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Microbial community development in the traps of aquatic *Utricularia* species

Dagmara Sirová [a,b,*], Jakub Borovec [a,b], Barbora Černá [a], Eliška Rejmánková [c], Lubomír Adamec [d], Jaroslav Vrba [a,b]

[a] Department of Ecosystem Biology, University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic
[b] Institute of Hydrobiology, Biology Centre AS CR, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic
[c] Department of Environmental Science and Policy, University of California Davis, One Shields Avenue, Davis, CA 95616, USA
[d] Section of Plant Ecology, Institute of Botany AS CR, Dukelská 135, CZ-37982 Třeboň, Czech Republic

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ABSTRACT

We examined trap fluid of three aquatic carnivorous species of *Utricularia* (Lentibulariaceae) to assess the role of microbial community within their traps in plant nutrient acquisition. In the context of increasing trap age, we characterized microbial community composition using phospholipid fatty acid (PLFA) analysis and microscopy. Nutrient content in various fractions of the trap fluid was analyzed and the abundance of free-suspended bacteria estimated. The activities of extracellular phosphatase in the trap fluid were determined using fluorometry and the contribution of the microbial community to phosphatase production assessed by epifluorescence microscopy. The trap microbial community seems to be largely derived from *Utricularia* associated periphyton. PLFA analysis revealed that trap fluid contained all components of a complex microbial food web with bacteria forming more than 58% of the viable microbial biomass in the trap. Trap age seems to be the key factor in determining the patterns of microbial community development as well as enzyme production. The amount of nutrients increases with increasing trap age, and the total amounts of C, N, and P accumulated within traps during their lifetime are relatively large—of the order of 100 mg L^{-1} for C and N, and between 0.2 and 0.6 mg L^{-1} for P. A significant part of the nutrient pool is present in the dissolved form. Trap fluid stoichiometry (molar N:P ratios about 100) as well as the presence of nutrient limited microbial cells (molar N:P ratios 25–61) indicates the importance of phosphorus rather than nitrogen for the nutrition of *Utricularia*. Our findings support the hypothesis that mutualism, apart from the predator–prey interaction, is an important association in aquatic *Utricularia* traps and that the trap-associated microbial community may be of benefit to the rootless aquatic *Utricularia* species facing problems with P acquisition due to the loss of roots in their evolution.

1. Introduction

Of the approximately 250,000 species of angiosperms, less than 1% are classified as carnivorous and almost half of these belong to *Utricularia* (Lentibulariaceae) (Juniper et al., 1989). Within the rootless genus, about 50 species are aquatic or amphibious plants growing usually in standing, nutrient-poor and often humic waters (Juniper et al., 1989; Guisande et al., 2007). *Utricularia* populations can be an important component of these habitats and many aspects of their ecology and carnivorous habit have been researched previously (Friday, 1989; Bern, 1997; Richards, 2001). The plants are thought to supplement photoautotrophic nutrition by trapping and utilizing prey, typically aquatic crustaceans, mites, nematodes, rotifers, and protozoa (Jobson and Morris, 2001; Richards, 2001) in small suction traps or utricles. The importance of carnivory for growth of *Utricularia* has, however, been disputed, especially for species growing under nutrient poor conditions where zooplankton is not a significant source of nutrients for the plants due to its low abundance (Bern, 1997; Richards, 2001). Previous works have concentrated on nutrient uptake from artificially fed prey (Friday and Quarmby, 1994; Jobson and Morris, 2001) as well as qualitative prey composition of plants growing *in situ* (Gordon and Pacheco, 2007). Traps were found to support diverse communities of bacteria and algae, and organisms previously considered as prey, specifically rotifers and various protozoa, have been found alive, even reproducing within

* Corresponding author at: Department of Ecosystem Biology, University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic. Tel.: +420 387 7775872; fax: +420 385 310248.
E-mail address: dagmara_sirova@hotmail.com (D. Sirová).

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the traps (Botta, 1976; Jobson and Morris, 2001; Richards, 2001; Sirová et al., 2003). A mutualistic, rather than predator–prey interaction has therefore been suggested between *Utricularia* and the trap-associated community (Richards, 2001).

Compared to other carnivorous genera such as *Sarracenia*, *Utricularia* trap content is difficult to analyze. The trap is a hollow utricle usually 1–4 mm long, mostly two cells thick, and filled with water. It is one of the most intricate structures in the plant kingdom (Juniper et al., 1989), with a variety of glands and trichomes both on the inner and outer surfaces, the function of which still remains unresolved. After the prey irritates trigger hairs situated close to the trap door, it is sucked in as a result of negative pressure maintained inside the utricle. As the trap can be fired by any mechanical irritation (wind, larger invertebrates, fish, *etc.*), "prey" frequently includes various detritus and particles of suitable size, including algae and bacteria (Richards, 2001). After firing, the trap restores negative pressure by rapidly removing *ca.* 40% water from the lumen (while leaving dissolved organic matter behind) until the original compressed shape is reached. This process lasts about 30 min and the trap is ready to fire again (Sydenham and Findlay, 1975). The trap environment is completely sealed for particles, therefore once inside the trap, prey cannot leave the lumen unless trap walls are damaged or the trap is no longer functional.

Information regarding the conditions within the trap environment, however, is lacking and little is also known about the mechanisms of digestion in *Utricularia*. Recently, Adamec (2007) confirmed close to zero oxygen concentration in the fluid of mature traps of six aquatic *Utricularia* species, possibly due to rapid respiration of the trap glands. An alternative explanation may be intensive mineralization of accumulated organic matter, especially in older traps. Evidence for the presence of active extracellular enzymes in the trap fluid in four aquatic *Utricularia* species was provided by Sirová et al. (2003) and the activity of phosphatases was detected both inside the terminal gland cells and on the surface of quadrifid glands in the traps of 26 *Utricularia* species (Płachno et al., 2006). Extracellular enzymes therefore likely play an important role in nutrient, especially phosphorus (P), acquisition of *Utricularia*.

All mature traps seem to contain detritus and living communities of microorganisms, most probably originating in *Utricularia*-associated periphyton (Mette et al., 2000; Richards, 2001). Microbial food webs are a vital component of aquatic systems, involving the recycling of nutrients through phytoplankton, bacteria and microzooplankton ("microbial loop", Azam et al., 1983). Bacteria consume dissolved organic material (DOM) that cannot be directly ingested by larger organisms. Flagellates and ciliates consume these bacteria, helping to recycle organic matter back into the food web. Bacteria also help to facilitate photoautotrophic growth by releasing nutrients when they absorb DOM (Coveney and Wetzel, 1995). As the trap environment is completely sealed and contains all components of a complex microbial food web, it is reasonable to assume that a significant proportion of enzyme activity as well as available nutrients in the trap fluid are derived from these communities.

Determining the role of utricles and their associated microorganisms in the nutrition of *Utricularia* is further complicated by the rapid changes in trap condition and trapping efficiency of the utricles with age. In *U. vulgaris*, for example, most utricles complete their life cycle in 30 days or less, and during this time, continuous changes in trap function occur. An individual utricle is able to trap for only 10–19 days of age, depending on its size and position, however, its trapping efficiency declines markedly after the fourth to sixth day (Friday, 1989). The age of traps is therefore a vital consideration in any study of the functional ecology of *Utricularia* (Friday, 1989; Sirová et al., 2003).

This study aims to elucidate structure and function of the microbial community in *Utricularia* traps of different ages. Phosphatase activity was measured in *U. vulgaris* and *U. foliosa* trap fluid. Traps of *U. vulgaris*, *U. foliosa,* and *U. purpurea* were assessed by epifluorescence microscopy to determine whether trap-associated communities are contributing to extracellular phosphatase production. Trap fluid and periphyton samples from *U. foliosa* and *U. purpurea* plants collected at the same field location were analyzed using phospholipid fatty acid (PLFA) analysis. This technique provides a broad-based description of the entire microbial community with information obtained on viable biomass concentrations, community composition, and nutritional status. Algal and microfaunal species composition in the trap fluid and periphyton of *U. foliosa* and *U. purpurea* was assessed. In an attempt to characterize nutrient relations inside the trap, we measured carbon (C), nitrogen (N) and P contents in various fractions of the trap fluid collected from the above two species.

2. Materials and methods

2.1. Plant material

Adult plants of *U. vulgaris* L. (collected in the Czech Republic) were cultivated outdoors in a plastic container which simulated natural conditions (for details see Sirová et al., 2003). The pH of cultivation medium was approximately 7.0, total alkalinity 0.62 mequiv. L^{-1}, free CO_2 concentration 0.14 mM, and humic substances (humic acids + tannins) 4.2 mg L^{-1}. Based on the concentrations of nutrients (NH_4–N 34 μg L^{-1}, NO_3–N 4 μg L^{-1}, PO_4–P 2 μg L^{-1}), the water was considered oligotrophic. Addition of fine zooplankton prey to the container was interrupted 2 weeks before sampling.

The *U. foliosa* L. and *U. purpurea* Walt. plants were collected from a shallow, oligotrophic, carbonate-based marsh "Hidden" (Orange Walk province, Belize, Central America; Rejmánková et al., 1996) in August 2004. The following water chemistry parameters were measured at the location by standard methods (see Rejmánková et al., 1996) at the time of plant material collection: pH 7.8, electrical conductivity 14.3 mS m^{-1}, total alkalinity 1.14 mequiv. L^{-1}, NH_4–N 6 μg L^{-1}, NO_3–N 0 μg L^{-1}, and PO_4–P 2 μg L^{-1}.

All *Utricularia* plants were transported submerged in filtered water collected at the sites to prevent the trapping of air and detritus by the traps.

2.2. Collection of trap fluid, pH measurements

Trap fluid from utricles of the same size class (>2 mm) and age was collected using a glass pipette with a fine, 0.4-mm wide tip, attached to a plastic syringe (Sirová et al., 2003). In all experiments, only traps without visible zooplankton prey were selected for analysis. Fluid collected from 200–500 traps (~0.5 mL) of each of the *Utricularia* species in various treatments was pooled into Eppendorf vials. Samples for the phosphatase visualization under the epifluorescence microscope were processed immediately; samples for enzyme assay were filtered through 0.2 μm filter to remove bacterial and algal cells as well as detritus and were frozen at −20 °C till analysis. Ambient water samples were processed in the same way. Samples for bacterial counting and species identification were fixed in 2% formaldehyde; samples for PLFA analysis and nutrient content were processed as described below. The pH of trap fluid was estimated using a pH paper (Lachema, Brno, Czech Rep.; for detailed description see Sirová et al., 2003).

In order to determine the influence of trap age on the selected parameters studied, we collected fluid from young, intermediate,

and old traps of *U. vulgaris*, *U. foliosa*, and *U. purpurea*. The age of the traps was determined by the differences in trap pigmentation and by the distance of leaf whorls or segments from the apex (starting with the first segment or whorl bearing mature functional traps). In *U. vulgaris*, young traps were slightly pink and were cut from the 2nd–6th leaf segments, intermediate traps cut from the 8th–12th segments were pink, while old traps cut from the 13th–18th segments were pink to purple. In *U. purpurea,* young traps were cut from the 2nd leaf whorl, intermediate from the 5th–6th whorls, and old traps from the 8th–10th whorls. The young traps of *U. foliosa* were collected from the 1st–2nd, intermediate from the 5th–9th, and old from the 12th–15th mature trap pinnae.

2.3. Nutrient analyses

For the measurements of nutrient content in the trap fluid, field grown *U. foliosa* and *U. purpurea* were chosen, because they grow in close proximity to each other on the highly P-limited site in Belize described above. Ambient water samples for the NH_4–N, NO_3–N and PO_4–P analyses were filtered through a 0.45-μm membrane filter and analyzed colorimetrically according to standard procedures (Hunter et al., 1993).

Approximately 0.3–0.5 mL of trap fluid was collected from functional young, intermediate, and old traps of both species without macroscopic prey and processed immediately. The fluid collected was diluted into 20 mL of double-distilled water. A part of this volume was then used for the measurements of total nutrient content in the trap fluid. The remaining volume was filtered through Whatman GF/G glass fibre filter, a portion of the filtrate was again collected for analysis, the remainder passed through Machery-Nagel glass fibre filter (pore size 0.4 μm) and analyzed for nutrient content. We obtained four trap fluid fractions in this way which we designated as total C, N, and P, "nanomicrobial" C, N, and P (this fraction included algae, protozoa, rotifers, and larger bacterial flocks), "picomicrobial" C, N, and P (represented only free bacteria suspended in the trap fluid), and dissolved C, N, and P. Each fraction was evaluated microscopically after DAPI staining (Porter and Feig, 1980).

Carbon and N content in the trap fluid fractions was analyzed using Skalar Formacs TOC/TN analyzer (Skalar analytical B.V., The Netherlands). Phosphorus content was analyzed by standard methods using perchloric acid digestion according to Kopáček and Hejzlar (1993).

2.4. Microbial community structure—PLFA analysis

Approximately 300 μL of the trap fluid from the young, intermediate, and old traps of the field-grown *U. foliosa* and *U. purpurea* was collected for PLFA analysis. In addition, periphyton associated with both species was collected by vigorous shaking of the plants in a plastic bag with 250 mL of double-distilled water for 15 min. A pooled periphyton sample from 10 plants (2nd, 5th, and 10th leaf whorls for *U. foliosa*, 2nd–8th leaf whorls for *U. purpurea*, respectively) was obtained, 25 mL of which was filtered (0.2 μm membrane filters) and the filters processed identically to trap fluid samples. All samples were kept at 4 °C and processed within 24 h following collection. Microbial community structure and biomass were estimated from total phospholipid fatty acid content determined by the mild alkaline trans-esterification extraction method (Frostegård et al., 1993). Different fatty acids were determined by gas chromatography (HP 6890, FID, capillary column HP-5, 30 m, 0.25 mm, 0.25 μm) and identified using bacterial standard fatty acid methyl esters (a mixture of FAME 37, 24 bacterial FAMEs Supelco; Larodan Fine Chemicals AB). Peak identification was verified by comparison of the MS EI spectra with those from standards on a GC–MS Finnigan GCQ (Finnigan MAT, TX, USA). The PLFA nomenclature follows the pattern of A: BωC, described by Oravecz et al. (2004). An increasing ratio of total monounsaturated/total saturated fatty acids (MUFA/TSFA) in the trap fluid was used as an indicator of higher substrate availability (Bossio and Scow, 1998). We used the following five microbial groups to characterize trap microbial community: Bacteria (Bossio and Scow, 1998), Actinomycetes (Kroppenstedt, 1985), Microfungi (Findlay et al., 1990), Microflora (includes algae and cyanobacteria, Dunstan et al., 1994), Microfauna (includes Protozoa, Rotatoria, Nematoda, and small Arthropoda, White, 1983).

2.5. Species identification and the counting of bacteria

Approximately 300 μL of trap fluid from the traps of the field-grown *U. foliosa* and *U. purpurea* and 10 mL of the periphyton sample (see above) was used for algal, cyanobacterial and microfaunal species identification under the light microscope (Olympus CX41, Tokyo, Japan). For determining the number of utricles containing metazoan prey, five plants each of *U. foliosa* and *U. purpurea* were collected randomly at the field site and their utricles examined under the stereomicroscope (Olympus SZX16, Tokyo, Japan). In *U. foliosa*, 100 traps were examined from each of the first 10 mature trap pinnae. In *U. purpurea* 150 mature traps per plant were observed.

The fluid for bacterial counting from 200–300 traps (~0.25 mL) of the same age was pooled into Eppendorf vials. The samples were stained with DAPI as described above and filtered through 0.2 μm membrane filters. Bacterial cells were counted under the epifluorescence microscope (magnification 1000×) using a counting grid. For the observation of bacteria on trap walls, individual traps were dissected with a scalpel and large fragments were stained with DAPI.

2.6. Enzyme assay

A common fluorometric method was adopted for the microplate assay and modified to determine phosphatase activity in both trap fluid and ambient water (for detailed description, see Sirová et al., 2003). The 4-methylumbelliferyl phosphate fluorogenic substrate (Glycosynth, Warrington, UK) was used. Samples were analysed in black 96-well microplates for fluorescence detection (Nunc, Roskilde, Denmark) and fluorescence was measured at 365 nm excitation and 445 nm emission wavelengths with FluoroMax-3/MicroMax (Jobin Yvon/Spex Horiba, Irvine, CA, USA) or Thermo Labsystems Fluoroskan (Ascant, FL, USA).

Fluorescently labelled enzyme activity technique was used to detect phosphatase activity of microorganisms from *Utricularia* traps. ELF®97 phosphate (ELFP), in our case supplied in the Endogenous Phosphatase Detection Kit (Molecular Probes, Eugene, OR, USA), forms insoluble fluorescent precipitates of ELF-alcohol (ELFA) at the site of phosphatase activity. The fluid from 200 to 300 traps (0.25 mL) of the same age from each *Utricularia* species was pooled into Eppendorf vials. ELFP was diluted 20× in detection buffer and 10 μL of the solution was added to 0.25 mL of trap fluid. The samples were incubated in the dark at room temperature for 20 min. Counterstaining was then performed with fluorescent DAPI stain. The samples were subsequently filtered through 0.2 μm membrane filters and immediately evaluated under the epifluorescence microscope (Olympus AX-70, Tokyo, Japan) using the UV-excitation filter set with a long pass emission filter (excitation/emission: 360–

370 nm/420 nm) (Nedoma et al., 2003). Green ELFA fluorescence, red chlorophyll *a* autofluorescence, and blue DAPI fluorescence are visible using this filter set.

2.7. Statistical treatment

Due to the extremely small volume of fluid in single traps as well as the particulars associated with trap fluid collection and processing (see Sirová et al., 2003) it was not possible to work with independent samples, only pseudoreplicates. Thus, the variability shown (±2 S.D., ±S.D.) represents only the analytical variability (*i.e.* nutrient content and PLFA analyses as well as enzyme activity measurements), not the variability of the material used. Nevertheless, we believe that by averaging the contents of hundreds of traps of comparable age in a single volume we are able to work with a good representative sample. Standard statistical analyses were performed using Prism 4.0 (GraphPad Software Inc.) software and STATISTICA 6.0 (StatSoft Inc.); non-parametric tests (Mann–Whitney, Kruskal–Wallis, Friedman ANOVA, and Wilcoxon tests) were used to compare parameters among the different trap ages.

3. Results

3.1. Analyses of trap-fluid nutrient content

The results of nutrient (C, N, and P) content analyses in *U. foliosa* and *U. purpurea* traps without macroscopic prey (Table 1) indicate that, in both species, the total content of C and N that accumulate within traps during their lifetime are relatively large—of the order of 100 mg L^{-1}, while within 1–4 mg L^{-1} for P. The largest proportion of all three nutrients is in the nanomicrobial fraction, although significant amounts are also present in the suspended bacteria alone and in the dissolved form. In *U. purpurea*, the content of nutrients in the various fractions increases highly significantly ($N = 6$, $\chi^2_{(\text{d.f.}=2)} = 22.4$, $p = 0.00002$) with increasing trap age. Young and medium traps of *U. foliosa* do not differ significantly in nutrient content, the old traps, however, contain higher concentrations ($N = 6$, $\chi^2_{(\text{d.f.}=2)} = 5.67$, $p = 0.049$) of all nutrients analyzed.

The molar ratios of analyzed nutrients in the trap fluid vary with each fraction and trap age (Table 2). Of the three nutrients analyzed, carbon undergoes the largest quantitative changes with increasing trap age in both species, most probably due to the accumulation of plant exudates within the trap. The C:P ratio markedly increases in the dissolved fraction of old traps, while both the nano- and picomicrobial fractions show an opposite trend. The most dynamic fraction are the free suspended bacteria of *U. foliosa* traps. Their cellular stoichiometry, especially the C:P ratio in the picomicrobial fraction, seems to change markedly as the trap ages. This trend, however, is not as well pronounced in the traps of *U. purpurea*.

Table 2
Molar ratios of C, N, and P in various fractions of trap fluid from *U. foliosa* and *U. purpurea* traps of different age

	Tot	Nano	Pico	Dis
C:N				
U. foliosa				
Young	12	13	41	9
Intermediate	17	28	10	12
Old	23	38	1	16
U. purpurea				
Young	22	30	19	10
Intermediate	19	22	11	13
Old	23	25	9	20
C:P				
U. foliosa				
Young	729	699	914	814
Intermediate	710	665	155	1008
Old	565	515	16	1765
U. purpurea				
Young	1125	1224	819	910
Intermediate	1024	1149	171	1233
Old	955	1086	124	2389
N:P				
U. foliosa				
Young	61	55	22	91
Intermediate	43	24	16	87
Old	25	14	16	109
U. purpurea				
Young	51	41	42	90
Intermediate	53	53	16	92
Old	42	44	14	119

Data based on the results from Table 1. Tot, total; nano, nanomicrobial; pico, picomicrobial; dis, dissolved.

3.2. Analyses of trap-fluid microbial community

Our data indicate that traps are heavily colonized by living bacteria, microfungi, algae, and microfauna (Table 3, Fig. 1). According to the PLFA analysis, the largest proportion of viable microbial biomass in the trap fluid of both *U. foliosa* and *U. purpurea* traps of all ages constitutes of G− bacteria (>58%, usually >75%), followed by microflora and microfauna with similar proportional representation (4–25%), microfungi (3–14%), and G+ bacteria (Actinomycetes) with less than 5% of the total (Table 3). The biomass of microbial groups in *U. purpurea* traps showed a significant increase with trap age ($N = 5$, $\chi^2_{(\text{d.f.}=2)} = 7.68$, $p = 0.021$). This pattern did not occur in *U. foliosa*, where the changes in microbial biomass were not significant. The total PLFA, however, was highest in the old traps (Table 3). The MUFA/TSFA ratio was lowest in young *U. foliosa* traps and young and intermediate traps of *U. purpurea* (Table 3).

Table 1
Nutrient content (mg L^{-1}) in various fractions of trap fluid in field-grown *U. foliosa* and *U. purpurea* traps of different ages, without macroscopic prey

	C_{tot}	C_{nano}	C_{pico}	C_{dis}	N_{tot}	N_{nano}	N_{pico}	N_{dis}	P_{tot}	P_{nano}	P_{pico}	P_{dis}
U. foliosa												
Young[a]	632	467	26	139	62	43	1	18	2.2	1.7	0.1	0.4
Intermediate[a]	565	302	15	249	40	13	2	25	2.1	1.2	0.2	0.6
Old[b]	854	542	5	307	44	17	5	22	3.9	2.7	0.7	0.4
U. purpurea												
Young[a]	403	330	8.7	64	21	13	0.5	7	0.9	0.7	0.03	0.2
Intermediate[b]	1065	888	24	152	64	48	3	13	2.7	2.0	0.4	0.3
Old[c]	1569	1231	48	290	81	58	6	17	4.2	2.9	1.0	0.3

Tot, total; nano, nanomicrobial; pico, picomicrobial; dis, dissolved. Means of two (P) or three (C and N) parallel determinations are shown (±S.E.M. was less than 5% in all cases); distinct superscripts associated with trap age categories within each species indicate statistically significant difference (Wilcoxon test, $P < 0.05$).

Table 3
The relative abundance of selected microbial groups expressed as PLFA concentration (nmol PLFA mL^{-1}) in the fluid from *U. foliosa* and *U. purpurea* traps of different age

Microbial group	*U. foliosa*			*U. purpurea*		
	Young[a]	Intermediate[a]	Old[b]	Young[a]	Intermediate[b]	Old[c]
Bacteria (G−)	32.2 ± 7.8	25.8 ± 6.1	39.3 ± 10.9	8.2 ± 3.6	11.5 ± 0.7	41.7 ± 1.2
Actinomycetes (G+)	0.4 ± 0.3	1.2 ± 0.4	0.8 ± 0.2	0.2 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
Microfungi	1.8 ± 0.9	1.6 ± 0.4	1.6 ± 0.1	0.5 ± 0.02	1.0 ± 0.3	7.5 ± 1.6
Microflora	1.7 ± 0.1	1.6 ± 0.1	2.2 ± 0.1	0.4 ± 0.2	1.0 ± 0.4	10.5 ± 0.4
Microfauna	0.9 ± 0.4	2.1 ± 0.3	2.0 ± 0.7	0.5 ± 0.1	0.5 ± 0.3	11.7 ± 0.7
Total PLFA	37.6	32.2	45.3	9.8	14.6	71.4
MUFA/TSFA	0.2	0.5	0.4	0.5	0.5	0.8

Means (±S.D.) of three parallel determinations are shown; distinct superscripts associated with trap age categories within each species indicate statistically significant difference (Wilcoxon test, $P < 0.05$).

A relatively high number of viable algal and microfaunal species was found in the fluid from intermediate *U. foliosa* and *U. purpurea* traps: 136 and 140, respectively (Table 4). All cyanobacterial and most of diatom (Bacillariophyceae) species were also identified in the plant-associated periphyton. Euglenophyceae and Desmidiales, however, occurred almost exclusively in the traps. Overall, more than 70% of species from trap fluid were found in the periphyton. The counts of bacteria suspended in the trap fluid of *U. vulgaris* and *U. foliosa* were one or two orders of magnitude higher than those in the ambient water ($5.2 \pm 0.4 \times 10^6$ mL^{-1} and

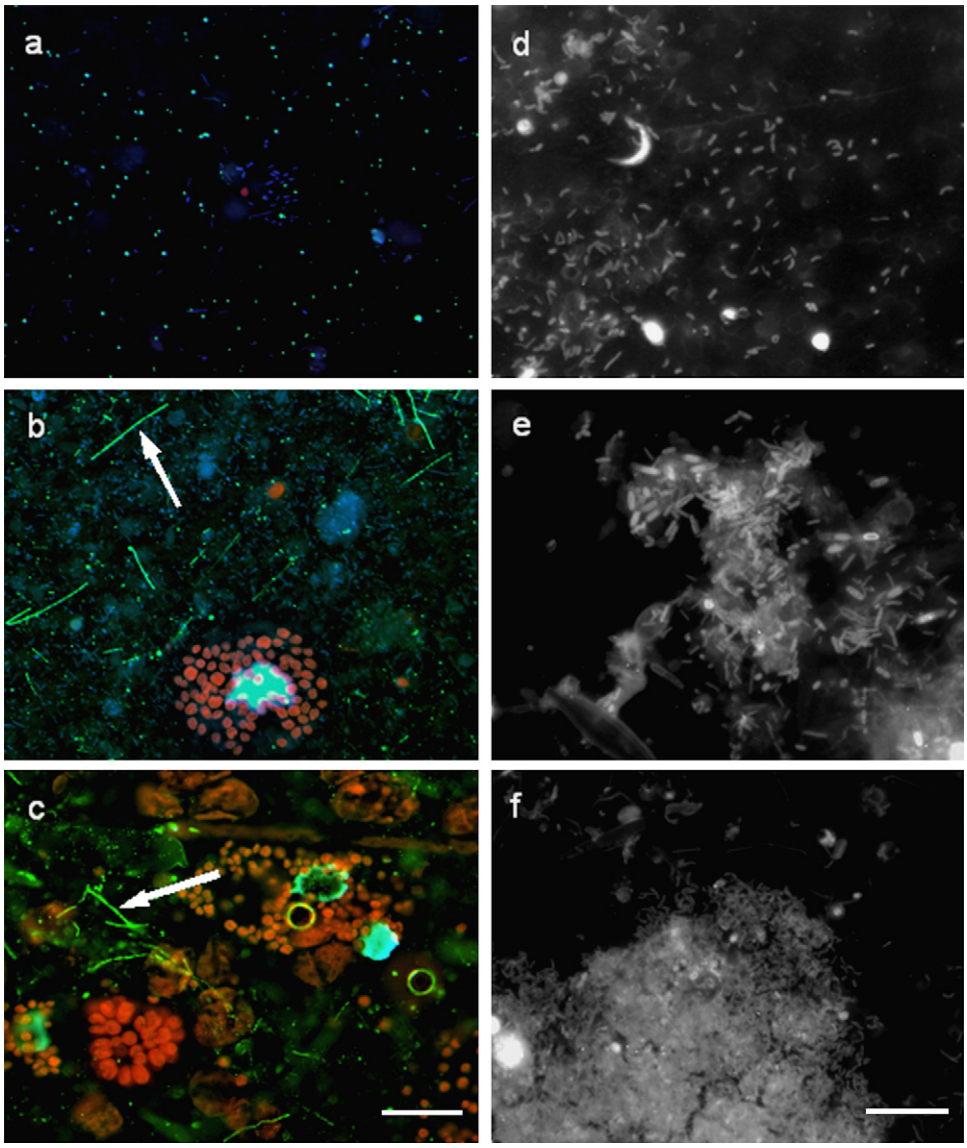

Fig. 1. Microphotographs of ELFA-labelled (left) and DAPI-stained (right) samples of young (a and d), intermediate (b and e) and old (c and f) *U. vulgaris* traps (outdoor culture, Czech Republic). Green ELFA fluorescence signifies a site of active phosphatase enzyme. Red chlorophyll *a* autofluorescence of algae and cyanobacteria is also visible. Arrows indicate filamentous bacteria exhibiting membrane-bound phosphatase activity. Note that left and right panels are different samples. Scale bar represents 20 μm.

Table 4
Number of algal and microzooplankton species found in the traps of field-grown *U. foliosa* and *U. purpurea*

	U. foliosa	*U. purpurea*
Bacillariophyceae	26 (19)	22 (20)
Cryptophyceae	0 (1)	1 (1)
Chrysophyceae	0 (0)	1 (1)
Desmidiales	22 (4)	28 (3)
Chlamydomonadales	2 (0)	1 (0)
Chlorococcales	14 (9)	12 (11)
Euglenophyceae	3 (0)	3 (0)
Dinophyceae	1 (0)	1 (0)
Cyanobacteria	58 (58)	63 (63)
Rotifera	6 (3)	4 (3)
Nematoda	1 (1)	0
Cilliata	3 (1)	3 (2)
Undetermined	24	12
Total	136	140

Parentheses indicate the number of species also identified in plant periphyton.

$2.8 \pm 0.8 \times 10^6$ mL^{-1}, respectively) and decreased with increasing age due to the increasing degree of bacterial aggregation in older traps (Fig. 1d–f). Due to this aggregation, it was practically impossible to perform accurate counting of the total number of bacteria per trap. Among the most conspicuous organisms within the traps of all studied *Utricularia* species are *Euglena* spp. and so far unidentified mixotrophic ciliates, which tended to gather in high numbers around or within detrital clusters and animal remains, possibly utilizing the accumulated organic matter. Relatively low percentage of traps containing larger zooplankton prey was found in the field-grown *U. foliosa* and *U. purpurea* plants (0.9% and 1.4%, respectively; data not shown).

3.3. Phosphatase activity

The activity of free dissolved phosphatase measured fluorometrically was highest in young traps of both *U. vulgaris* and *U. foliosa* species, 16 and 3.7 μmol L^{-1} h^{-1}, respectively, and markedly decreased with trap age (Table 5). In *U. vulgaris*, the old traps displayed approximately 26% of phosphatase activity observed in young traps.

Phosphatase activity in the traps of all studied species as revealed by epifluorescence microscopy was greatly influenced by trap age and followed a similar pattern in all species studied. Phosphatase in young traps, which harboured the smallest number of microorganisms, was active mainly in the free dissolved form. The number of microorganisms as well as the proportion of phosphatase activity bound to cell membranes of microorganisms increased with increasing trap age, being highest in the old traps (Fig. 1a–c). Bacterial as well as algal cells displayed extracellular phosphatase activity.

There was no difference in the trap fluid pH between the young and intermediate traps of both *U. vulgaris* and *U. foliosa*, however, the pH was significantly higher in *U. foliosa* traps (Table 5). In the old *U. vulgaris* traps, we observed a marked and significant decrease in pH (median 4.2) compared to the younger traps (median 5.1).

4. Discussion

According to our results, trap age seems to be a key factor influencing the extracellular enzyme activity as well as the microbial community development in the trap fluid of *Utricularia* plants. The nutrient concentrations and microbial biomass in traps of specific age follow a similar pattern in both species. The dissolved phosphatase activity decreased with increasing trap age in both *U. vulgaris* and *U. foliosa*, accompanied by a change in bacterial abundance and pH. Similar trends were previously observed by Sirová et al. (2003) for enzyme activity in culture-grown *U. vulgaris*. Enzyme activity in the young traps was present mainly in the free dissolved form, whereas bacterial and algal counts were lower and so was the activity of their cells (Fig. 1a and d). It is therefore possible to conclude that the phosphatase activity in young traps originates mainly from the plant itself. As the trap ages, the number of microorganisms increases and so does phosphatase activity bound to their cells. The number of phosphatase sites localized by the FLEA technique is, in fact, highest in the old traps where they are mostly associated with microbial cells (Fig. 1b–f; see also Płachno et al., 2006). A significant part of the phosphatase activity in older traps will therefore originate from the microbial community. These results together with a significant drop in pH in the old traps of *U. vulgaris* (Table 5; Sirová et al., 2003) indicate a change in the trap function after it reaches a certain age. Although the old traps studied here were still fully functional, it is possible that the enzyme production by the plant was reduced and that the internal glands have shifted their function to take up nutrients accumulated within the trap (Owen et al., 1999). Vintéjoux and Shoar-Ghafari (2005) have observed ultrastructural changes in the quadrifid glands within *Utricularia* traps at different stages of development.

According to our observations, *Utricularia* traps are heavily colonized by bacteria whose numbers in the trap fluid per unit volume exceed by 1–3 orders of magnitude those in the ambient water. Counts of free suspended bacteria in the trap fluid were found to be an order of magnitude higher in *U. foliosa* than in *U. vulgaris*. Counts within a similar range (up to 10^8 cells per mL) are also reported by Gray et al. (2006) in the pitchers of carnivorous *Sarracenia purpurea*. Lower number of suspended bacteria in older traps caused by the increased degree of bacterial aggregation may be a result of changing nutrient conditions within the traps or an adaptation to the increasing numbers of bacterivorous microfauna. The excess of dissolved organic carbon accumulating in *Utricularia* traps without metazoan prey most probably consists of plant exudates and provides an abundant carbon source for these bacteria. In many ecosystems bacteria play a key role in the

Table 5
Comparison of enzyme activity, bacterial abundances, and fluid pH in the *U. vulgaris* and *U. foliosa* trap fluid from traps of different age

	U. vulgaris			*U. foliosa*	
	Young	Intermediate	Old	Young	Intermediate
Phosphatase (μmol L^{-1} h^{-1})	16.0 ± 0.7	12.2 ± 1.1	4.11 ± 0.23	3.74 ± 1.46	1.78 ± 0.64
Bacterial abundance (10^7 mL^{-1})	7.3 ± 2.9	8.2 ± 2.6	4.7 ± 2.2	102 ± 23[a]	63 ± 33[a]
Trap fluid pH	5.1 (5.1–5.1)[a]	5.1 (4.8–5.1)[a]	4.2 (3.9–4.8)[b]	7.1 (6.8–7.9)[a]	7.2 (6.7–7.7)[a]

Means (±2 S.D.) of four (phosphatase activity) or three (bacterial abundance) parallel determinations are shown. Medians and ranges of values are shown for pH ($n = 5$); distinct superscripts associated with trap age categories within each species indicate statistically significant difference at $P < 0.05$. Note that bacterial counts represent abundances of free-swimming bacteria in the trap fluid only, not the total count in the trap.

mineralization of organic matter, potentially controlling nutrient flow to other components of the food web (Sterner and Elser, 2002). Their large surface area to volume ratio enables bacteria to acquire nutrients such as P at concentrations far below those available for plant uptake (Currie and Kalff, 1984). This, together with their ability to produce additional extracellular enzymes, may well make them valuable in aiding nutrient acquisition for the plant, especially in nutrient-poor environments such as Belizean wetlands. Thus *Utricularia* traps, in analogy with corals, can concentrate the microbial food from the ambient water and create a microenvironment enriched both in food and nutrients ("gardening", Ferrier-Pagès et al., 1998).

The nutrient concentrations in the trap fluid without metazoan prey are surprisingly high, even comparable to those commonly found in waste waters. A typical 2 m long *U. foliosa* plant with a trap of 2 mm diameter, approximated as a cylindrical shape 0.8 mm high, with 80,000 traps, and the surface area of 1.4 m^2 will have estimated 1.1 m^2 of internal trap absorption surfaces. These surfaces are therefore permanently in contact with a medium where the concentrations of potentially utilizable nutrients exceed those in the ambient water by 2–3 orders of magnitude (note particularly the pools of dissolved N and P in Table 1). Once inside the trap, nutrients accumulated in the closed trap are no longer available for *Utricularia* competitors. For a rootless "pelagic" plant, this undoubtedly is an advantage that may have contributed to the success of *Utricularia* within the carnivorous plant group and to the ubiquitous distribution of the genus in a variety of environments (Müller and Borsch, 2005). The overall stoichiometry of the trap content suggests P rather than N limitation (C_{tot}:P_{tot} *vs.* C_{tot}:N_{tot} in Table 2). Cellular C:P ratios in the bacterial fraction as well as the MUFA/TSFA ratio seem to support this conclusion. Nutrient limitation of the microbial community appears highest in young traps and decreases with increasing trap age and nutrient accumulation.

Utricularia plants are known to harbour rich periphyton communities compared to other aquatic macrophytes (e.g., Friday, 1989). The associated periphyton may aid in nutrient acquisition by attracting phytophilous zooplankton or periphyton grazers as potential prey (Mette et al., 2000; Guisande et al., 2000). Our data indicate that the microbial community inside *Utricularia* traps is derived largely from its periphyton. There are some species, however, that are found almost exclusively in the traps. These include Desmidiales, which were previously reported to be a significant part of trap algal community (Mette et al., 2000; Richards, 2001) and may favour the lower pH found inside (Sirová et al., 2003; this study). Euglenophyceae, on the other hand, may prefer the trap environment due to high concentrations of organic nutrients (Palmer, 1969).

As interactions at the microbial level can cascade up to the higher trophic levels, the microbes can be considered as integral parts and fully interacting members of the trap community (Cochran-Stafira and von Ende, 1998). Our findings seem to support the hypothesis that the important association in aquatic *Utricularia* traps is mutualism rather than a predator–prey interaction and that the major benefit to the rootless *Utricularia* (at least under prey absence) is derived from the microbial community (Richards, 2001; Sirová et al., 2003). The strategy of trapping and utilizing microorganisms, either directly as prey or indirectly via microbial food web interactions, may have become of ecological relevance to the aquatic *Utricularia* species that were facing problems with P acquisition due to the loss of roots in their evolution. This plant–microbe interaction may present a stoichiometric benefit similar to mycorrhizal interactions among terrestrial plants. Whether or not this is truly the case remains to be confirmed by evaluating plants from sites varying in nutrient and zooplankton availability; nevertheless rootless aquatic *Utricularia* species form a functional unit with the microbial community inside their traps and the processes within this community should not be neglected in future studies of *Utricularia* ecology.

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