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Ecological implications of organic carbon dynamics in the traps of aquatic carnivorous *Utricularia* plants

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Abstract. Rootless aquatic carnivorous *Utricularia* exude up to 25% of their photosynthates into the trap lumen, which also harbours a complex microbial community thought to play a role in enhancing *Utricularia* nutrient acquisition. We investigated the composition of organic carbon in the trap fluid, its availability for microbial uptake, the influence of plant nutrient status and trap age on its biodegradability, and the composition of prokaryotic assemblages within the traps of three aquatic *Utricularia* species. Using ion chromatography and basal respiration rate measurements we confirmed that up to 30% of total dissolved organic carbon in *Utricularia* trap fluid in oligotrophic conditions was easily biodegradable compounds commonly found in plant root exudates (mainly glucose, fructose and lactate). The proportion of these compounds and their microbial utilisation decreased with increasing mineral nutrient supply and trap age. Fluorescence *in situ* hybridisation analyses showed that microbial trap assemblages are dominated by alpha and beta Proteobacteria, and that the assemblage composition is affected by changes in the ambient mineral nutrient supply. We suggest that organic carbon dynamics within the traps, involving both the plant and associated microbial assemblages, underlies the acquisition of key nutrients by *Utricularia* and may help explain the evolutionary success of the genus.

Aditional keywords: amino acids, biodegradability, exudate composition, nutrient availability, organic acids, plant–microbe interactions, sugars.

Introduction

Utricularia (Fig. 1) is the largest, most cosmopolitan and perhaps the most successful genus among carnivorous plants. All members of the genus are rootless and have small bladder-like traps that can capture allochthonous material of suitable size (Taylor 1989). These rather sophisticated organs have a lumen filled with fluid, which is sealed to the surrounding environment, except for events where mechanical irritation triggers the opening of the trap (for details on trap operation, see Sydenham and Findlay 1975; Sasago and Sibaoka 1985*a*, 1985*b*). Traps are known to capture small animals such as crustaceans or protozoa (Harms 1999; Richards 2001), and the uptake of N and P from artificially fed prey has been detected previously (Friday and Quarmby 1994). It has been widely accepted that the benefit for investing in bladders is the enhanced uptake of nutrients from captured and digested organisms (e.g. Darwin 1875; Sorenson and Jackson 1968; Juniper *et al.* 1989). However, the trapping rates reported from natural environments are highly variable, and several studies have suggested limited importance of prey-derived nutrients for the growth of aquatic *Utricularia* (Sorenson and Jackson 1968; Kosiba 1992; Englund and Harms 2003; Adamec 2008*a*; Adamec *et al.* 2010), especially in highly oligotrophic environments, where detrital organic matter or phytoplankton seems a more probable nutrient source (Richards 2001; Peroutka *et al.* 2008).

Recently, the microbial community inside *Utricularia* traps was evaluated and viable components of a complete microbial food web were found in the trap fluid, including large numbers of bacteria (Sirová *et al.* 2009). The micro-organisms, especially bacteria, were found to produce extracellular phosphatases. Considering their large biomass and high metabolic potential, they were suggested to play an important role in the transformation of organic matter inside the traps. Furthermore, high concentrations of nutrients were detected in the trap fluid without microzooplankton prey, specifically large amount of dissolved nitrogen (N), phosphorus (P), and particularly carbon (C) (Sirová *et al.* 2009). Sirová *et al.* (2010) showed that a significant proportion of *Utricularia* primary production was supplied into the trap lumen by the plant itself, in the form of C exudates. It was hypothesised that C exudates fuel microbial

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Fig. 1. Experimental *Utricularia reflexa* plants on a Petri dish. Traps or bladders of various sizes can be seen in each leaf whorl: a distance between two bars (scale in the centre) represents 1 mm.

degradation of trapped organic matter within the traps, making N or P more readily available for plant uptake, in a manner similar to the rhizosphere interactions of rooted plants. However, there was no information on the composition of trap C compounds, and their availability for microbial uptake also needed further confirmation.

In the rhizosphere of rooted plants, micro-organisms take advantage of nutrients provided by the plant, and C-rich organic compounds in root exudates are continuously metabolised in the narrow zone of soil at close proximity to the root. In return, microbes may assist the plant by making nutrients more readily available. In general, microbes that inhabit the rhizosphere serve as an intermediary between the plant, which requires soluble mineral nutrients, and the soil, which contains the necessary nutrients mostly in complex inaccessible forms (Brimecombe *et al*. 2007). Further, the composition and consequently the biodegradability of root exudates changes with changing properties of the surrounding environment such as nutrient availability (Neumann and Römheld 2007).

Utricularia species are rootless; however, it has been speculated that traps of aquatic species harbour a complex microbial community inside their traps with mutualistic benefits similar to those in the rhizosphere of rooted plants (Sirová *et al*. 2009, 2010). These could enable *Utricularia* to grow and utilise organic nutrients (either dissolved or particulate) in the water column without the need for attachment to a substrate or sediment. Inhabiting this ecological niche may provide advantages such as reduced competition with rooted aquatic macrophytes for mineral nutrients and light and also increased diffusivity of available substrate; it may also explain the ecological success and wide distribution of the genus.

The aim of our work was to investigate the composition of organic C in the trap fluid, determine its availability for microbial uptake, and assess the influence of nutrient status of the plants on its biodegradability, as well as on the composition of the bacterial community within the traps.

Materials and methods

Experimental plants

Utricularia reflexa Oliver plants were used to determine the effect of trap age on trap fluid composition and biodegradability. Plants were collected in the Okavango region of Botswana. They were cultivated in 3 L indoor aquaria, under natural light, with sedge litter as a substrate (Sirová *et al*. 2003). This species was specifically chosen for its comparably larger traps growing in easily defined leaf nodes and linear shoots with low frequency of branching, which allows for an accurate determination of trap age.

The experimental species selected for the nutrient enrichment experiment differ markedly in their morphology and ecology. Dimorphic *Utricularia stygia* Thor usually grows affixed to the sediment or surface by the non-photosynthetic trap-bearing (i.e. carnivorous) shoots, with photosynthetic shoots floating freely in the shallow water column, whereas *Utricularia australis* R.Br. is a free-floating species with linear monomorphic shoots (Taylor 1989). Adult plants of *U. stygia* (collected in the Czech Republic) were pre-cultivated outdoors in a 2.5 m^2 plastic container that approximately simulated natural conditions (for details see Adamec 1997, 2008*a*; Sirová *et al*. 2003). The pH of cultivation medium was 7.5 at the time of material collection. Based on the concentrations of nutrients, the water was considered oligotrophic. Adult plants of *U. australis* ranging from 20–25 cm in length were collected from Ruda fishpond at Branná (Třeboňsko Biosphere Reserve, Czech Republic).

Trap fluid collection from U. reflexa *traps of different age*

Eight *U. reflexa* shoots of approximately equal length (25–30 cm) were collected and old shoot parts bearing non-functional traps were discarded. Remaining shoots were divided into six equal segments with the apical segment representing the youngest functional traps and the basal segment the oldest. Each of the six segments contained two leaf nodes with 2–4 traps. Trap fluid from traps without any macroscopic prey was collected by a glass capillary (Sirová *et al*. 2003) into an Eppendorf (Hamburg, Germany) vial attached to a peristaltic pump. In order to avoid contamination, plant surfaces were gently dried with a tissue before trap fluid collection. Each sample represented the fluid pooled from traps in respective segments in the eight plants described above. This resulted in six mixed samples representing the fluid from traps of increasing age, in which dissolved nutrient content and biodegradability was determined (see below).

Nutrient addition experiment setup and trap fluid collection

Three 320 L plastic containers filled with water were placed into a naturally lit greenhouse in equal light conditions and served for water cooling of the smaller experimental aquaria. Three aquaria each holding 35 L of tap water, 10 L of filtered water (20 µm) from the Branná location, and 20 g of dried *Carex elata* A. Bennett litter as substrate were placed into each of the cooling containers.

Approximately 15 *U. australis* plants (shortened to 10–12 cm in length) and 20 fully grown *U. stygia* plants of similar size with both shoot types were added into each aquarium. *U. australis* was allowed to float freely in the water column, but *U. stygia* plants were placed into the sedge litter and remained at the bottom of the experimental aquaria.

Three experimental aquaria received a 25 μg L^{-1} addition of PO_4-P, other three aquaria received 2 mg L^{-1} NH_4-N, and the remaining three represented controls with no nutrients added. The nutrient additions were repeated every 3 weeks; the last nutrient addition took place 2 weeks before the experiment end. Water inside the experimental aquaria was kept at a constant level during the experiment and the average water temperature for the duration of the experiment was 23°C (17–29°C). The chemical characteristics of the cultivation water in the experimental aquaria at the end of the experiment are shown in Table 1. Macroscopic prey was not available for the plants.

At the end of the 15 weeks incubation period, all *U. australis* and *U. stygia* plants (10–20 per species and container) from each of the three replicates of each treatment were pooled. Growth apices were collected, dried to constant weight, ground and analysed for tissue nutrient content. Trap fluid from medium-aged traps (6th–10th leaf nodes) from all the plants of each species in each experimental container was collected as described above (tens to hundreds of traps were used, depending on the species) and pooled into a single replicate (~0.5 mL per replicate). In addition, periphyton associated with the plants was collected for community composition analyses by vigorous shaking of eight plants from each experimental container and each species (entire carnivorous shoots for *U. stygia*, 6th–12th leaf nodes for *U. australis*) in a plastic vial with 100 mL of phosphate buffered saline (PBS) for 10 min. This procedure removed surface-attached algal and bacterial cells. A pooled periphyton sample from each experimental container and for each species was thus obtained and processed as described below. Concentrations of dissolved C, N, and P in the trap fluid samples were determined, and the number of bacteria counted and trap fluid biodegradability assessed as described below.

Analysis of organic matter and inorganic ion composition in the trap fluid and the cultivation water

Fresh samples for the analysis of trap fluid composition and cultivation water were filtered immediately after collection (0.2 μm pore size, Millipore Corporation, Billerica, MA, USA) and analysed on the dual channel ion chromatograph ICS 3000 (Dionex, Sunnyvale, CA, USA). The volume injected was 200 μL for cultivation water and 5 μL for trap fluid for each channel. Mono- and disaccharides, sugar alcohols, and amino acids were analysed using amperometric detection with separation on an AminoPack (Dionex) PA10 analytical column. Organic acids and inorganic ions were separated using an AS11-HC column and detected on a conductivity detector. Results were expressed as μmol C L^{-1} in the case of organic substances or as μmol L^{-1} in the case of inorganic anions.

Table 1. Chemical characteristics of cultivation water in the different treatments at the end of experiment

TDP, total dissolved phosphorus; DOC, dissolved organic carbon; TA, total alkalinity. Means (±s.e.) of three parallel determinations are shown. Statistically significant differences are indicated (*, $P < 0.05$; $n = 3$)

Characteristic	Control	P-addition	N-addition
TDP (μmol L^{-1})	403 ± 62	372 ± 62	217 ± 31
PO_4-P (μmol L^{-1})	93 ± 31	186 ± 93	124 ± 31
NH_4-N (μmol L^{-1})	294 ± 56	238 ± 42	840 ± 378*
NO_3-N (μmol L^{-1})	39 ± 11	43 ± 0.5	47 ± 4.0
DOC (mmol L^{-1})	288 ± 12	336 ± 12*	288 ± 24
TA (mmol L^{-1})	0.8 ± 0.3	1.1 ± 0.0	1.1 ± 0.1
pH	7.2 ± 0.4	7.3 ± 0.1	7.3 ± 0.0

Microbial community composition in the trap fluid and periphyton of U. australis and U. stygia traps and periphyton

Trap fluid and periphyton samples (0.1 mL) were shaken for 30 min on a shaker in a PBS buffer with paraformaldehyde (2% final concentration). Aliquots of both trap fluid and periphyton samples were subsequently filtered onto white polycarbonate filters (Millipore, type GTTP, 0.2 μm pore size, 47 mm diameter), and stored at –20°C until further processing. Catalysed reporter deposition-fluorescence *in situ* hybridisation (CARD-FISH) was conducted as previously described by Sekar *et al*. (2003) using the following horseradish peroxidase-labelled probes targeting main groups commonly occurring in aquatic environments: EUB I-III for all bacteria (Daims *et al*. 1999); the probes ALF968 (Neef 1997), BET42a (Manz *et al*. 1992), and GAM42a (Sekar *et al*. 2003) for the alpha, beta and gamma subgroups of Proteobacteria, respectively; probe CF319 for the Cytophaga-Flavobacterium-Bacteroides phyllum (Manz *et al*. 1996); the actinobacterial probe HGC69a (Roller *et al*. 1994); and probe ARCH915 (Stahl and Amann 1991) targeting Archaea. Hybridised filters were analysed according to Jezbera *et al*. (2006). We did not include cultivation water in our detailed analyses of microbial community composition, as the trap microorganisms tend to originate, according to available literature (Sirová *et al*. 2009; Mette *et al*. 2000), from the periphyton associated with *Utricularia* surfaces rather than from the surrounding water.

Incubation experiment – trap fluid biodegradation test

Measurement of microbial respiration rate is a common method in environmental studies and can be used as a measure of organic matter biodegradability (Joergensen and Emmerling 2006). The CO_2 evolution rate, also called basal respiration, gives an estimate of total microbial activity in the studied environment, in our case the *Utricularia* trap lumen. We chose *in vitro* analysis of the trap fluid, with the plant itself excluded from the sample.

Trap fluid (0.1 mL, filtered using a 0.2 μm pore size spin filter tube) of different trap age or from the various treatments and plant species was added to 12 mL Vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) vials containing 1.1 mL of distilled water and mixed. One μL of a bacterial inoculum containing a mixed bacterial community from the medium-aged traps of either *Utricularia* species was added into each vial and samples were incubated in darkness at 25°C for 260 h in three replicates. The chosen temperature of 25°C is in the growth optimum range of the *Utricularia* species studied (Adamec and

Kovářová 2006; Adamec 2010*a*, 2010*b*; Adamec *et al*. 2010). The Vacutainer head space sample in equilibrium with the liquid was collected by an air tight syringe at 24, 140 and 260 h of incubation, and CO_2 was determined using a HP 6850 gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with a 0.53 mm × 15 m HP-plot Q column and a 0.53 mm × 15 m HP-plot molecular sieve 5A column, with a thermal conductivity detector, using helium as the carrier gas. The respiration rate after 260 h was considered the basal respiration rate (biodegradability) at these temperatures. Results were normalised to dissolved organic carbon (DOC) content in the trap fluid and expressed as μmol CO_2 $(mol\ C)^{-1}\ h^{-1}$.

Nutrient analyses

The C and N content in the filtered trap fluid was analysed using Skalar Formacs TOC/TN analyser (Skalar Analytical BV, Breda, The Netherlands). Plant biomass C and N content was measured on the NC 2100 soil analyser (ThermoQuest Italia SpA, Milan, Italy) according to Nelson and Sommers (1996). P concentration in trap fluid and tissue P content in plant biomass were analysed by standard methods using perchloric acid digestion according to Kopáček and Hejzlar (1993).

Statistical treatment

The differences in nutrient concentration in the cultivation water, tissue nutrient content, and prokaryotic community composition were evaluated by 1-way ANOVA, using Prism 4.0 (GraphPad Software Inc, San Diego, CA, USA) software and Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA).

To perform multivariate statistical analyses, we used the program CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA). The concentrations of analytes in the trap fluid were square-root transformed before analysis. Since the gradient length determined by the detrended correspondence analysis was smaller than 2, we used redundancy analysis (linear method) in further processing of the dataset. A step-forward regression was performed to determine nutrient addition treatment with statistically significant effect on the trap fluid composition (Monte Carlo test with 500 permutations).

Results

Nutrient addition experiment

The cultivation water in nutrient addition treatments had slightly higher concentrations of PO_4-P, and significantly higher concentrations of NH_4-N, compared with the control treatments. The DOC values were significantly higher in the P-addition treatments, and all treatments had similar pH and total alkalinity (Table 1). The simple organics analysed using ion chromatography was below the detection limit of the method in all cultivation aquaria.

During the course of the experiment, *U. australis* plants in the N-addition treatment had largely stopped producing traps or lost them completely. Therefore, presented data do not show the results for *U. australis* trap fluid analyses. Slightly higher, but statistically insignificant P contents were found in plant tissue in the P addition treatments (Fig. 2). Total numbers of the trap fluid bacteria were in the order of $10^9\ L^{-1}$, markedly but insignificantly higher in the P addition treatment, due to large variation both within and among treatments (Fig. 3*a*).

Trap fluid composition of simple organic compounds and ions

The stoichiometry of the trap fluid was very similar in all three species and we did not find statistically significant differences among the treatments of the nutrient addition experiment (Fig. 2). Dissolved organic carbon in the trap fluid ranged from 38 to 43 mmol L^{-1} in control and P treatments of *U. australis* and was slightly lower in *U. stygia* (35 to 39 mmol L^{-1}). The trap fluid of all species, regardless of treatment or age, was rich in dissolved N and P, which ranged from 1.2 to 2.8 mmol L^{-1} and 0.04 to 0.23 mmol L^{-1}, respectively (Fig. 2).

We were able to identify 51 different analytes in the trap fluid of all three species (Table 2). These included five inorganic anions, six sugar alcohols, nine simple sugars, 16 amino acids,

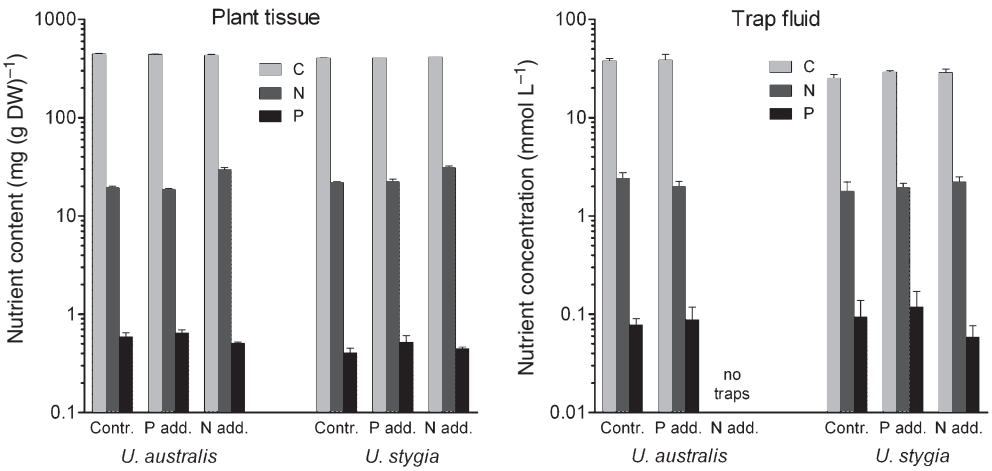

Fig. 2. Nutrient content in plant tissue and nutrient concentration in the trap fluid of *Utricularia australis* and *Utricularia stygia* plants in the different nutrient addition treatments. Means (±s.e.) of three parallel determinations are shown. Contr., control; P add., P addition; N add., N addition.

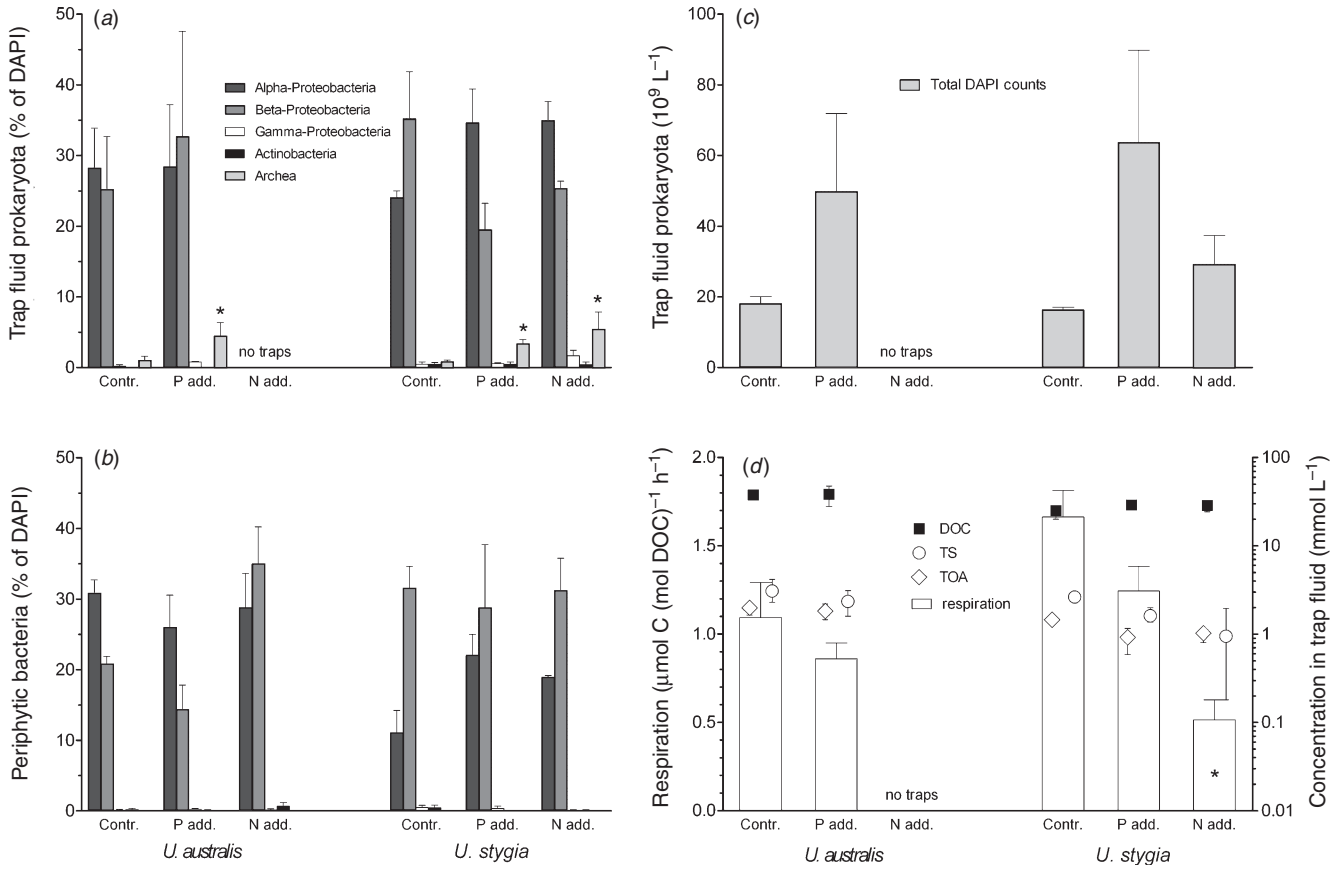

Fig. 3. Microbial community composition in the (*a*) trap fluid, (*b*) periphyton, (*c*) the numbers of prokaryotic (bacterial and archaeal) cells, and (*d*) the concentration of dissolved organics and microbial respiration in *Utricularia australis* and *Utricularia stygia* in the different nutrient addition treatments. Means (±s.e.) of three parallel determinations are shown; asterisk indicates statistically significant difference ($P < 0.05$). DOC, dissolved organic carbon; OA, organic acids; TS, total sugars; Contr., control; P add., P addition; N add., N addition.

and 15 organic acids. There was considerable variation in analyte concentration among the plant species both among and within different treatments and age; however, sugars and organic acids generally constituted the largest proportion of the identified organic compounds (26–60% and 11–38%, respectively), specifically, glucose and lactic acid were present in highest concentrations (up to 0.42 and 0.52 mmol C L^{-1}, respectively; averages for each treatment and values for different traps age shown in Table 2). The most important amino acids with regards to concentration were alanine and glycine (0.008–0.14 and 0.004–0.1 mmol C L^{-1}, respectively). Xylitol constituted the largest proportion of sugar alcohols (up to 0.21 mmol C L^{-1}). Large quantities of chloride (up to 6.9 mmol L^{-1}) were also detected in all traps. Total identified analytes constituted between 5 and 33% of total DOC inside traps (Table 2).

Assessment of *U. reflexa* traps revealed marked differences in trap fluid from traps of different age. The youngest traps contained studied analytes at the highest concentrations, which decreased with progressing age, the exception being the oldest traps, where a slight rise in concentrations was observed again (Fig. 4).

Redundancy analysis revealed a statistically significant ($P < 0.05$; $n = 3$) effect of N addition on the composition of trap fluid organic compounds, with higher concentrations of amino acids as well as nitrate (Table 2; Fig. 5). In contrast, trap fluid from control treatments was dominated by sugars and organic acids. Phosphorus treatments showed conspicuously higher concentrations of ribose and sorbitol. A positive correlation ($r^2 = 0.63$) was found between the plant tissue C : N ratio and the total identified exudates in the trap fluid.

Bacterial community composition in U. australis *and* U. stygia *traps and periphyton*

Out of the five bacterial groups studied, alpha and beta Proteobacteria represented the largest proportion in the trap-associated communities (Fig. 3*a*) as well as in the periphyton (Fig. 3*b*) in both species and all treatments studied. Although both the trap fluid and periphyton in the control treatments had similar ratios of alpha and beta Proteobacteria, the ratio differed markedly in the nutrient addition treatments (Fig. 3*b*). Gamma Proteobacteria and the gram positive Actinobacteria constituted a minority (<1.7% of DAPI counts). Members of the Cytophaga-Flavobacterium-Bacteroides phyllum were not detected in this study. Archaea were found only inside the traps, represented up to 10% of DAPI counts and increased significantly in numbers in both the P and N-addition treatments in *U. stygia* ($P < 0.05$; $n = 3$), and the P-addition treatment in *U. australis* ($P < 0.05$; $n = 3$).

Table 2. Concentrations of major analytes determined in the trap fluid

Means (± s.e.) of three parallel determinations are shown (except for single measurements in *Utricularia reflexa*); concentrations in μmol C L^{-1} for organics or μmol L^{-1} for inorganic ions. *U. r.* – 1, . . . *U. r.* – 6 = *U. reflexa* trap fluid from traps growing on leaf nodes of increasing age. *U. a.* – C, *U. a.* – P = *Utricularia australis* trap fluid from the control and P-addition treatments, respectively; *U. s.* – C, *U. s.* – P, *U. s.* – N = *Utricularia stygia* trap fluid from the control, P and N-addition treatments, respectively. Note that the age of traps analysed in the nutrient addition experiment is roughly comparable to the two youngest age categories in *U. reflexa*. Values significantly different from those in the control treatment are indicated (*t*-test, *, $P = 0.05$)

Analytes	*U. r.* – 1	*U. r.* – 2	*U. r.* – 3	*U. r.* – 4	*U. r.* – 5	*U. r.* – 6	*U. a.* – C	*U. a.* – P	*U. s.* – C	*U. s.* – P	*U. s.* – N
Inorganic anions											
Chloride	2019	999	138	1198	1925	1885	2640 ± 281	2584 ± 568	1869 ± 267	3073 ± 2704	1605 ± 304
NO_3-N	55	13	1	6	7	9	21 ± 8	10 ± 4	5 ± 2	6 ± 5	12 ± 5
NO_2-N	0.8	0.0	0.0	0.2	0.0	0.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 1
PO_4-P	69	15	1	6	10	24	12 ± 4	36 ± 22	38 ± 33	40 ± 46	1 ± 0
Sulfate	308	143	30	166	129	94	168 ± 22	197 ± 6	67 ± 23	97 ± 55	159 ± 112
Sugar alcohols											
Arabitol	28	5	10	5	5	9	47 ± 17	56 ± 1	47 ± 24	37 ± 11	35 ± 9
Glycol	37	12	11	8	9	16	104 ± 29	78 ± 3	58 ± 43	34 ± 24	38 ± 28
Mannitol	34	4	5	4	5	22	224 ± 58	334 ± 163	635 ± 177	359 ± 55*	276 ± 36*
myo-Inositol/arginine	149	51	51	36	39	69	159 ± 24	195 ± 17	272 ± 14	192 ± 28*	160 ± 54*
Sorbitol/adonitol/dulcitol	35	5	6	6	6	11	34 ± 6	28 ± 4	13 ± 2	17 ± 5	11 ± 2
Xylitol	187	60	66	76	71	113	274 ± 18	645 ± 417	237 ± 19	264 ± 51	219 ± 53
Total sugar alcohols	470	137	148	136	136	240	842	1337*	1263	901*	738*
Sugars											
Arabinose	0	2	3	2	1	4	8 ± 1	9 ± 3	9 ± 3	8 ± 2	4 ± 3
Fructose/glutamine	807	82	75	43	51	149	506 ± 418	562 ± 140	529 ± 57	352 ± 144	129 ± 119*
Galactose	41	15	13	11	10	13	40 ± 9	57 ± 39	481 ± 298	202 ± 154	39 ± 7*
Glucose	3471	637	585	450	462	1429	1883 ± 437	1431 ± 395	1305 ± 74	931 ± 119*	684 ± 570
Maltose	347	11	43	10	10	91	87 ± 28	67 ± 5	11 ± 16	8 ± 7	0 ± 0
Rhamnose	0	0	0	0	0	0	2 ± 2	4 ± 2	1 ± 0	1 ± 1	1 ± 1
Ribose	43	7	5	4	5	16	54 ± 2	92 ± 44	26 ± 2	38 ± 4*	23 ± 7
Saccharose	600	38	46	25	50	86	501 ± 139	119 ± 119*	258 ± 27	62 ± 9*	66 ± 57*
Xylose	0	0	0	0	0	0	1 ± 1	3 ± 3	13 ± 1	10 ± 5	1 ± 1*
Total sugars	5309	791	769	545	590	1788	3082	2345	2634	1614*	948*
Amino acids											
Alanine	135	25	26	16	20	51	113 ± 13	155 ± 44	80 ± 24	103 ± 7	86 ± 26
Asparagine	0	1	1	2	3	8	12 ± 5	5 ± 5	6 ± 4	7 ± 6	4 ± 6
Aspartic ac.	50	9	9	6	5	13	36 ± 6	45 ± 24	34 ± 12	47 ± 6	59 ± 3*
Cysteine	0	25	0	0	0	0	8 ± 9	0 ± 0	11 ± 16	12 ± 10	17 ± 4
Glycine	100	18	24	15	24	55	98 ± 8	113 ± 44	60 ± 26	78 ± 13	86 ± 17
Histidine	58	9	6	5	6	18	38 ± 11	42 ± 10	37 ± 18	44 ± 8	39 ± 9
Isoleucine	41	10	10	5	8	21	30 ± 8	31 ± 13	31 ± 14	40 ± 8	62 ± 10*
Leucine	89	20	19	14	11	29	37 ± 9	30 ± 11	39 ± 22	33 ± 8	33 ± 6
Methionine	6	2	1	1	1	3	7 ± 2	9 ± 2	14 ± 4	14 ± 12	9 ± 2
Phenylananine	0	12	0	7	0	0	22 ± 8	16 ± 4	25 ± 16	35 ± 14	46 ± 7
Proline/serine	149	23	22	20	20	50	124 ± 23	154 ± 71	82 ± 43	115 ± 15	96 ± 27
OH-Proline/lactose	97	9	9	4	7	34	13 ± 9	80 ± 52	5 ± 4	22 ± 13	23 ± 9
Threonine	76	16	18	13	14	27	94 ± 15	110 ± 17	77 ± 41	87 ± 17	78 ± 22
Tryptophane	57	9	12	6	10	24	37 ± 4	58 ± 18	32 ± 8	50 ± 3*	55 ± 17
Tyrosine	38	13	12	12	10	19	32 ± 3	28 ± 2	33 ± 17	49 ± 14	94 ± 30*
Valine	62	12	14	7	11	45	67 ± 19	96 ± 43	45 ± 18	65 ± 4	100 ± 30*
Total amino acids	959	213	184	134	151	398	769	972	613	803	887
Organic acids											
Acetic acid/glycolic acid	171	76	8	45	57	50	88 ± 8	65 ± 65	36 ± 25	28 ± 7	154 ± 132
Adipic acid	12	1	0	0	0	1	8 ± 3	21 ± 15	4 ± 2	8 ± 11	26 ± 25
Butyric acid	0	0	0	0	0	0	0 ± 0	0 ± 0	0 ± 0	6 ± 9	0 ± 0
Citric acid	80	14	1	7	7	10	50 ± 5	44 ± 16	42 ± 11	35 ± 20	40 ± 25
Formic acid	195	3	0	2	2	8	45 ± 40	12 ± 0	8 ± 8	3 ± 3	18 ± 22
Gluconic acid	60	72	14	130	154	122	107 ± 9	147 ± 52	48 ± 14	47 ± 18	80 ± 34
Isocitric acid	5	1	0	2	2	2	21 ± 3	14 ± 1*	4 ± 2	5 ± 1	4 ± 1
Lactic acid	1364	467	108	276	303	562	1409 ± 188	1292 ± 150	1048 ± 205	579 ± 119*	543 ± 240*
Maleic acid	3	1	0	0	1	0	1 ± 0	1 ± 0	1 ± 0	2 ± 2	5 ± 5

Table 2. (*continued*)

Analytes	*U. r.* – 1	*U. r.* – 2	*U. r.* – 3	*U. r.* – 4	*U. r.* – 5	*U. r.* – 6	*U. a.* – C	*U. a.* – P	*U. s.* – C	*U. s.* – P	*U. s.* – N
Malic acid/succinic acid	23	23	1	3	2	2	96 ± 15	100 ± 83	199 ± 20	151 ± 62	56 ± 9*
Malonic acid	15	7	1	5	4	4	28 ± 12	22 ± 1	16 ± 3	13 ± 3	14 ± 5
Oxalic acid	29	22	3	14	14	9	83 ± 29	71 ± 17	37 ± 13	36 ± 14	48 ± 31
Phytic acid	0	1	0	0	0	0	1 ± 1	0 ± 0	1 ± 1	0 ± 0	0 ± 0
Propionic acid	0	0	0	0	0	0	0 ± 0	6 ± 5	4 ± 6	1 ± 0	17 ± 24
Pyruvic acid	12	0	0	0	0	1	47 ± 7	32 ± 1*	15 ± 2	6 ± 3*	21 ± 22
Total organic acids	1970	688	137	484	546	774	1983	1827	1462	921*	1026*
Sum of organic analytes	8707	1829	1238	1299	1422	3201	6677	6481	5972	4239*	3599*
Sugar alcohols (% sum)	5%	7%	12%	10%	10%	8%	13%	21%	21%	21%	21%
Sugars (% sum)	61%	43%	62%	42%	41%	56%	46%	36%	44%	38%	26%
Amino acids (% sum)	11%	12%	15%	10%	11%	12%	12%	15%	10%	19%	25%
Organic acids (% sum)	23%	38%	11%	37%	38%	24%	30%	28%	24%	22%	28%
Total trap DOC	27 158	14 836	18 756	25 427	22 202	26 755	37 705	43 329	24 902	28 707	28 967
Sum of organic analytes (% total DOC)	32%	12%	7%	5%	6%	12%	18%	15%	24%	12%	15%

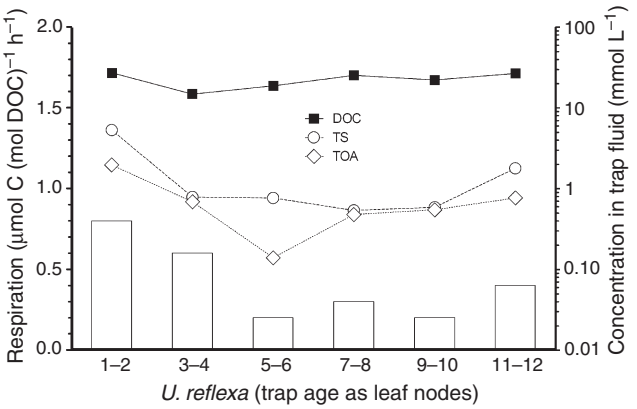

Fig. 4. Concentration of dissolved organic carbon (DOC), total organic acids (TOA), total sugars (TS), and microbial respiration in the *Utricularia reflexa* trap fluid of different age (leaf nodes 1–2, represent the youngest part of the shoot bearing mature traps).

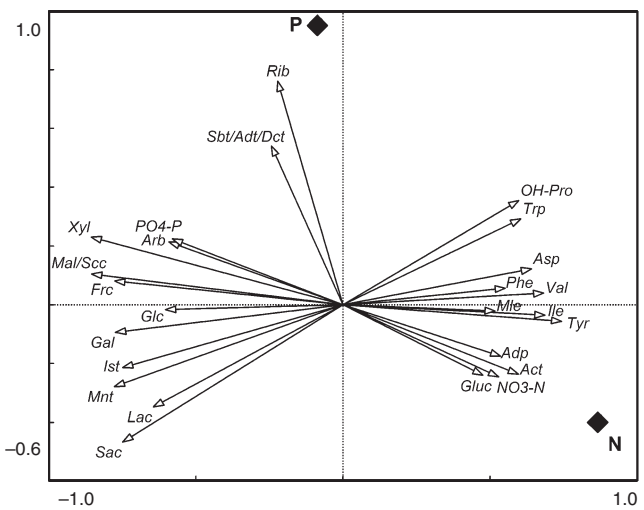

Fig. 5. Direct gradient analysis (redundancy analysis) of trap fluid composition in *Utricularia stygia* plants of different nutrient status. Nutrient addition explained 23.7% (N) and 7.1% (P) of the variability; the effect of N was statistically significant ($P = 0.04$). Only 25 analytes with best relationship to canonical axes are displayed: Act, acetic acid/glycolic acid; Adp, adipic acid; Arb, arabinose; Asp, aspartic acid; Frc, fructose/glutamine; Gal, galactose; Glc, glucose; Gluc, gluconic acid; Ile, isoleucine; Ist, myo-inositol/arginine; Lac, lactic acid; Mal/Scc, malic acid/succinic acid; Mle, maleic acid; Mnt, mannitol; NO_3, nitrate; OH-Pro, OH-proline/lactose; Phe, phenylananine; PO_4, phosphate; Rib, ribose; Sbt/Adt/Dct, sorbitol/adonitol/dulcitol; Sac, saccharose; Trp, tryptophane; Tyr, tyrosine; Val, valine; Xyl, xylose.

The biodegradation of trap fluid

Microbial respiration was detected in the trap fluid of all three species, ranging from 0.34 to 0.96 µmol CO_2 (mol C)$^{-1}$ h^{-1} (Figs 3*c*, 4). Looking at trap age (Fig. 4), the youngest traps of *U. reflexa* were found to have the most utilisable trap fluid C compounds, followed by the oldest traps, whereas traps of intermediate age showed a markedly lower degradability of trap fluid. Total sugar and organic acid concentrations followed the same pattern, despite the fact that DOC remained relatively constant during trap ageing (Fig. 4).

In the nutrient addition experiment, the biodegradability of the trap fluid was highest in control treatments, markedly lower in P addition treatments and significantly lower in N-enriched plants ($P < 0.05$; $n = 3$). This corresponds very well with the above described trends in the concentration gradients of the identified analytes, as a strong positive correlation ($r^2 = 0.78$) was found between the proportion of simple organic C (expressed as % of total trap DOC) and the microbial respiration (biodegradability of trap fluid) measured.

Discussion

Research on microbial–plant mutualisms has largely focussed on N_2-fixing rhizobia or arbuscular mycorrhizal fungi, whereas plant associations with endophytic or free-living rhizosphere microbes

have received little attention in comparison (Kiers and Denison 2008). It is becoming increasingly apparent that plants utilise a much broader range of nutrient sources than previously thought; these include the recently discovered ability of plant roots to directly utilise bacterial cells from the culture medium (Paungfoo-Lonhienne *et al*. 2010). Although *Utricularia* plants are rootless, their traps, according to recent findings, behave in a strikingly similar way to roots: the inner trap surfaces absorb nutrients (Friday and Quarmby 1994) and secrete various extracellular enzymes (Sirová *et al*. 2003), the trap biomass and morphology change according to the supply of mineral nutrients (Englund and Harms 2003; Kibriya and Jones 2007; Adamec 2008*b*; Adamec *et al*. 2010), and, importantly, significant amounts of photosynthate are exuded into the trap lumen (Sirová *et al*. 2010). To the best of our knowledge, the results in this study represent a first insight into the C exudate composition, utilisation by microbes, and its possible ecological significance in *Utricularia*, as well as carnivorous plants in general.

We have confirmed that a significant proportion of the DOC pool found in *Utricularia* traps consists of diverse and simple C-rich compounds, such as are commonly found in plant root exudates (cf. Uren 2007). Most of the compounds were found in all three species, in all nutrient addition treatments, and in traps of all ages; the concentration and proportion of these compounds, however, all varied considerably.

As we were working with a dynamic plant–microbe system, it was not possible to distinguish directly between the products of plant and microbial metabolisms with the methods used in this study. Likewise, the concentrations of different exudate compounds measured represent a certain dynamic equilibrium that reflects the rate of utilisation by either the microbial community or the trap itself and the rate of exudation by traps at the time of sampling. However, due to the relatively high concentrations of most analytes and based on previous results (Sirová *et al*. 2010), we are confident that most of these compounds are exuded by the plant itself and supplied well in excess of microbial C utilisation rates found in this study. Moreover, most of these compounds occur at highest concentrations within the youngest traps, which were reported to have the lowest bacterial numbers (Sirová *et al*. 2009) on the one hand, but the largest proportion of freshly produced photosynthates (Sirová *et al*. 2010) on the other.

The trap fluid DOC was found to be utilisable by the trap-associated microflora, with respiration rates comparable with those commonly measured in the soil (Macdonald *et al*. 2009; Bruun *et al*. 2010). The degradability of trap fluid decreased with increasing trap age, along with the concentrations of most of the simple organics measured, which corresponds well with the decrease in photosynthate supply into older *Utricularia* traps as reported by Sirová *et al*. (2010). It also adds further support to the hypothesis that the traps change in function at a certain age, perhaps entering a phase of rapid nutrient absorption before senescing (Sirová *et al*. 2009).

It has been well established that plant rhizodeposition is influenced by N and P supply, and that elevated nutrient levels tend to decrease the amount of DOC exuded by the roots (Darwent *et al*. 2003). Our results confirm that allocation of simple organic C into the *Utricularia* traps is determined by the ambient mineral nutrient concentrations, which also, in the case of N in *U. australis*, has a pronounced effect on trap biomass (see Fig. 2, cf. Adamec 2008*b*). In oligotrophic environments (control), the *Utricularia* plants enhance trap microbial activity and nutrient mobilisation by supplying easily available C, mainly glucose and lactic acid. This stimulation of trap-associated microbes is no longer necessary in systems with higher nutrient supply, and C is probably allocated elsewhere in plant tissue, which results in observed decrease in available C concentrations and the reduction or even loss of traps. However, *Utricularia* generally grow poorly in nutrient-rich conditions, possibly due to competition with microbial or macrophyte populations in the water column.

The significantly elevated concentrations of organic acids in the control treatments may also play a role in maintaining the very stable, species-specific pH values of the *Utricularia* trap fluid found in previous studies (Sirová *et al*. 2003, 2009).

We found a significant rise in amino acid concentrations in *U. stygia* traps from the N-addition treatments. It has been demonstrated that the rate of amino acid export from the photosynthetic tissues to the phloem is dependent on the N available to the plant. In wheat, for example, amino acids are used for protein synthesis when N supply is low, exported to the phloem when supply is adequate, and accumulated in the storage pool when supply is above plant demand (Caputo and Barneix 1997). In *Utricularia*, it seems that excess amino acids are exuded into the trap lumen. Amino acids are rapidly turned over by microbes in the soil and rhizosphere (Owen and Jones 2001); however, the biodegradability of trap fluid from N-addition treatments was significantly lower compared with the control, despite similar C : N : P ratios in all treatments. This, together with significantly lower concentrations of sugars and organic acids, suggests a C limitation of trap microbial communities under elevated ambient N supply. Higher concentrations of amino acids in the phloem exudate have also been reported to regulate plant mineral N uptake rates (Muller and Touraine 1992). Therefore, it is possible that elevated amino acid content in *Utricularia* traps inhibits mineral N uptake to match the demands in the plants. The elevated concentrations of ribose found in the traps of the P-enriched *Utricularia* plants may partly represent a product of the fast growing microbial community (microbial numbers inside traps were 2–3 times higher in the P-addition variant compared with the N-addition and control treatments; Fig. 3*c*).

Sugar alcohols were also found to be prevalent in all *Utricularia* trap types. Xylitol, a compound found at highest concentrations in the trap fluid, has been shown to act as an antimicrobial agent in studies with pathogenic bacteria. Its effectiveness lies in reducing the adherence abilities of many bacteria to surfaces and, hence, hinders biofilm formation (Tapiainen *et al*. 2004; Soderling and Hietala-Lenkkeri 2010). It is possible that the same mechanism prevents the bacterial communities from excessively colonising the inner surfaces of the traps, which assures a more efficient absorption and/or secretion of compounds from or into the trap lumen. Xylitol appears to be most abundant in the youngest traps, where the lowest degree of bacterial aggregation and biofilm formation has been observed previously (Sirová *et al*. 2009). Fungi are able to produce significant amounts of xylitol by fermenting D-xylose (Chiang and Knight 1960; Dahija 1991), and low dissolved oxygen (DO)

concentrations found in the traps would favour this process; however, D-xylose fermentation is not very likely due to low xylose concentration in the trap fluid, even in the youngest traps with lowest microfungal biomass (Sirová *et al*. 2009).

Utricularia trap function rests essentially on the water transport out of their traps via a two-step ATP-driven ion pumping process, initiated by respiration-dependent transport of Cl^- ions against an electrochemical gradient (Sydenham and Findlay 1973, 1975; Fineran and Lee 1980; Juniper *et al*. 1989). Relatively large concentrations, in the order of mmol L^{-1}, of trap fluid Cl^- found in this study are most probably associated with this process. Sydenham and Findlay (1975) reported similar concentrations of Cl^- in the trap lumen of aquatic *Utricularia* sp., with marked differences between set traps and traps that have fired. Due to functional peculiarities of *Utricularia* traps, each trap is able to suck in ~40% of its resetting volume during the firing event, thus, diluting the inner trap fluid or increasing the concentrations of other solutes. The variability in the Cl^- concentrations, as well as that of the other analytes measured in this study, can be explained by the fact that traps were in different stages of the resetting process at the time of sampling – they may have been processed anywhere within the 'right after firing' and 'set' range.

Proteobacteria were the most abundant bacterial group in the *U. australis* and *U. stygia* traps, as seems to be the case in the traps of other carnivorous plants, namely *Sarracenia purpurea* (Siragusa *et al*. 2007). The total numbers of both alpha and beta Proteobacteria did not exhibit any significant changes following nutrient addition, although it is possible that changes occurred in proteobacterial assemblage composition.

Archaea were also found in the traps of *U. australis* and *U. stygia*, constituting a substantial proportion of the total prokaryotic assemblage in both nutrient addition treatments based on FISH results. This group was not detected in the periphyton, which provides further evidence that trap-associated microbial communities are distinct from those in the ambient environment. Recent studies indicate that the Archaea are ubiquitous, growing in environments with a wide range of nutrient and organic C concentrations, as well as within wide temperature and pH ranges. They are the dominant ammonia-oxidisers in many of these environments and some of the key players in N cycling (Erguder *et al*. 2009). Many Archaea are probably more suited for low DO conditions (Erguder *et al*. 2009), which have been shown to occur in *Utricularia* traps (Adamec 2007). Herrmann *et al*. (2008, 2009) reported that Archaea form an important part of the ammonia-oxidising Prokaryotes associated with the rhizosphere of several aquatic macrophytes, where they showed abundances comparable with our results (up to 5% of total microbial community). Until now, only one other study has reported an association of Archea with carnivorous plants: Cadillo-Quiroz *et al*. (2010) found Archaea inhabiting the rhizoplane of *S. purpurea*. Although there is little information on their P requirements, Herfort *et al*. (2007) demonstrated a positive correlation between crenarchaeotal 16S rRNA gene copies and phosphate concentrations in sea water. Herrmann *et al*. (2009) showed that the composition of ammonia-oxidising Archaea in the rhizosphere of aquatic macrophytes is generally determined by lake trophy, displaying positive correlation between the number of detected operational taxonomic units and nutrient availability. These results correspond with the significantly larger abundance of Archaea in *Utricularia* traps from the N and P nutrient addition treatments observed in this study. Although more research is needed to confirm the ecological role of Archaea in the *Utricularia* traps, it can be speculated that this association, like that in the rhizosphere, has an impact on the N budget for the plants.

The traps of carnivorous plants have often been successfully used as model systems in ecological studies (e.g. Cochran-Stafira and von Ende 1998; Sota *et al*. 1998; Mouquet *et al*. 2008). We suggest that traps of aquatic *Utricularia* with the associated microbial communities, which can be relatively easily manipulated for experimental purposes, are useful study systems which can help uncover novel aspects of plant–microbe interactions, especially in relation to plant nutrient acquisition.

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