Aging of *Utricularia* traps and variability of microorganisms associated with that microhabitat

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ABSTRACT

Various authors have described algae in aquatic *Utricularia* traps as commensals, as stress factors or as prey. This study examined the diversity and abundance of organisms (prey, algae, protozoa and bacteria) in the traps of aquatic *Utricularia reflexa* in relation to prey occurrence and trap age. The number of organisms increased with the trap age. In both young and old traps, phytoplankton dominated of all organisms found. In young traps, *Scenedesmus* spp. and *Characiopsis* sp. were the most abundant algae, while *Scenedesmus* spp. and the palmelloidal form of *Euglena* spp. dominated in the old traps. Most of the algal species found stayed alive in the trap environment. The number of living algae and ciliates inside the traps increased with the increasing trap age, too. As the number of *Paramecium bursaria* inside traps consistently increased with the trap age and number of bacteria, which serve as a food for them, ciliates can be regarded as commensals, but not as prey for the plant. The predominant organisms in the traps were those that can be considered either commensals or intruders, exceeding captured macroscopic prey.

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1. Introduction

Aquatic and amphibious species of the genus *Utricularia* attract and trap fine zooplankton ("The real use of the bladders is to capture small aquatic animals, and this they do on a large scale." Darwin, 1875: p. 404), mainly crustaceans, and later they utilize nutrients from these animals (Harms, 1999; Mette et al., 2000; Friday and Quarmby, 1994; Sanabria-Aranda et al., 2006; Guisande et al., 2007). According to Darwin, organisms trapped by *Utricularia* died as a result of oxygen depletion (see also Płachno, 2010). Recently, Adamec (2007) showed in six aquatic *Utricularia* species that the trap fluid is anoxic, and postulated that the captured zooplankton cannot survive under these conditions. He also suggested that organisms that do not tolerate anoxia are the plants' prey, while those that can live without oxygen may be commensals. Richards (2001) suggested for *U. purpurea* growing in barren waters that the mutualistic relationships inside the traps are more important than pure carnivory based exclusively on the predator-prey relationship. Since then the role of carnivory for *Utricularia* has been the subject of lively debate among several authors (Sirová et al., 2003, 2009,

2010; Adamec, 2007, 2011; Peroutka et al., 2008; Alkhalaf et al., 2009), but the exact role of commensals in plant nutrient uptake has never been determined precisely.

Darwin was one of the first to notice that many living organisms are not killed by the plant in the bladders of *Utricularia*: "In all cases the bladders with decayed remains swarmed with living Algae of many kinds, Infusoria, and other low organisms, which evidently lived as intruders" (Darwin, 1875: p. 405). Despite many years of research, the environment inside *Utricularia* bladders as well as the digestion mechanisms in these traps have not yet been fully understood and explained, but there has been much progress recently. For example, it is now known that *Utricularia* traps are heavily colonized by bacteria, which form the main live microbial biomass in the traps (Sirová et al., 2009), and that the plant supplies the microbial community with organic C (Sirová et al., 2010). Alkhalaf et al. (2009) suggested that ciliates inside the bladders might feed on the debris and control the abundance and biomass of bacteria. Other authors (Mette et al., 2000; Seine et al., 2002) consider protozoa to be prey for bladderworts. Another and somewhat controversial group within *Utricularia* traps consists of algae (e.g., Darwin, 1875; Adamec and Komárek, 1999; Mette et al., 2000; Richards, 2001; Gordon and Pacheco, 2007; Peroutka et al., 2008, Alkhalaf et al., 2009; Wołowski et al., 2011), whose role has not been determined so far. Algae (phytoplankton) in aquatic *Utricularia* traps

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have been considered as organisms having a positive influence on the plant (Alkhalaf et al., 2009; Sirová et al., 2009), as intruders (Darwin, 1875), as stress factors (parasites), or as prey (Adamec and Komárek, 1999; Mette et al., 2000; Gordon and Pacheco, 2007; Peroutka et al., 2008). Płachno et al. (2006) found that cyanobacteria and algae in *Utricularia* traps showed phosphatase activity. Later, Sirová et al. (2009) supported these results and additionally showed that bacteria in the traps also secreted exogenous enzymes. In the related genus *Genlisea*, traps are microhabitats for many species of algae, some of which produce digestive enzymes and help in prey digestion (Płachno and Wołowski, 2008; Płachno et al., 2009). Płachno and Wołowski (2008) suggested that the plants can use nutrients from algae in the trap; the nutrients are released by lysis of algal cells due to senescence or other processes.

The environmental conditions inside *Utricularia* traps change as the traps age. According to Sirová et al. (2009), the activity of enzymes, the pH of trap fluid, and also the microorganism community change with increasing trap age and nutrient accumulation. The relationship between prey functions and the trap community remain unclear. Sirová et al. (2009) found the decrease of the number of bacteria in the fluid of the older traps. In another work, no significant differences in number of *Euglena* between empty traps and those with prey were found (Sirová et al., 2003). Recently it has even been suggested that "vegetarian" or detritivorous modes of nutrition dominate over true carnivory in aquatic *Utricularia* species growing in nutrient-poor waters with low prey availability (Peroutka et al., 2008; Alkhalaf et al., 2009).

In this study we examined the diversity and abundance of organisms in the traps of greenhouse-grown *Utricularia reflexa* in relation to prey occurrence and trap age. We tested the correlation between the abundance of protozoa in the traps and the number of captured animals and their dead remains. We discuss the role of trap commensals for *Utricularia* ecology.

2. Materials and methods

2.1. Plant material

Adult plants of *U. reflexa* Oliv. (see Fig. 1) (collected in the Okavango Delta, Botswana) were cultivated in an open, naturally lit glasshouse in the Institute of Botany (Czech Academy of Sciences, Třeboň, Czech Rep.) in a white plastic container (area 0.8 m², 300 l; for details see Adamec et al., 2010) for three weeks. Irradiance at plant level was about 42% of its value in the open (i.e., 350–700 μmol m^{-2} s^{-1} PAR during sunny days). Water depth was kept at 35 cm and tap water was used. During the growth period, maximum daily water temperature ranged from 22 to 28 °C, and night minima from 20 to 24 °C. *Carex* litter was used as substrate. The water in these cultures was considered oligotrophic and humic. The pH of the media ranged from 7.7 to 7.8, and free CO_2 concentration from 0.05 to 0.06 mM. Fine zooplankton was added repeatedly to the container to promote plant growth. The sampled material included two age categories of traps: young (traps collected from the sequence of leaf nodes near the apex of the plant) i.e., fully developed and capable of capturing prey; and old traps (traps collected from the sequence of leaf nodes near the middle part of the plants), containing a large amount of prey. Totally, 10 plants (shoots) were collected and 1–2 traps from each plant and age category were sampled but the data were not kept in pairs for both trap ages. They were fixed in 2.5% glutaraldehyde and in 0.05 M cacodylate buffer (pH 7.0). The fixation immediately killed both the plant material and all the organisms inside the traps. Theoretically, dead traps may not be fully closed and if so, a part of dead organisms could be released out of the traps to the fixation liquid. However, because all traps were identically processed and the counts of dead

organisms found inside the traps were relatively high, this hypothetical artifact could have only a minor influence on our results. Plant material and the fixed ambient water were sampled on 20 August 2009. Additional samples of the water and traps were fixed in September.

For investigation of living commensals, living plants (8 shoots) of *U. reflexa* were transported to the Department of Phycology of the Institute of Botany (Polish Academy of Sciences, Kraków, Poland) and cultivated. They were cultivated in the culture water in which they were transported from the Institute of Botany at Třeboň, in a large plastic bag. After several days (to avoid the stress connected with the transport), their traps were examined. The ambient water of the August samples contained similar microorganisms as in the traps.

2.2. Processing of material

Single fixed traps of *U. reflexa* represented two different ages. Old traps ranged in size from 2 to 5 mm, and young ones from 2 to 3 mm and 10 randomly selected traps of both ages were examined under a light microscope. They were washed thoroughly with distilled water before examination in order to get rid of organisms that might have been present on the external surface of the trap. After adding a drop of distilled water, the prepared trap was halved with a razor blade, covered with a 20 mm × 20 mm coverslip. All visible organisms under the coverslip were counted and the counts of those remaining inside the cut halves were added. Individual species were examined using a Zeiss Jenaval microscope. Live traps were checked for living commensal organisms inside the traps under a light microscope. They were then fixed as above and later prepared for SEM observations of attached bacteria inside the traps. The dried tissues were sputter-coated with gold and viewed in a Hitachi S-4700 microscope in the Scanning Microscopy Laboratory of Biological and Geological Sciences (Jagiellonian University, Cracow).

Identification of algae and ecological data follow Hirano (1956, 1957), Crosdale and Flint (1988), Förster (1982), Hindák (1990), John et al. (2002) and Coesel and Meesters (2007). Algae from traps were counted as dead when the protoplast of the algae showed any damage or when chloroplasts were contracted (Peroutka et al., 2008). The number of organisms (or taxa) identified are shown as the number of individuals or colonies per trap, ignoring trap volume, which varied slightly. Bacteria were quantified from quadrifid-glands and epidermal surface of traps using SEM micrographs.

2.3. Trap fluid pH measurement

Three replicate plants of *U. reflexa* were used for trap fluid pH measurement (see Sirová et al., 2003) in three trap categories: empty young traps (3rd–10th mature leaf nodes), old empty traps (20th–30th mature leaf nodes), and old traps of the same age with prey. About 30–40 μl trap fluid was collected using a fine pipette from 4 to 6 traps of the same trap category from one plant for one pH measurement. The pH of the trap fluid was determined with pH paper from 4 independent measurements for each trap category.

2.4. Statistical treatment

Statistical analyses employed Statistica 9.0 (StatSoft, 2009). The following analyses were performed: the Student *t*-test for independent samples, evaluation of statistically significant differences in the abundance of systematic groups of organisms in traps of different ages (the paired *t*-test was not possible), and Spearman's rank correlations to check the correlation between the abundance of the

Fig. 1. An example of specimen of *Utricularia reflexa* plant used in our study. Note young traps near apex (white arrow) and older traps (black arrow).

protozoa *Paramecium bursaria* and the number of animals and their remains in the traps. The pH values were not transformed.

3. Results

In *U. reflexa* traps, animals from different taxonomic groups were identified: rotifers, nematodes and arthropods, including crustaceans (ostracods) and arachnids (mites). There were also numerous partially digested remains of animals that could not be determined. Living protozoa dominated by *Vorticella* sp. and *P. bursaria* were found in the live traps (Fig. 2a). *P. bursaria* reproduced

in the trap fluid (not shown). There were also numerous living algal species: *Euglena* sp., *Epithemia argus* Kützing, *Characiopsis* conf. *longipes* (Rabenhorst) Borzi, *Ankistrodesmus fusiformis* Corda, *Coelastrum astroideum* De-Not, *C. microporum* Nägeli, *Cosmarium connatum* (Bréb.) Ralfs, *C. impressulum* Elfving, *C. paragrantoides* Skuja, *C. phaseolus* Brebisson, *C. regnellii* Wille, *Pediastrum tetras* (Ehrenb.) Ralfs, *Scenedesmus ellipticus* Corda, *S. obtusus* Meyen, *Tetraedron minimum* (A.Br) Hanseg, *Oedogonium* sp. (Fig. 2b) and also some dead diatoms. The most variable group were green algae (Chlorophyta) represented by seven genera, among them five *Cosmarium* species. Numerous chlorococcal taxa and cyanobacteria were observed, both in clusters and as single palmelloidal forms (*Euglena* spp.). Figs. 3 and 4 summarize the abundance of individual organisms in the traps, counted in the fixed material. High standard deviation is caused by big differences between the individual investigated traps e.g., the abundance of *Scenedesmus* sp. in old traps varied up to 9 times. In young traps, 85% of all organisms were algae (*Scenedesmus* spp. and *Characiopsis* sp. were the most numerous) and 13% protozoa (mainly *P. bursaria*). In old traps, 60% of all organisms were algae (green algae *Scenedesmus* spp. and palmelloidal form of *Euglena* spp. were the most numerous) and 39% were protozoa (mainly *P. bursaria*). *P. bursaria* gathered near animal remains. Bacteria occurred both in young (Fig. 2c) and old traps, but in old traps they were very abundant (Fig. 2d) and covered the surface of the trap glands (four-armed trichomes) and inner walls of the traps (Fig. 2d). In the samples collected in September, *P. bursaria* were not found in the water, however, the ciliates occurred inside the traps of *U. reflexa*.

For all taxa except *Characiopsis* sp. and *Oedogonium* sp., there were statistically significant differences ($p < 0.05$) in the number of individuals between young and old traps. The Pearson's correlation coefficients between the number of prey and animal remains pooled together against ciliates were 0.12 ($p < 0.05$) for young traps
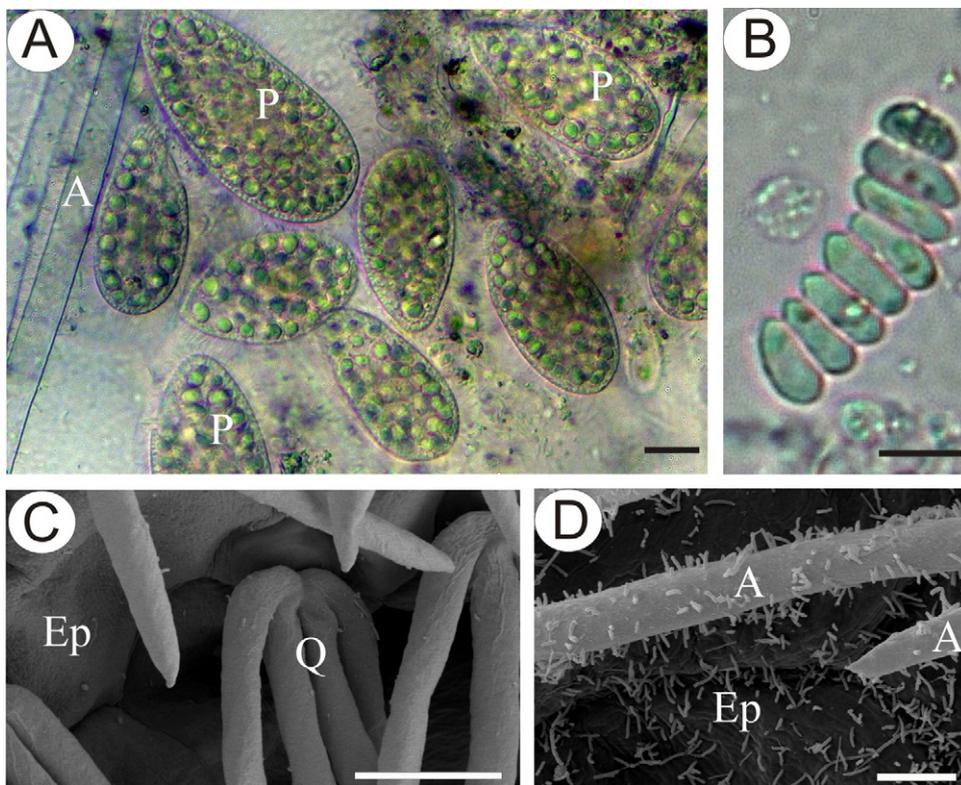

Fig. 2. Organisms living inside *Utricularia reflexa* traps and morphology of the internal trap surface: (A) Numerous ciliates *Paramecium bursaria* (P) inside old *U. reflexa* trap, A – arm of quadrifid (four-armed trichome/gland), bar = 20 μm. (B) *Scenedesmus* sp. from trap, bar = 10 μm. (C) Rare bacteria in the internal wall of young trap, Q – quadrifid, Ep – epidermal cell, bar = 20 μm. (D) Numerous bacteria on old trap internal surface, A – arm of quadrifid, Ep – epidermal cell, bar = 10 μm.
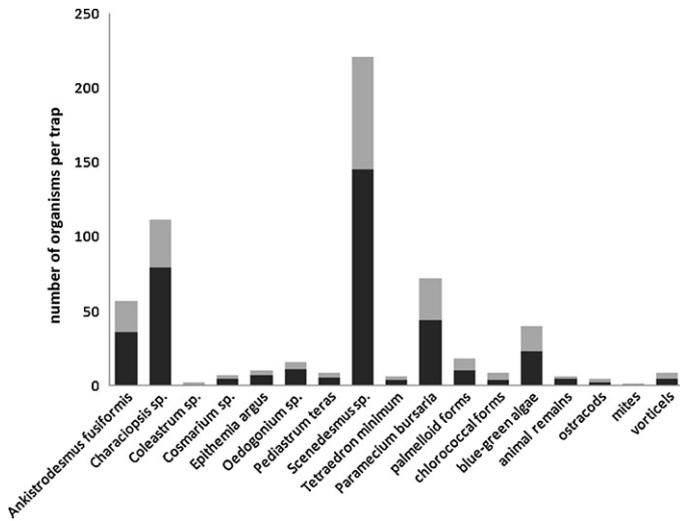
Fig. 3. Average abundance of organisms in investigated young traps of *U. reflexa*. Error bars (standard deviation) was shown in grey color.

and 0.79 ($p < 0.05$) for old traps. Trap fluid pH was 5.4 (SD = 0.0; data not shown) in all three trap categories (young empty traps, old traps with or without prey).

4. Discussion

We found that the number of living organisms within the main taxonomic groups (algae, ciliates, bacteria) inside the traps increased with trap age. Some of these organisms (e.g., bacteria, ciliates, some algae) can reproduce in the trap fluid (Hegner, 1926, cf. Płachno and Wołowski, 2008; Płachno et al., 2009). Spontaneous trap firings (Adamec, 2011) could also explain how phytoplankton regularly enter traps without any prey and concentrate here during trap aging. It has been suggested that aquatic *Utricularia* traps might provide cyanobacteria and eukaryotic algae with a favourable environment for their growth (Wagner and Mshigeni, 1986; Alkhalaf et al., 2009; cf. *Genlisea* traps rich in phytoplankton, Płachno and Wołowski, 2008; Płachno et al., 2009). This *Utricularia* microenvironment (niche) is especially suitable for some Chlorophyceae
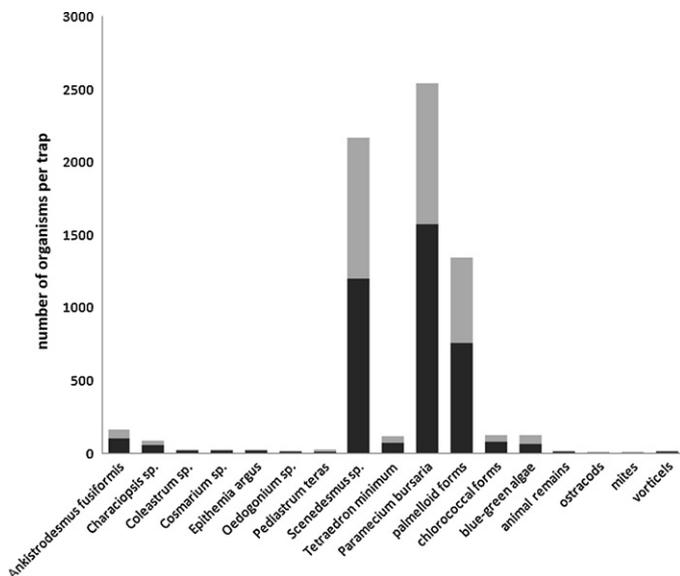

Fig. 4. Average abundance of organisms in investigated old traps of *U. reflexa*. Error bars (standard deviation) was shown in grey color.

genera (e.g., *Scenedesmus* spp., *Ankistrodesmus* sp.; Alkhalaf et al., 2009; our results) and mixotrophic phytoplankton such as *Euglena*. Abundant populations of *Euglena* spp. have been found in traps of some aquatic *Utricularia* species (e.g., Adamec and Komárek, 1999; Sirová et al., 2003, our results). However, Gordon and Pacheco (2007), Peroutka et al. (2008), Alkhalaf et al. (2009) and Sirová et al. (2009) noted low abundance of Euglenophyceae in traps. The traps with high abundance of Euglenophyceae were collected only from *Utricularia* cultures of the Institute of Botany at Třeboň, Czech Republic, so we cannot rule out regional or habitat-related differences in the composition of algae inside the traps. Similar local differences in the composition of algae in *Utricularia* traps were found in Austria (Peroutka et al., 2008).

According to Peroutka et al. (2008), *Utricularia* plants can be considered partial vegetarians because algae found in the traps of European aquatic *Utricularia* were usually dead and degraded to some extent. They noted that other authors who had found live algae in *Utricularia* traps had failed to distinguish between active functional traps and old inactive ones, or had even analyzed dead ones. However, we found that even young functional *Utricularia* traps were colonized by both living algae and ciliates. Living algae inside traps increase over time. In the case of phytoplankton we do not think it is possible to make a strict general conclusion that algae are prey or commensals or parasites of *Utricularia* traps. There are algal species that remain alive and even reproduce in the traps of aquatic *Utricularia* (e.g., *Euglena* spp.), while others are killed and digested in the trap environment.

Sirová et al. (2009) found that the bacterial community freely suspended in trap fluid decreased with increasing trap age, but in our work we found by SEM that old traps were heavily colonized by attached bacteria. Especially, it should be noted that bacteria heavily colonized the surface of quadrifids (the four-armed trichomes – name coined by Darwin (1875)), which occur on the internal trap wall and play a substantial role in enzyme secretion and nutrient absorption (Fineran and Lee, 1975; Płachno and Jankun, 2004; Płachno et al., 2006). Sirová et al. (2009) analyzed bacteria only in trap fluid, and not those attached to the trap surface. In future studies, bacteria freely suspended in the trap fluid and those attached to the inner trap surface should both be counted.

Like Hegner (1926), we found large numbers of protozoa in *Utricularia* traps. In *U. reflexa* traps, *P. bursaria* remained alive and were not digested. Since these ciliates were not digested and their number inside the traps increased with the trap age, with the quantity of animal prey (and thus with the amount of nutrients released from animal remains) and the bacterial number (prey for ciliates), these ciliates can be regarded clearly as commensals and not prey for the plant. *Utricularia* behaves like a farmer who provides fertilizer, that is, products of photosynthesis (Sirová et al., 2010) for bacteria, which in turn serve as a food for specific predators (ciliates) within this miniature food web. Another plausible explanation of the increasing number of *Paramecium* during trap aging might be based on the spontaneous trap firings (Adamec, 2011). However, in the samples collected repeatedly in September, *P. bursaria* were only observed in the traps which demonstrates that the *Paramecia* have a tight affinity to the traps and that they really live and reproduce inside the traps as commensals. Our results support the suggestions of Sirová et al. (2009, 2010) and Alkhalaf et al. (2009) about the role of the microbial commensal community and ciliates in *Utricularia* traps, but do not support Mette et al.'s (2000) statement that ciliates are prey for aquatic *Utricularia*. In regard to ciliates, our results differ from those of Peroutka et al. (2008), who observed only a low proportion of protozoa (3.2%) in traps in naturally grown *Utricularia* species and considered them prey.

Acknowledgements

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