UNIVERSITY OF SOUTH BOHEMIA

FACULTY OF BIOLOGICAL SCIENCES

BACHELOR THESIS

Enzymatic activities in traps of four aquatic species of the carnivorous genus *Utricularia*

Dagmara Sirová

Supervisor: Prof. RNDr. J. Komárek, DrSc.

Consultants: RNDr. L. Adamec, CSc.
RNDr. J. Vrba, CSc.

2003

D. Sirová, 2003: Enzymatic activities in traps of four aquatic species of the carnivorous genus *Utricularia*, p. 30

UNIVERSITY OF SOUTH BOHEMIA, FACULTY OF BIOLOGICAL SCIENCES, DEPARTMENT OF BOTANY, ČESKÉ BUDĚJOVICE, CZECH REPUBLIC

ANNOTATION

Enzymatic activity of five common hydrolases was measured in the trap fluid of four aquatic species belonging to the carnivorous genus *Utricularia* and in the ambient water. A pH optimum of aminopeptidases and phosphatases was estimated. A common fluorimetric method (Hoppe 1983, 1993) was adopted, 4-methylumbelliferyl (MUF) phosphate was used as a substrate.

In this thesis I present the results of 2 years of work of RNDr. L. Adamec, CSc., Institute of Botany AS CR, Section of Plant Ecology; RNDr. J. Vrba, CSc., Hydrobiological Institute AS CR; Faculty of Biological Sciences, University of South Bohemia, and D. SIROVÁ, Faculty of Biological Sciences, University of South Bohemia. It is an extended manuscript to New Phytologist with those above as co-authors.

This study was partly funded by the GA AS CR project (No. A6017202, awarded to J. V.) and by the Research Programme of the Academy of Sciences of the Czech Republic (No. AV0Z6005908).

STATEMENT

I state that I have elaborated this bachelor thesis individually based only on experimental results and the literature cited.

In České Budějovice, 2003-5-12

Dagmara Sirová

D. Sirová

ACKNOWLEDGEMENTS

I would like to thank RNDr. L. Adamec, CSc. and RNDr. J. Vrba, CSc. for their patience and kind guidance and for their understanding during my illness. Thanks are due to prof. RNDr. J. Komárek, DrSc.; prof. RNDr. K. Beneš, CSc., RNDr.V. Němec, CSc. for helpful comments and to RNDr. M. Studnička, CSc. for providing experimental plant material. I also aknowledge F. Radzo for his encouradgement, and T. Skopec for his patience and support.

CONTENTS

1. INTRODUCTION

The genus *Utricularia* (Lentibulariaceae), with over 210 species, is the most widespread of the carnivorous plants (Juniper *et al.*, 1989; Taylor, 1989). Many species from this rootless genus are aquatic and form floating shoots in quiet, pollution-free ponds and humic waters where they are important components of the freshwater flora. Like other carnivorous plants, *Utricularia* supplements normal photolithotrophic nutrition by trapping and utilising prey, typically aquatic crustaceans, mites, rotifers, and protozoa (Jobson and Morris, 2001; Richards, 2001). The trap is a hollow utricle, mostly two cells thick, filled with water. Traps are usually 1–4 mm in lenght. They have a doorway obstructed by a trapdoor which opens inwards after irritation. The trapdoor is surrounded by trigger hairs and other appendages. After the prey brushes against the trigger hairs, it is sucked in due to the underpressure maintained inside the utricle. After firing, the trap restores the underpressure by removing water from the lumen until the original compressed shape is reached. After this process, lasting about 30 min, is completed, the trap is ready to fire again (Sydenham and Findlay, 1975).

Little is known about the mechanisms of digestion in *Utricularia*. There have been no recent or detailed studies on *in-situ* enzymatic activity in the actual trap fluid. Standard biochemical techniques have provided evidence for the presence of proteases in the traps (Luetzelberg, 1910; Adowa, 1924; Hada, 1930). Protease (Parkes, 1980; Vintéjoux, 1973, 1974), acid phosphatase, and esterase (Heslop-Harrison, 1975; Parkes, 1980) were localized cytochemically in the quadrifid digestive glands.

Utricles also support a diverse community of microorganisms, including many species of living bacteria, algae, rotifers, and protozoa (Cohn, 1875; Hegner, 1926; Schumacher 1960; Botta, 1976; Jobson and Morris 2001; Richards, 2001). Species of *Euglena* (Euglenophyta) apparently even reproduced in this environment (Hegner, 1926; Botta, 1976). It is therefore reasonable to assume that a considerable proportion of enzymatic activity in the

trap fluid is derived from this community. This supports the hypothesis that *Utricularia* plants benefit more from the by-products of this community than from carnivory itself (Richards, 2001).

The aim of our study was to measure *in situ* activity of five common hydrolases in the trap fluid collected from four aquatic *Utricularia* species (*U. aurea* Lour., *U. australis* R.Br., *U. foliosa* L., *U. vulgaris* L.) and in the water in which the plants were cultured, in order to estimate pH optima and changes in activity of these enzymes at time intervals following prey capture. In this way, we determined the direct availability of the enzymes in the traps for prey digestion.

1.1. Aims of study

- Measure *in-situ* enzymatic activity of five common hydrolases in the trap fluid collected from four aquatic *Utricularia* species (*U.aurea* Lour., *U.autralis* R.Br., *U. foliosa* L., *U.vulgaris* L.) and in the ambient culture water.

- Estimate pH optima of these enzymes.

- Measure possible changes in their activity at time intervals following prey capture using a common fluorimetric method with 4-methylumbelliferyl substrates (Hoppe 1983).

- In this way determine the direct availability of the enzymes in the traps for prey digestion.

- Visualize phosphatase activity in the traps of *Utricularia* species using enzyme-labeled fluorescence.

1.2. Characteristics of *Utricularia* species used in this study

U. foliosa (Appendix, Fig.10, 12) is a large species with robust stolons, up to several meters long, flattened in cross section and covered with mucilage. Leaves are usually dimorphic, some with fewer segments and numerous traps, others with more numerous segments and fewer or no traps. Traps are broadly ovoid, 1-2 mm long, with 2 usually unequal appendages. *U. foliosa* occurs throughout tropical Africa, Madagascar, southern states of USA, Central and South America. It grows in still or slowly flowing waters, in lakes, rivers and marshes at low altitudes.

U. vulgaris (Appendix, Fig.11) is a medium sized to large perennial species with numerous 1,5–6 cm long leaves and dimorphic traps usually 1,5-5 mm long. Its habitat includes lakes, pools, ditches and river backwaters. It grows in still or slowly moving waters usually at low altitude. Its distribution range covers most of Europe, North Africa and temperate Asia to western Siberia and Tibet.

U. australis is similar to *U. vulgaris*, stolons are branched, 50 cm long or longer, leaves are very numerous, 1,5-4 cm long, bearing usually moderately numerous dimorphic traps. Traps are ovoid and 0,5-2,5 mm long. It is distributed throughout most of Europe, temperate Asia to China and Japan, tropical and South Africa, tropical Asia, Australia and New Zealand in habitats similar to *U. vulgaris*.

U. aurea is the commonest and most widespread Asian suspended aquatic species, it also occurs in Australia. It varies considerably in size, but most plants are intermediate. Stolons are up to 1 m long bearing very numerous leaves 1-8 cm long. Traps are dimorphic, mostly lateral on the secondary and tertiary segments and 1-4 mm long. This species can be found in still waters, usually pools, tanks, river backwaters and swamps, often intermixed with other aquatic vegetation. (Taylor,1989).

1.3. Carnivory in plants

Although plants are autotrophic with respect to chemical energy and reduced carbon, they need to obtain nutrients from their environment, usually through uptake from soil by their roots. Carnivory has developed as an evolutionary adaptation to nutrient poor environments, it presents an alternative and efficient means to acquire nutrients, mainly reduced nitrogen and phosphorus and has evolved independently across the plant kingdom (Albert et al., 1992). Since carnivorous plants are able to survive and grow without prey, it is thought that carnivory provides an important competitive advantage, especially in environments where levels of nitrogen, phosphorus and other minerals are low.

There is however cost to carnivory, as carnivorous plants need to invest in complex morphological and physiological adaptations to attract, capture, digest, and absorb prey. This must yield a greater return than its original investment to justify the expenditure. The investment in carnivory may vary with environmental conditions and prey availability (Friday 1992, Knight & Frost, 1991). There are means by which these species can improve the cost:benefit ratio – the regulation of hydrolase expression in *Sarracenia purpurea* is an example of such a mechanism (Gallie, 1997).

1.4. The ecology of *Utricularia*

Submerged macrophytes such as *Utricularia* play important roles in aquatic communities, especially in the littoral zone, where they often form dense stands. Their photosynthesis influences a range of chemical and physical characteristics of the environment such as levels of nutrients, oxygen content, pH, temperature and light conditions (Carpenter & Lodge, 1986; cited in Harms, 2002).

Unlike other carnivorous plants, *Utricularia* does not seem to actively attract its prey (Juniper *et al.* 1989). Instead, the plant makes use of epiphytic algae and bacteria that grow on its surface (Appendix, Fig. 4). The plant surface in good light conditions provides its epiphytes with leaking nutrients (Wallace, 1978; Carpenter & Lodge, 1986) and by facilitating epiphytic primary production in this way, these macrophytes help in making littoral zones and wetlands some of the most productive areas in the world (Brown & Lomolino, 1998; Harms, 2002). The epiphytes become an important food source for many grazing microcrustaceans and rotifers (Fryer, 1968, 1974; Wallace, 1977; Jeppesen *et al.*, 1998 cited in Harms, 2002). In this way, *Utricularia* indirectly facilitates the recruitment of periphyton grazers and at the same time has the prey close to its bladders. In littoral zones, up to 80% of the observed microcrustaceans can be associated with macrophytes. Although most prey are microcrustaceans (Cladocera, Copepoda, Ostracoda), other invertebrates such as Bivalvia, Annelida, Acari, Isopoda and insect larvae (Diptera, Coleoptera, Ephemeroptera, Trichoptera, Odonata) can be found inside bladders. By utilizing grazers, the plant regains nutrients once lost to the epiphytes and also gains nutrients that periphyton took up from water (Harms, 2002). *Utricularia* thus creates a positive feedback loop in which periphyton and grazing invertebrates serve as nutrient catalysators for *Utricularia* (Ulanowicz, 1995).

1.5. The trap of *Utricularia*

1.5.1. General structure of the trap

Traps arise from both laminae and petioles of the leaves, as well as from the stolons and their branches. Shape and size of the traps and appendages at the trap door differ considerably between species and are often used as an aid for species identification. Similarly, the size and number of trap glands exhibit great variations (Taylor, 1964). The traps range in size from 0.2 to 6 mm (Taylor, 1989).

The trap (Fig.1; Appendix, Fig.5) is a thin walled sack mostly two cells thick, obstructed by a door opening inwards. In most species, the edge of the doorway carries a pair of branched appendages, which are frequently called 'antennae'. These appendages around the doorway form a funnel to direct potential prey towards the door (Darwin, 1875; Meyers & Strickler, 1979). The threshold forms a massive thickening which preserves the shape of the opening. The upper surface of the threshold is called '*pavement epithelium*' and the door lies on it when the trap is closed. The shape of the pavement epithelium fits precisely the shape of the lower door and prevents, with the aid of cuticular *velum*, the leakage of water from the trap when the trap is set. The trigger hairs are placed immediately below the central hinge. They are stiff, sharply pointed bristles extending upwards.

Internal glands in the bladders pump water out, so that set traps are under tension with the door lodged against the velum. Mechanical stimulation such as brushing of the prey against the trigger hairs causes the door to flex open and water and material are sucked into the bladder. The prey capturing process takes about 30 ms and is one of the fastest movements in plant kingdom (Sydenham & Findley, 1973).

1.5.2. The inner side of the trap and the external glands

The inner trap surface of all *Utricularia* species is covered with typical divided glands with either two or four arms. The bifid glands in most species are restricted to the underside of the threshold while the quadrifid glands (Appendix, Fig. 6,7) are scattered all over the inner walls. Both types of glands are morphologically different, but no ultrastructural differences have been found (Fineran & Lee, 1975).

The glands of the internal surface seem to have three main functions:

(i) The removal of water during the resetting phase

(ii) The secretion of digestive enzymes

(iii) The absorbtion of digested products

Some authors (Sasago & Sibaoka, 1985a; Sydenham & Findlay, 1975) claim that glands of different morphology may perform different functions – the bifids are transferring water and the quadrifids are purely digestive. This has still to be confirmed.

Utricularia traps bear typical external glands which might be the sites of water extrusion from the traps (Sydenham & Findlay, 1975).

The surface of most *Utricularia* species is also covered with glands secreting mucilage (Appendix, Fig.8). For review of *Utricularia* trap structure and function see Juniper *et al.*, 1989.

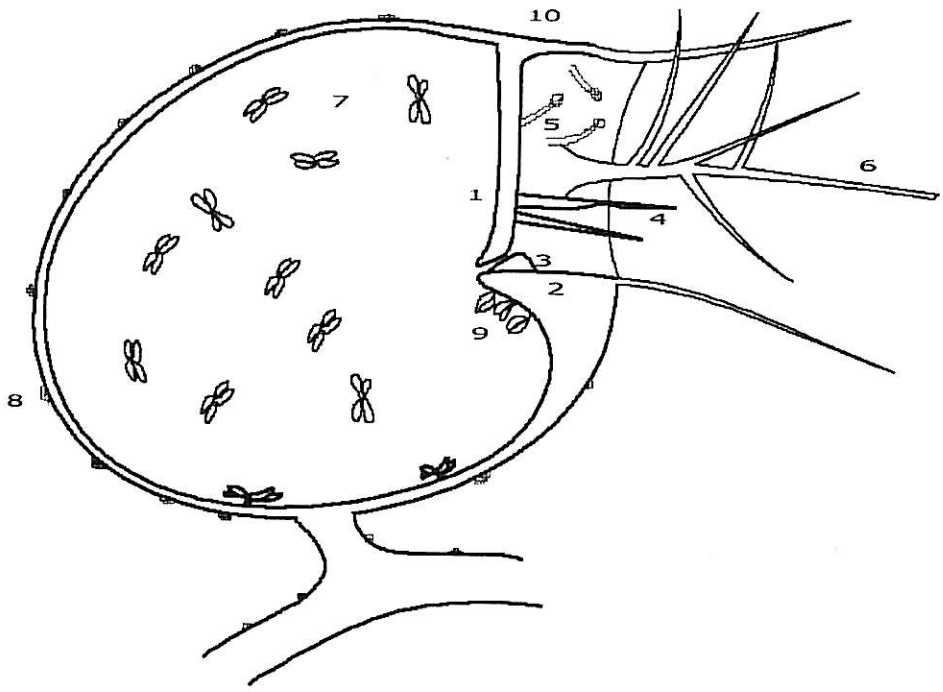

Fig.1. Schematic transverse section through the trap of *Utricularia.* (1) door; (2) pavement epithelium; (3) velum; (4) trigger hairs; (5) stalked mucilage glands; (6) antennae; (7) inner chamber with quadrifid glands; (8) spherical sessile glands; (9) bifid glands; (10) rostrum. (Redrawn after Juniper et al., 1989)

2. METERIAL AND METHODS

2.1. Plant material

Adult plants of *U. vulgaris* and *U. australis* (collected in the Czech Republic) were cultivated outdoors in plastic containers (area 2 m^2, 750 l for the former species; area 0.8 m^2, 220 l for the latter species; Adamec 1997a,b). *U. foliosa* (collected in northern Florida, USA) was grown in a greenhouse in a similar container as *U. australis*. In these cultures, water depth was kept at 25–35 cm and tap water (NO_3^--N 0.7-1.1 mg l^{-1}; NH_4^+-N<10 µg l^{-1}; PO_4-P<10 µg l^{-1}) was added to compensate for water loss. *U. aurea* (collected in Malaysia) was grown indoors in 3 l aquaria (Adamec 1999).

Litter of robust sedge species was used as a cultivation substrate in all of the above cultures, and approximately simulated natural conditions. According to the concentrations of NH_4^+ (NH_4^+-N 5-10 µg l^{-1}), NO_3^- (NO_3^--N 28-40 µg l^{-1}), HPO_4^{2-} (PO_4-P 12-15 µg l^{-1}), and humic substances (humic acids + tannins 4-10 mg l^{-1}), the water in these cultures was considered oligotrophic and humic (Adamec 1997b). The pH of the cultivation media ranged from 7.3–7.9, dissolved oxygen concentration from 0.22-0.3 mM, and free CO_2 concentration from 0.03–0.1 mM.

Cultivation methods used were analogous to those described for the aquatic carnivorous plant *Aldrovanda vesiculosa* (Adamec 1997a,b; 1999). Fine zooplankton (*Chydorus* sp., *Bosmina* sp., *Cyclops* sp. was added repeatedly to the cultures to promote plant growth. Feeding was interrupted approximately 2 weeks before trap sampling.

2.2. Collection of the trap fluid, pH measurements

Trap-bearing leaves of the four studied *Utricularia* species were cut from adult plants and placed into plastic jars (0.2 l) with filtered (mesh size 44 µm) culture water. The cuttings were collected from younger parts of the plants to ensure full trap functionality. A part of the material was used immediately to

collect trap fluid for estimation of enzymatic activity in empty traps. Fine zooplankton, which is commonly found in habitats of all aquatic *Utricularia* species, (*Chydorus* sp., size 0.5-0.6 mm, fresh weight ca. 50-100 µg) was added to the remaining material and was carefully removed after 8 h. By this time, most of the traps had fired and contained prey. Trap fluid from the fed traps of *U. foliosa, U. australis,* and *U. vulgaris* was collected 2 d later (and also after 4 d in *U. vulgaris*) during which the plastic jars were placed in the same light and temperature conditions as those under which the plants were cultivated. No zooplankton was added to traps of *U. aurea.*

Trap fluid from the largest traps (usually >2 mm) was collected by glass pipettes with fine, 0.4 mm wide tips attached to a plastic syringe. The trap door was carefully pushed inwards by the pipette tip to avoid damage to plant cells and consequent contamination. The fluid was sucked into the tip by capillary action and forced into plastic 0.5 ml Eppendorf filtration vials placed on ice. The fluid (volume ca. 0.5 ml) collected from 90–300 traps of each *Utricularia* species was pooled into a vial equipped with a filter (0.2 µm) and centrifuged at 600–1200 g for 10–15 min. This procedure filtered out thousands of algal cells, mainly *Euglena* sp., which could burst upon freezing and contaminate the samples. Several vials with filtrate were prepared in this way and frozen at –20°C. The abundance of *Euglena* cells in parallel fluid samples was estimated using a Petroff-Hausser counting chamber (depth 0.04 mm) and a light microscope (magnification 200×). This was repeated 10 times for each *Utricularia* species.

The pH of the trap fluid was estimated roughly using a pH paper (Lachema, Brno, Czech Rep.). A piece of the pH paper of 3×3 mm was placed on clean white plastic pad and soaked in the fluid pooled from 10 traps (volume ca. 20 µl). Colour change of the pH paper was compared with the standard colour scale. This was repeated 5 times for each of the *Utricularia* species.

2.3. Enzymatic assay

A common fluorometric method (Hoppe 1983, 1993) was adopted for a microplate assay (Marx et al., 2001) and further designed to determine enzyme activity in both trap fluid and ambient water samples. We used five fluorogenic substrates, L-leucine 7-amino-4-methylcoumarin hydrochloride, 4-methylumbelliferyl (MUF) phosphate, MUF N-acetyl-β-D-glucosaminide, MUF α-D-glucoside, and MUF β-D-glucoside (Glycosynth, Warrington, UK), to estimate the activity of aminopeptidases, phosphatases, β-hexosaminidases, α-glucosidases, and β-glucosidases, respectively. We used special black 96-well microplates for fluorescence detection (Nunc, Roskilde, Denmark) in two different microplate set-ups as follows: (i) For activity in traps, all wells were filled with 300 µl of 2 mM acetate buffer (pH 4.7). Then 24 wells were filled with the same trap fluid at 10 µl each. (ii) For activity in ambient water, 300 µl of an ambient water sample and 20 µl of 100 mM acetate buffer (pH 4.7) were added in each of 48 wells. Then, respectively, tetrads or octets of the wells were supplemented with one of the five above substrates at 50 µl each (300 µM final concentration) and mixed afterwards; distilled water at 50 µl each was added to one tetrad or octet of control wells as background fluorescence.

Immediately after adding all substrates, the microplate was inserted into the microplate reader to detect the rate of hydrolysis of each substrate during the next two hours (= incubation time, t). Relative fluorescence in each well was measured (excitation/emission: 365 nm/445 nm wavelengths, 1 nm/5 nm band passes, respectively) at five-minute intervals with the spectrofluorometer FluoroMax-3/MicroMax (Jobin Yvon/Spex–Horiba, USA–France). A linear increase in fluorescence over time indicated hydrolysis of a particular substrate. Using linear regression of the mean of 4 or 8 replicates, corrected for the average background fluorescence, we determined a relative fluorescence rate (r_{RF}) as a slope of the regression line.

For calibration of the method, we filled a microplate in either set-up with appropriate amounts of sample and buffer but, instead of the corresponding substrate, we added 50 µl of 7-amino-4-methylcoumarin or 4-

methylumbelliferone as the standard at the following final concentrations: 0, 10, 20, 40, 60 and 80 µM. We determined a calibration factor (f_C) as a slope of the regression line. Finally, we calculated the rate of hydrolysis (v, in µmol l^{-1} h^{-1}) according to the following equation:

$$v = \frac{r_{RF}}{f_c \cdot t}$$

2.4. Determination of pH optimum

To determine enzymatic pH optimum, we estimated activities of aminopeptidase and phosphatase both in the fluid from the traps and in the ambient water. The media were buffered to pH values between 4 and 9. We adjusted acetate buffers to pH values of 4.0, 4.7, and 5.5, and Tris/HCl buffers to pH 7.0 and pH 9.0 and used them at the same concentrations as those used above. We also used the corresponding microplate set-ups and substrates for detection of r_{RF} as described above. Values measured at each pH were expressed as % of the maximum r_{RF} within the pH range of 4–9.

2.5. Statistical treatment

The small volume of trap fluid collected from single traps and the method of fluid filtration allowed us to collect only one mixed sample for each *Utricularia* species and time point. Thus, it was not possible to collect independent samples of the fluid and the tetrads of single mixed samples (i.e., pseudoreplicates) therefore represented only the variability of activity measurements, not the variability of the material used. In consequence, we could not evaluate differences among species or variants statistically. Relationships between the enzymatic activities in the empty traps and culture water were tested using the nonparametric Spearman correlation (Prism 3.0; *www.graphpad.com*).

3. RESULTS

Estimated pH values in trap liquids varied between 4.9–5.4 in both empty and fed traps of all four *Utricularia* species, although old plants of *U. vulgaris* had a lower pH (Table 1). Tables 2 and 3 present hydrolysis rates for five artificial substrates estimated in traps of four *Utricularia* species and in ambient water from the particular cultures, respectively, expressed in the same units (enzymatic activity per litre of trap fluid or water). Among empty traps of growing plants, phosphatases always exhibited the highest activity (6.1–29.8 μmol l^{-1} h^{-1}) followed by β-glucosidases (1.35–2.95 μmol l^{-1} h^{-1}), while the activities of α-glucosidases, β-hexosaminidases, and aminopeptidases were usually lower by one or two orders of magnitude (0–1.60, 0–0.356, and 0.076–0.116 μmol l^{-1} h^{-1}, respectively; Table 3). Empty functioning traps of old *U. vulgaris* plants (forming turions) exhibited nothing but phosphatase activity, which was 30 times lower than in the empty traps of growing plants of the same species. While phosphatase activity expressed per unit volume in the trap fluid was always lower than that in the ambient water, activities of the other enzymes at the same pH of 4.7 were usually higher in the ambient water than in the trap fluid (cf. Tables 2 and 3). Correlation analyses did not show any clear relationship between the activities in the trap fluid and those in the ambient water, although an overall correlation of all enzymatic activities in empty traps of all four species with those in ambient waters was significant (r_s=0.65; P=0.0004; n=25). Its significance indeed was mainly due to the high phosphatase activities in the traps (Fig. 2). Both correlations of all five enzymes (with phosphatase being an outlier) for one *Utricularia* species (i.e., one cultivation container or aquarium) and of one enzyme for all four species were insignificant in all but one cases.

Aminopeptidases in the empty traps of *U. vulgaris* and in its ambient water responded similarly to ascend pH, while phosphatases showed very distinct pH optima (Fig. 3). Phosphatase activity in traps exhibited maximum activity (12.6 μmol l^{-1} h^{-1}) at pH 5.5; the values at both pH 4.0 and pH 9.0 were close to zero. On the contrary, in the culture water, no phosphatase activity was detected at low pH (4.0–5.5), 13 % of the maximum activity (7.4 μmol l^{-1} h^{-1}) was

detected at pH 7.0 (pH close to that in the culture water), while considerable activity of 58 µmol l^{-1} h^{-1} was observed at pH 9.0 (Fig. 3a). The comparison of data in Table 3 and Fig. 2a shows that activity of phosphatases (at the pH 4.7) in the culture water was greatly variable in time. The aminopeptidases, both in the trap fluid and in the ambient water, were inactive at lower pH values (4.0 and 4.7) but showed high activity at higher pH values (Fig. 3b).

After addition of prey, the fed traps of *U. foliosa* and *U. australis* showed conspicuous decreases in all enzymatic activities after 2 d. In contrast, all activities increased remarkably in the fed traps of *U. vulgaris* after 2 d, though activities of some enzymes subsequently declined (Table 3).

	U. aurea	***U. foliosa***	***U. australis***	***U. vulgaris***
empty growing traps	5.20 (5.1–5.4)	5.05 (5.0–5.1)	4.95 (4.9–5.1)	4.97 (4.9–5.1)
empty old traps	n.d.	n.d.	n.d.	4.24 (4.2–4.3)
fed traps after 2 days	n.d.	5.06 (5.0–5.1)	4.93 (4.9–5.1)	4.93 (4.9–5.1)
fed traps after 4 days	n.d.	n.d.	n.d.	4.96 (4.9–5.1)

Table 1. Estimated pH values of the fluid sucked out from *Utricularia* traps. Means (ranges) of five parallel determinations are shown; n.d., not determined.

Enzymes	**Activity in the water samples (µmol l^{-1} h^{-1})**				
	U. aurea	***U. foliosa***	***U. australis***	***U. vulgaris***	***U.vulgaris Old plants***
Aminopeptidase	0.000	0.117±0.005	0.088±0.003	0.085±0.003	0.000
Phosphatase	1.82±0.27	3.17±0.12	3.63±0.15	3.11±0.11	0.425±0.063
β-hexosaminidase	0.104±0.007	1.44±0.05	2.57±0.10	3.40±0.11	0.000
α-glucosidase	0.000	0.841±0.058	2.53±0.10	3.52±0.25	0.000
β-glucosidase	0.289±0.119	1.92±0.07	2.10±0.09	5.04±0.20	0.045±0.003

Table 2. Activity (at pH 4.7) of five enzymes in ambient samples of the water in which plants were cultured. Means (±2SD) of eight parallel determinations are shown.

Enzymes	Activity in the fluid from the traps (μmol l^{-1} h^{-1})								
	U. aurea	*U. foliosa*		*U. australis*		*U. vulgaris*			*U. vulgaris* old plants
	0 d	0 d	2 d	0 d	2 d	0 d	2 d	4 d	0 d
Aminopeptidase	0.076±0.010	0.022±0.001	0.000	0.055±0.003	0.026±0.001	0.048±0.002	0.116±0.007	0.066±0.006	0.000
Phosphatase	29.6±8.2	6.12±0.28	0.511±0.021	7.62±0.31	0.521±0.018	29.8±4.1	99.9±6.9	65.3±9.2	0.933±0.050
β-hexosaminidase	0.356±0.015	0.000	0.000	0.096±0.003	0.000	0.286±0.008	1.95±0.07	2.19±0.09	0.000
α-glucosidase	0.000	1.60±0.07	0.847±0.048	0.774±0.053	0.000	0.043±0.001	0.821±0.062	2.85±0.22	0.000
β-glucosidase	1.81±0.23	2.95±0.17	1.53±0.10	1.35±0.06	0.000	1.40±0.05	15.0±0.6	4.77±0.26	0.000

Table 3. Activity of five enzymes at pH 4.7 in the fluid sucked out from Utricularia traps and ultrafiltered. Old plants of U. vulgaris formed turions; 0 d, empty traps; 2 d, traps with prey after 2 d; 4 d, traps with prey after 4 d. Means (±2SD) of 4 parallel determinations are always shown

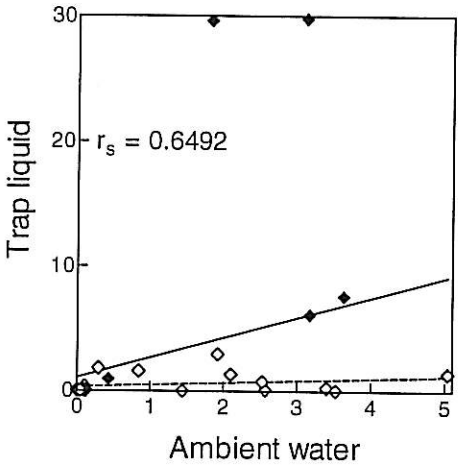

Fig. 2. Correlation of all enzyme activities in the empty traps of four *Utricularia* species and in the ambient waters (both in µmol l^{-1} h^{-1}). Spearman rank coefficient and solid regression line for all data (n=25); solid symbols are the phosphatases, dashed regression line applies only for the other enzymes (open symbols, n=20).

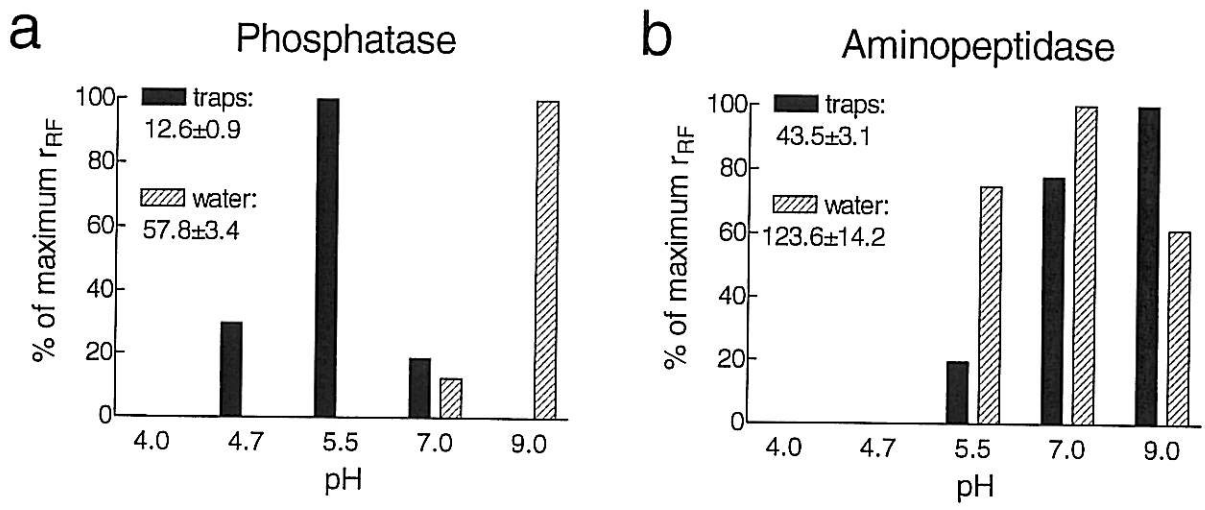

Fig. 3. pH optima of phosphatases (a) and aminopeptidases (b) in the trap fluid of *U. vulgaris* and its ambient water. Absolute values (mean ±2SD; in µmol l^{-1} h^{-1}) of each maximum activity are given in the legends.

4. DISCUSSION

Among digestive enzymes of carnivorous plants, acid peptidases and acid phosphatases have been reported commonly to occur in traps of various terrestrial or aquatic species (e.g., Tökés et al., 1974; Clancy and Coffey, 1976; for the review see Juniper et al., 1989). Yet, this study is, as far as we know, the first attempt to measure in parallel activities of five hydrolytic enzymes both in the trap fluid collected from the most common aquatic *Utricularia* species and in the ambient water. Surprisingly, we did not observe remarkable aminopeptidase activities in either empty or fed traps of four *Utricularia* species under study (Table 2). Moreover, the ambient aminopeptidase activity was usually several times higher than that in the trap fluid in the plants (cf. Tables 2 and 3). This fact, together with the similar pH optima of the aminopeptidases both in the ambient water and in the empty traps of *U. vulgaris*, suggested that production of aminopeptidases in the *Utricularia* traps was very low. Furthermore, considering that ca. 40 % of the resting trap volume was sucked in from the ambient water as a result of firing (Juniper et al., 1989) before trap collection, a part of the trap aminopeptidase activity could come from the ambient water. Overall peptidase activities found in all *Utricularia* traps, however, were 1–2 orders of magnitude lower than those determined in the pitcher fluid of carnivorous *Heliamphora tatei* (Jaffe et al., 1992): 50–175 mg l^{-1} h^{-1} of bovine albumin, i.e., 0.76–2.65 μmol $l^{-1}h^{-1}$.

On the other hand, our results clearly showed important release of the acid phosphatase and its accumulation in the empty traps of all the *Utricularia* species tested. Its activity in the trap fluids exceeded the phosphatase activity in the ambient water about 2–18 times (both measured at pH 4.7). Moreover, typical acid phosphatases were active at pH 4.7 in the trap fluid of *U. vulgaris*, whereas those in the ambient water were of alkaline nature (Fig. 2). Clancy and Coffey (1976) also reported release of various acid phosphatases by a terrestrial carnivorous plant, *Drosera rotundifolia*; some of them with similar pH optima around 5.0. However, they detected the first enzyme release 1 to 2 days after feeding, the maximum activity on day 4, and then a gradual decrease. In two

aquatic *Utricularia* species, conversely, we usually observed a marked decrease in activities of all five enzymes in the traps 2 days after feeding. The only exception was *U. vulgaris*, with a similar response like in *D. rotundifolia* (Clancy and Coffey, 1976) after feeding (Table 3). No information exists whether the enzyme secretion by *Utricularia* traps is permanent or stimulated by prey (Juniper et al., 1989). However, the former case is much more probable, as suggested by the relatively high enzyme activities in empty traps (Table 3).

Such a distinct course of enzyme activities between *Utricularia* species after feeding might be due to a quite different digestion rate among the species or a different abundance of comensals in the traps (e.g. *Euglena*) facilitating prey digestion. As opposed to direct cytochemical detection of certain enzymes in the quadrifide gland cells of *Utricularia* traps (Vintéjoux, 1973, 1974; Heslop-Harrison, 1975; Parkes, 1980), determination of enzyme activities in the trap fluid does not prove the plant origin of these enzymes but it shows the in-situ enzyme capacity for prey digestion. Although the role of *Euglena* and other comensals in prey digestion has been assumed in *Utricularia* traps (Hegner, 1926; Jobson et al., 2000; Richards, 2001) direct evidence is lacking. Jobson et al. (2000) observed that feeding on *Euglena* sp. significantly reduced the growth of terrestrial *U. uliginosa*. It might indicate that *Euglena* in the traps likely competed for limited nutrient sources, thus behaving as parasites rather than comensals (Appendix, Fig.9). Estimated abundance of *Euglena* cells in the fluid averaged about 600 cells per trap. There were no significant differences in cell numbers between empty and fed traps, however, differences were observed between young and older traps.

The very low trap activity of aminopeptidases, in contrast to phosphatases found in all four *Utricularia* species under study, is in a discrepancy with a greatly efficient and rapid uptake of both N and P from prey in *U. vulgaris* traps (Knight, 1988; Friday and Quarmby, 1994). On the other hand, *U. foliosa* under natural conditions took up 1.75 % of its seasonal P gain, while only 0.44 % of that of N from carnivory (Bern, 1997). It can imply that the P

uptake from prey in *Utricularia* traps is more important than that of N in P-limited waters.

We also tried to detect directly the activity of phosphatases in the quadrifide glands of eight aquatic *Utricularia* species (*U. aurea, U. australis, U. foliosa , U. vulgaris, U. bremii* Heer ex Kölliker, *U. floridana* Nash, *U. ochroleuca* R. Hartman, *U. purpurea* Walter). Using enzyme labeled fluorescence, a phosphatase activity in the glands was clearly detected only in *U. australis* and *U. ochroleuca* (see Appendix, Fig. 13 for details).

Glycolytic enzymes have never been tested in *Utricularia* traps so far (Juniper et al., 1989). It follows from the comparison of activities of these three enzymes (β-hexosaminidase, α- and β-glucosidase) in empty traps and in the ambient water in our study that these enzymes were produced, at least partly, inside the traps. Though β-hexosaminidase (i.e., chitinase) was detected in traps of some carnivorous plant species the origin of this activity and its physiological importance for prey digestion remain uncertain (see Juniper et al., 1989).

Extracellular enzyme activities usually used to be high in humic freshwaters. However, except for aminopeptidase, all enzyme activities estimated in the ambient water samples at pH of 4.7 were by one or two orders of magnitude higher compared to any values published from various natural freshwaters so far (for review, see Münster and De Haan, 1998). Moreover, we determined only dissolved activities, i.e., in 0.2 µm filtrates of ambient waters. Only Bittl et al. (2001) detected similar high values of acid phosphatases in atmospherically acidified forest mountain lakes.

Though overall production of acid phosphatase and its release into the *Utricularia* traps was obvious, the origin of other enzyme activities observed in the traps remained unclear. In those cases, when a trap enzyme activity was lower than that in the ambient water, we could suppose rather an allochthonous origin of the enzyme activity in the trap. After firing, an empty trap could suck in ambient water with dissolved enzymes. Then, the plant, restoring the underpressure by removing the water, could even concentrate the ambient enzymes in its traps to activities slightly higher than in the environment.

5. SUMMARY

Enzymatic activity of five hydrolases was measured fluorometrically in the fluid collected from traps of four aquatic Utricularia species and in the water in which the plants were cultured.

In empty traps, the highest activity was always exhibited by phosphatases (6.1–29.8 μmol l^{-1} h^{-1}) and β-glucosidases (1.35–2.95 μmol l^{-1} h^{-1}), while the activities of α-glucosidases, β-hexosaminidases, and aminopeptidases were usually lower by one or two orders of magnitude. Two days after addition of prey (*Chydorus* sp.), all enzymatic activities in the traps decreased conspicuously in *U. foliosa* and *U. australis* but markedly increased in *U. vulgaris.*

Phosphatase activity in the empty traps was 2–18 times higher than that in the culture water at the same pH of 4.7, but activities of the other trap enzymes were usually higher in the water. Correlative analyses did not show any clear relationship between these activities.

Trap comensals (*Euglena*) could partly be responsible for production of some trap enzymes. The traps can produce phosphatases independently of catching prey. Taking into account the enzymatic activities in traps, phosphorus uptake from prey might be more important than that of nitrogen for the plants.

6. APPENDIX

Fig.4. The trap of *U.gibba* attached filaments of the epiphytic green alga *Oedogonium*. (Magnification 100x)

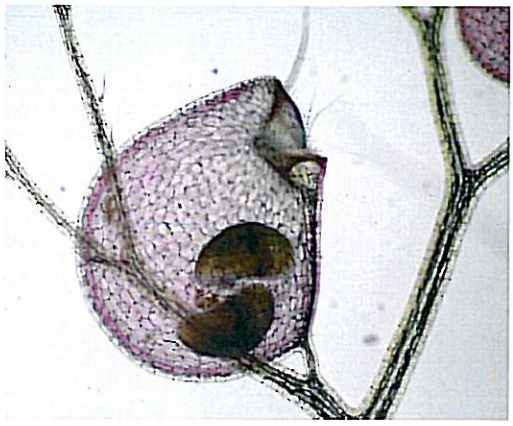

Fig.5. Side view of the *U. aurea* trap with microcrustacean prey visible inside. (Magnification 100x)

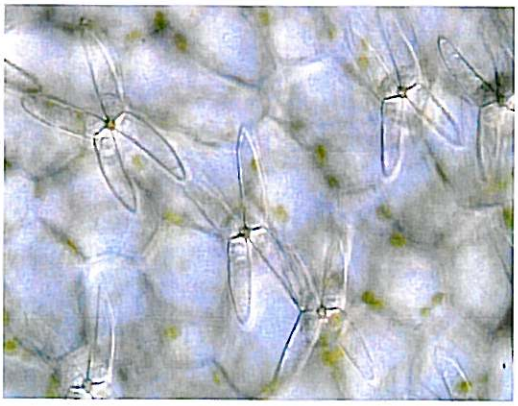

Fig.6. Quadrifid glands of *U.gibba*. (Magnification 400x)

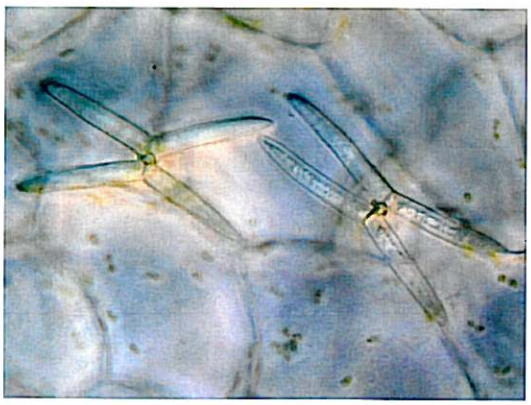

Fig.7. Quadrifid glands of *U. Floridana*. (Magnification 400x)

Fig.8. Stalked mucilage glands, *U.dimorphanta.* (Magnification 400x)

Fig.9. Microcrustacean prey inside the trap of *U.gibba* with feeding Euglena cells. (Magnification 400x)

Fig.10. Flowering plants of *U. foliosa*

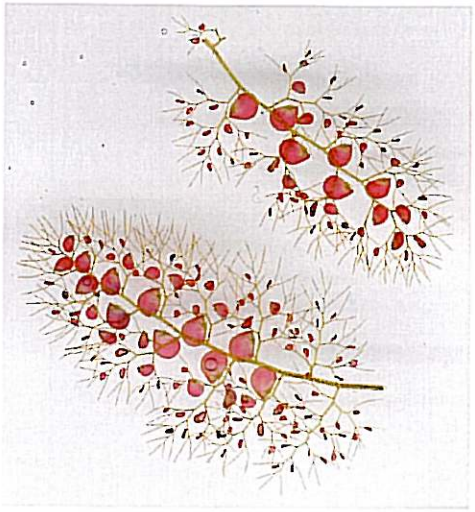

Fig.11. Trap-bearing branches of *U. vulgaris*

Fig.12. Trap-bearing branches of *U. foliosa*

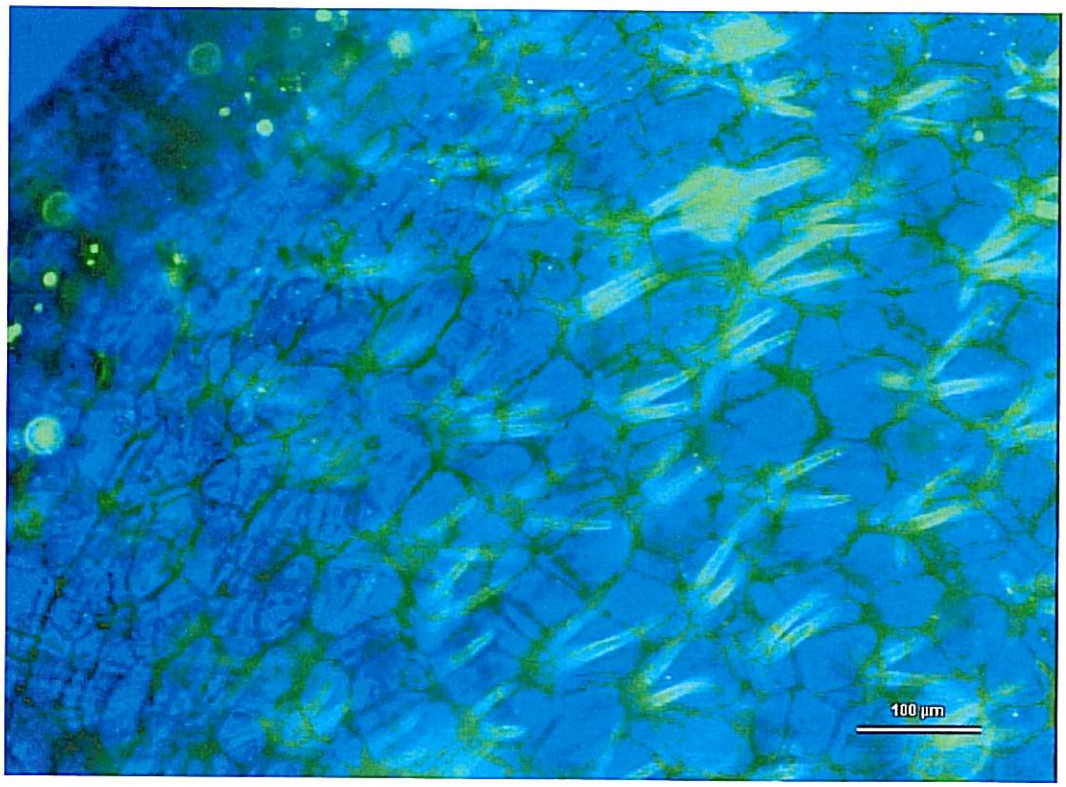

Fig.13. Microphotograph of the trap of *U. australis* showing localisation of phosphatase activity in the quadrifid glands using enzyme labelled fluorescence.

7. REFERENCES

Adamec L. 1997a. Photosynthetic characteristics of the aquatic carnivorous plant *Aldrovanda vesiculosa*. *Aquatic Botany* **59:** 297-306.

Adamec L. 1997b. How to grow *Aldrovanda vesiculosa* outdoors. *Carnivorous Plant Newsletter* **26:** 85-88.

Adamec L. 1999. The biology and cultivation of red Australian *Aldrovanda vesiculosa*. *Carnivorous Plant Newsletter* **28:** 128-132.

Adowa AN. 1924. Zur Frage nach den Fermenten von *Utricularia vulgaris* L. II. Der relative Gehalt der Blasen und Zweige von *Utricularia vulgaris* an Proteoklastischen Fermenten. *Biochemische Zeitung* **153:** 506-509.

Albert VA, Williams SE, Chase MV. 1992. Carnivorous plants: phylogeny and structural evolution. *Science* **257**: 1491-1495

Bern AL. 1997. *Studies on nitrogen and phosphorus uptake by the carnivorous bladderwort,* Utricularia foliosa, *in south Florida wetlands.* PhD thesis, Florida International University, Miami, USA.

Bittl T, Vrba J, Nedoma J, Kopáček J. 2001. Impact of ionic aluminium on extracellular phosphatases in acidified lakes. *Environmental Microbiology* **3:** 578-587.

Botta SM. 1976. Sobre las trampas y las víctimas o presas de algunas especies argentinas del género *Utricularia*. *Darwiniana* **20:** 127-154.

Brown JH and Lomolino MV. 1998. *Biogeography*, 2nd edn. Sinauer Associates, Inc., Sunderland, MA, USA

Carpenter SR and Lodge DM. 1986. Effects of submersed macrophytes on ecosystem processes. *Aquatic Botany* **26**: 341-370

Clancy FGA, Coffey MD. 1976. Acid phosphatase and protease release by the insectivorous plant *Drosera rotundifolia*. *Canadian Journal of Botany* **56:** 480-488.

Cohn F. 1875. Über die Function der Blasen von *Aldrovanda* und *Utricularia*. *Beiträge zur Biologie der Pflanzen* **1:** 71-92.

Darwin C. 1875: Insectivorous plants. John Murray, London

Friday LE. 1992. Measuring investment in carnivory: Seasonal and individual variation in trap number and biomass in *Utricularia vulgaris* L. *New Phytologist* **121**: 439-445

Friday LE, Quarmby C. 1994. Uptake and translocation of prey-derived ^{15}N and ^{32}P in *Utricularia vulgaris* L. *New Phytologist* **126:** 273-281.

Fryer G. 1968. Evolution and adaptive radiation in the Chydoridae (Crustacea:Cladocera): a study in comparative functional morphology and ecology. *Phil. Trans. R. Soc. Lond. B* **254**: 221-385

Fryer G. 1974. Evolution and adaptive radiation in the Macrothricidae (Crustacea:Cladocera): a study in comparative functional morphology and ecology. *Phil. Trans. R. Soc. Lond. B* **269**: 137-274

Gallie D, Chang S. 1997. Signal transduction in the carnivorous plant *Sarracenia purpurea*. Regulation of secretory hydrolase expression during development and in response to resources.
Plant Physiology **115**: 1461-1471

Hada Y. 1930. The feeding habits of *Utricularia. Transactions of the Sapporo Natural History Society* **11:** 175-183.

Harms S. 2002. The effect of bladderwort (*Utricularia*) predation on microcrustacean prey. *Freshwater Biology* **47**: 1608-1617

Hegner RW. 1926. The interrelationships of protozoa and the utricles of *Utricularia. Biological Bulletin* **50:** 239-270.

Heslop-Harrison Y. 1975. Enzyme release in carnivorous plants. In: Dingle JT, Dean RT, eds. *Lysozymes in biology and pathology.* Amsterdam, The Netherlands: North Holland Publishing Company, **4,** 525-578.

Hoppe HG. 1983. Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates. *Marine Ecology Progress Series* **11:** 299-308.

Hoppe HG. 1993. Use of fluorogenic model substrates for extracellular enzyme activity (EEA) measurement of bacteria. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ, eds. *Handbook of methods in aquatic microbial ecology.* Boca Raton, USA: Lewis Publishing, 423-431.

Jaffe K, Michelangeli F, Gonzales JM, Miras B, Ruiz MC. 1992. Carnivory in pitcher plants of the genus *Heliamphora* (Sarraceniaceae). *New Phytologist* **122:** 733-744.

Jeppesen E, Sondergaard Ma, Sondergaard Mo, Christoffersen K. 1998. *The structuring role of submerged macrophytes in lakes.* Springer, Berlin, Heidelberg, New York.

Jobson RW, Morris EC. 2001. Feeding ecology of a carnivorous bladderwort (*Utricularia uliginosa*, Lentibulariaceae). *Australian Ecology* **26:** 680-691.

Jobson RW, Morris EC, Burgin S. 2000. Carnivory and nitrogen supply affect the growth of the bladderwort *Utricularia uliginosa*. *Australian Journal of Botany* **48:** 549-560.

Juniper BE, Robins RJ, Joel DM. 1989. *The carnivorous plants.* London, UK: Academic Press.

Knight SE. 1988. *The ecophysiological significance of carnivory in* Utricularia vulgaris. PhD thesis, University of Wisconsin, Madison, USA.

Knight SE and Frost TM. 1991. control in *Utricularia macrorhiza*: Lake-specific variation in plant investment in carnivory. *Ecology* **72**: 728-734

Luetzelberg P. von 1910. Beiträge zur Kenntnis der *Utricularia*. *Flora* **100:** 145-212.

Marx MC, Wood M, Jarvis SC. 2001. A microplate fluorimetric assay for the study of enzyme diversity in soils. *Soil Biology & Biochemistry* **33:** 1633-1640.

Meyers DG, Strickler JR. 1979. Capture Enhancement in a Carnivorous Aquatic Plant: Function of Antennae and Bristles in *Utricularia vulgaris*. *Science* **203**: 1022-1025.

Münster U, De Haan H. 1998. The role of microbial extracellular enzymes in the transformation of dissolved organic matter in humic waters. In: Hessen DO, Tranvik L, eds. *Aquatic humic substances. Ecological studies, Vol.* 133: 199-257.

Nedoma J, Štrojsová A, Vrba J, Komárková J, Šimek K. 2003. Extracellular phosphatase activity of natural plankton studied with ELF97 phosphate: fluorescence quantification and labelling kinetics. *Environmental Microbiology* (in press).

Parkes DM. 1980. *Adaptive mechanisms of surfaces and glands in some carnivorous plants.* MSc thesis, Monash University, Clayton, Victoria, Australia.

Richards JH. 2001. Bladder function in *Utricularia purpurea* (Lentibulariaceae): is carnivory important? *American Journal of Botany* **88:** 170-176.

Sasago A, Sibaoka T. 1985. Water extrusion in the trap bladders of Utricularia vulgaris.2. A possible mechanism of water outflow.
Bot Mag Tokyo **98**: (1050) 113-124

Schumacher GJ. 1960. Further notes on the occurrence of desmids in *Utricularia* bladders. *Castanea* **25:** 62-65.

Sydenham PH, Findlay GP. 1975. Transport of solutes and water by resetting bladders of *Utricularia. Australian Journal of Plant Physiology* **2:** 335-351.

Sydenham PH, Findlay GP. 1973. The rapid movement of the bladder of Utricularia spec. *Aust. J. Biol. Sci. Melbourne* **26**,1115-1126

Taylor P. 1964. The Genus *Utricularia* (Lentibulariaceae) in Africa (South of the Sahara) and Madagascar. *Kew Bulletin* **Vol. 18** No. 1.

Taylor P. 1989. *The genus* Utricularia*: a taxonomic monograph.* Kew Bulletin Additional Series XIV. HMSO, London.

Tökés ZA, Woon WC, Chambers SM. 1974. Digestive enzymes secreted by the carnivorous plant *Nepenthes macfarlanei* L. *Planta* **119:** 39-46.

Ulanowicz RE. 1995. Utricularia`s secret: the advantage of positive feedback in oligtrophic environments. *Ecological Modelling* **79**: 49-57

Vintéjoux C. 1973. Études des aspects ultrastructuraux de certaines cellules glandulaires and raport avec leuc activité sécrétive chez l' *Utricularia neglecta* L. (Lentibulariaceae). *Comptes Rendues* **277D,** 2345-2348.

Vintéjoux C. 1974. Ultrastructural and cytochemical observations on the digestive glands of *Utricularia neglecta* L. (Lentibulariaceae). Distribution of protease and acid phosphatase activities.
Portugaliae Acta Biologica Series A **14:** 463-471.

Wallace RL. 1977. Distribution of sessile rotifers in an acid bog pond. *Archiv fűr Hydrobiologie* **79**: 478-505

Wallace RL. 1977. Substrate selection by larvae of the sessile rotifer Ptygura beauchampi. *Ecology* **59**: 221-227