probably highly nutritious for insects (Lamberti and Moore 1984). The consumption of bacteria by shredders, presumably from cells that adhere to the surface of ingested leaf fragments, has been reported (Meyer 1994, Hall et al. 1996). Hall and Meyer (1998) showed that the plecopteran shredder *Tallaperla* derived 20–40% of its carbon from bacteria. Friberg and Jacobsen (1999) found that larvae of the trichopteran *Sericostoma personatum* Spence fed on the filamentous green algae *Microspora* sp. showed greater increase in fat content than larvae fed on the macrophyte *Potamogetom perfoliatus* L. and *Alnus glutinosa* L. leaves, showing that algae may represent a more nutritious food resource than vascular plants.

The efficiency of conversion of ingested food (ECI = 3.0%) was low compared with other trichopterans [e.g., ECI = 13.7% to 24.5% for *Hesperophylax magnus* Banks, ECI = 6.6% to 17.1% for *Psychoglypha* sp (Arsuffi and Suberkropp 1986)]. This value suggests that senescent leaves represented a poor food source *per se*, and growth could be attributed to biofilm organisms such as bacteria, algae, and fungi. Some shredders can grow on a periphyton diet (Mihuc and Mihuc 1995), while others cannot (Albariño and Díaz Villanueva 2003). According to Merritt and Cummins (1996), when there is a mismatch between the acquisition system and the assimilation system, a low ECI is expected. The differences between the N-A and A treatments suggests that *V.* cf. *limnophilus* acquisition system corresponds to a shredder, and the ECI observed would suggest that attached microorganisms support secondary production.

Although fecal production was greater in N-A treatment, the percentage of feces in relation to ingested food was greater for treatment A (Figure 3). This result implies a great difference in the assimilation of both types of CPOM, with non-autoclaved leaf assimilated at a higher rate than autoclaved ones. The microorganisms present on leaves can be readily assimilated (42–97% assimilation rate, Hargrave 1970) and remain potential sources of carbon and nitrogen. However, fecal production could have been underestimated. On one hand, coprophagy could have occurred, as in some detritivores (e.g., *Sericostoma vittatum* Rambur, Feio and Graça 2000). On the other hand, fecal production could have been underestimated because a fraction of feces remain in the water as suspended and dissolved OM, leading to an overestimation of respiration and excretion.

As microbial biomass represents a small percentage of the ingested food of shredders (Cummins and Klug 1979), the shredders must process relatively large quantities of detritus to obtain sufficient nutrients for growth (Findlay and Tenore 1982). In this study, microbial biomass represented 0.22% of the ingested food (leaves), and *V.* cf. *limnophilus* had to ingest 100 mg of leaf litter to grow 3 mg. As a consequence, *V.* cf. *limnophilus* appear to play a key role in organic matter fragmentation in Fantasma pond, where coarse organic matter covers the entire bottom.

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