Annals of Botany **114**: 125–133, 2014
doi:10.1093/aob/mcu067, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Dinitrogen fixation associated with shoots of aquatic carnivorous plants: is it ecologically important?

Dagmara Sirová[1,*], Jiří Šantrůček[1,2], Lubomír Adamec[3], Jiří Bárta[1], Jakub Borovec[4], Jiří Pech[5], Sarah M. Owens[6], Hana Šantrůčková[1], Rudi Schäufele[7], Helena Štorchová[8] and Jaroslav Vrba[1,4]

[1]*Department of Ecosystem Biology, Department of Experimental Plant Biology, Faculty of Science, University of South Bohemia, Branišovská 31, CZ-37005 České Budějovice, Czech Republic,* [2]*Institute of Plant Molecular Biology, Biology Centre AS CR, Branišovská 31, CZ-37005 České Budějovice, Czech Republic,* [3]*Section of Plant Ecology, Institute of Botany AS CR, Dukelská 135, CZ-37982 Třeboň, Czech Republic,* [4]*Institute of Hydrobiology, Biology Centre AS CR, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic,* [5]*Institute of Applied Informatics, Faculty of Science, University of South Bohemia, Branišovská 31, CZ-37005 České Budějovice, Czech Republic,* [6]*Institute of Genomics and Systems Biology, Argonne National Laboratory, Argonne, and Computation Institute, University of Chicago, Chicago, IL, USA,* [7]*Department of Grassland Study, Technical University Munich, Alte Akademie 12, Freising-Weihenstephan, Germany and* [8]*Institute of Experimental Botany AS CR, Rozvojová 263, CZ-16502 Prague 6–Lysolaje, Czech Republic*

** For correspondence. Email dagmara_sirova@hotmail.com*

Received: 29 December 2013 Returned for revision: 17 February 2014 Accepted: 11 March 2014 Published electronically: 9 May 2014

• **Background and Aims** Rootless carnivorous plants of the genus *Utricularia* are important components of many standing waters worldwide, as well as suitable model organisms for studying plant–microbe interactions. In this study, an investigation was made of the importance of microbial dinitrogen (N_2) fixation in the N acquisition of four aquatic *Utricularia* species and another aquatic carnivorous plant, *Aldrovanda vesiculosa*.
• **Methods** 16S rRNA amplicon sequencing was used to assess the presence of micro-organisms with known ability to fix N_2. Next-generation sequencing provided information on the expression of N_2 fixation-associated genes. N_2 fixation rates were measured following $^{15}N_2$-labelling and were used to calculate the plant assimilation rate of microbially fixed N_2.
• **Key Results** *Utricularia* traps were confirmed as primary sites of N_2 fixation, with up to 16 % of the plant-associated microbial community consisting of bacteria capable of fixing N_2. Of these, rhizobia were the most abundant group. Nitrogen fixation rates increased with increasing shoot age, but never exceeded 1·3 μmol N g^{-1} d. mass d^{-1}. Plant assimilation rates of fixed N_2 were detectable and significant, but this fraction formed less than 1 % of daily plant N gain. Although trap fluid provides conditions favourable for microbial N_2 fixation, levels of *nif* gene transcription comprised $<0{\cdot}01$ % of the total prokaryotic transcripts.
• **Conclusions** It is hypothesized that the reason for limited N_2 fixation in aquatic *Utricularia*, despite the large potential capacity, is the high concentration of NH_4-N (2·0–4·3 mg L^{-1}) in the trap fluid. Resulting from fast turnover of organic detritus, it probably inhibits N_2 fixation in most of the microorganisms present. Nitrogen fixation is not expected to contribute significantly to N nutrition of aquatic carnivorous plants under their typical growth conditions; however, on an annual basis the plant–microbe system can supply nitrogen in the order of hundreds of mg m^{-2} into the nutrient-limited littoral zone, where it may thus represent an important N source.

Key words: *Aldrovanda vesiculosa*, aquatic carnivorous plants, *Utricularia vulgaris*, *U. australis*, *U. intermedia*, *U. reflexa*, daily nitrogen gain, N nutrition, $^{15}N_2$ labelling, nitrogen fixation, periphyton, traps.

INTRODUCTION

The ecological group of aquatic carnivorous plants consists of about 50 species of the genus *Utricularia* (Lentibulariaceae), plus *Aldrovanda vesiculosa* (Droseraceae; Taylor, 1989; Adamec, 2011*a*). With the exception of a few rheophytic *Utricularia* species growing in fast-flowing streams, aquatic carnivorous species grow in standing, usually humic (dystrophic) waters, often under limited nitrogen (N), phosphorus (P) or potassium supply (for reviews see Adamec, 1997, 2008, 2009; Guisande *et al*., 2007). Morphologically, most species of aquatic carnivorous plants have a linear, modular and fairly regular shoot structure, consisting of nodes with finely dissected leaves and thin cylindrical internodes; the nodes can bear branches (Friday, 1989; Taylor, 1989). Most species have undifferentiated, homogeneous green shoots bearing traps (e.g. *A. vesiculosa*, *U. vulgaris*), while in some species (e.g. *U. intermedia*) heterogeneous shoots occur, differentiated into green photosynthetic shoots without traps and pale carnivorous ones with traps. Unlike terrestrial carnivorous plants, all aquatic carnivorous species are rootless and many of them exhibit very rapid apical shoot growth (1–4·2 leaf nodes per day) and high relative growth rate (the time of biomass doubling is usually 7–20 d; Friday, 1989; Adamec, 2000, 2009, 2010, 2011*a*; Pagano and Titus, 2007). This very rapid growth in nutrient-poor waters requires several ecophysiological adaptations, including very

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high net photosynthetic rate of shoots, carnivory, efficient nutrient re-utilization (recycling) from senescent shoots, very high affinity for mineral nutrient uptake from ambient water and, possibly, the activity of microbial commensals inside traps in *Utricularia* (Adamec, 2000, 2006, 2008, 2009; Richards, 2001; Englund and Harms, 2003; Díaz-Olarte *et al*., 2007; Sirová *et al*., 2009).

In contrast to *Aldrovanda* with its snapping traps, the traps of aquatic *Utricularia* are closed hollow bladders with a negative pressure inside, usually 1–5 mm long and filled with trap fluid, which attract, capture and digest prey of suitable size (e.g. Richards, 2001; Englund and Harms, 2003; Díaz-Olarte *et al*., 2007; Peroutka *et al*., 2008; Sirová *et al*., 2009; Adamec, 2011*b*). Traps also support diverse microbial communities living in association with the plant and benefiting from carbon-rich plant exudates (Richards, 2001; Sirová *et al*., 2009, 2010, 2011; Płachno *et al*., 2012). Traps were shown to contain the members of a complex microbial food web, with bacteria forming more than 58 % of the viable microbial biomass in the trap fluid. Aquatic carnivorous plants are adapted to a preferential uptake of NH_4^+ over NO_3^- by their shoots from the ambient water. Under favourable conditions, however, the dominant part of their seasonal N gain can be covered from prey capture (Adamec, 1997, 2000, 2008, 2011*a*; Fertig, 2001).

Nevertheless, aquatic *Utricularia* species commonly grow in waters where both mineral nutrient concentrations and prey availability are low (Richards, 2001; Adamec, 2008, 2009; Peroutka *et al*., 2008; Sirová *et al*., 2009). Other means of N acquisition were therefore suspected. Wagner and Mshigeni (1986) estimated that the shoots of African *U. inflexa* with associated epiphytes and trap micro-organisms were capable of fixing N_2 at rates of up to 2·3 μmol g^{-1} [dry mass (DM)] h^{-1}. Plant-associated N_2 fixation has also been reported as a potential source of N for several terrestrial carnivorous plant species with opened pitcher or snapping traps (Prankevicius and Cameron, 2011; Albino *et al*., 2000), although direct utilization of microbially fixed N_2 by carnivorous plants has not yet been experimentally confirmed. Nitrogen-fixing bacteria have also been detected in a beneficial association with non-carnivorous aquatic plant roots and surfaces (Zuberer, 1982; Reddy, 1987).

This information pointed to an additional pathway for aquatic carnivorous plants to acquire N in nutrient-poor environments – microbial N_2 fixation. The objective of our study was to detect the presence and activity of microbial N-fixing species associated with the shoots of four aquatic *Utricularia* species and *A. vesiculosa*, to measure their N_2 fixation rate using $^{15}N_2$ labelling, and to assess the significance of symbiotic N_2 fixation for the nutrition of the plants. The activity of diazotrophs was evaluated using the relative abundance of transcripts of genes responsible for the encoding of proteins related to the fixation of atmospheric N_2 (henceforth denoted collectively as *nif* genes). Furthermore, we assessed the dependence of the N_2 fixation rate on shoot age (i.e. density of periphyton and/or trap fluid colonization) and determined the relative contribution of traps and shoot surfaces to net shoot N_2 fixation. Ammonium concentrations in the trap fluid were measured in two *Utricularia* species. The comparison of *Utricularia* (traps with commensal micro-organisms) and *Aldrovanda* shoots (traps without commensals) was used to determine whether N_2 fixation is linked specifically to the microbial community inherent to *Utricularia*. Four *Utricularia* species cultivated under semi-natural conditions or collected in the field were compared, and sterile *Utricularia* shoots raised *in vitro* were used as controls. The ecological significance of the N_2 fixation rate for the daily N gain of the plants as well as for the littoral ecosystem is discussed.

MATERIALS AND METHODS

Plant cultivation

The experiment was conducted on the following aquatic carnivorous plants: *Aldrovanda vesiculosa* L. (originating from eastern Poland), *Utricularia vulgaris* L., *U. australis* R.Br., *U. intermedia* Hayne (all from the Czech Republic) and *U. reflexa* Oliv. (from Botswana). The shoot of *U. intermedia* is distinctly differentiated into a green photosynthetic part without traps and a pale carnivorous part bearing numerous traps (Taylor, 1989). Adult stock plants of *A. vesiculosa* (8–12 cm long) and *U. vulgaris* (60–80 cm long) were cultivated outdoors in a 2·5-m^2 (water surface) plastic container and *U. intermedia* plants (photosynthetic shoots 15–18 cm long, carnivorous shoots 8–15 cm long) in a 0·8-m^2 container which approximately simulated natural conditions (for details see Sirová *et al*., 2003, 2009). Adult stock plants of *U. reflexa* (20–40 cm long) were cultivated in a 0·8-m^2 plastic container, which stood in a naturally lit greenhouse with open lateral walls for cooling (see Borovec *et al*., 2012). Water depth in all these containers was 25–30 cm and *Carex acuta* litter was used as substrate. The pH of the cultivation media was 7·0–8·1, total alkalinity 0·43–1·04 meq L^{-1}, free CO_2 0·02–0·21 mmol L^{-1} and electrical conductivity 19·2–37·6 mS m^{-1}. Tap water was used as the source of water. Based on the nutrient concentration, the media were considered oligo-mesotrophic and slightly humic. The concentrations of NH_4-N were between 48 and 59 μg L^{-1}, while those of NO_3-N were always below detection. Small zooplankton were added to the containers to support plant growth. All containers were slightly shaded. Adult plants of *Utricularia australis* (40–70 cm long) were collected from a mesotrophic bog at Ruda fishpond at Branná (Třeboňsko Biosphere Reserve, South Bohemia, Czech Republic; see Sirová *et al*., 2010) 1 d before the start of the experiment. Here, the pH was 6·5, total alkalinity 0·51 meq L^{-1}, concentration of free CO_2 0·37 mmol L^{-1}, conductivity 9·0 mS m^{-1}, concentration of NH_4-N 62 μg L^{-1} and that of NO_3-N below detection. All experimental plants were transported to the laboratory in filtered culture or natural water so as not to fill their traps with air.

Trap fluid NH_4^+-N concentration measurements

Utricularia vulgaris and *U. reflexa* plants were chosen for the estimation of NH_4-N concentration in the trap fluid, due to their suitable trap size. Trap fluid from several dozen traps without any macroscopic prey from young (2nd–7th adult leaf node), middle (8th–13th leaf node) and old plant segments (13th–16th leaf node) was collected using a thin glass capillary attached to a peristaltic pump (Sirová *et al*., 2009). A pooled sample of approx. 250–300 μL from traps of the same age on a single plant was considered as a replicate; four independent samples from different plants were collected. Samples were

immediately filtered using microcentrifuge Eppendorf vials (0·22 μm pore size). Ammonium N concentrations were determined by a standard colorimetric bis-pyrazolon method on the flow injection analyser (QuickChem 8500, Lachat, Milwaukee, WI, USA).

Bacterial 16S rRNA amplicon and RNA sequencing, data analysis

To assess the presence and diversity of N_2-fixing bacterial species, *U. vulgaris* plants from the semi-natural culture were chosen, due to their abundant large traps from which we expected the highest yield of DNA and RNA necessary to obtain high-quality sequences. Whole trap-free shoots or excised traps (approx. 50 traps or 500 mg fresh weight) were collected, placed into liquid N and stored at −80 °C until further analysis. Nucleic acid extractions were conducted according to a modified bead-beating protocol (Urich *et al*., 2008). Total DNA was quantified fluorometrically using SybrGreen (Leininger *et al*., 2006). PCR primers (515F/806R) targeted the V4 region of the small subunit (SSU) rRNA gene, previously shown to yield accurate phylogenetic information and to have only few biases against any bacterial taxa (Liu *et al*., 2007; Bates *et al*., 2011; Bergmann *et al*., 2011). Each sample was amplified in triplicate, combined, quantified using Invitrogen PicoGreen (Carlsbad, CA, USA) and a plate reader, and equal amounts of DNA from each amplicon were pooled into a single 1·5-mL microcentrifuge tube. Once pooled, amplicons were cleaned up using the Ultra Clean PCR clean up kit (MO BIO Laboratories, Carlsbad, CA, USA). Amplicons were sequenced on an Illumina MiSeq platform at the Institute of Genomics and Systems Biology, Argonne National Laboratory, Argonne (Chicago, IL, USA). Paired-end reads were joined using Perl scripts yielding approx. 253-bp-long amplicons. Approximately 1·8 million paired-end reads were obtained with average 66 000 reads per sample. Quality filtering of reads was applied as described previously (Caporaso *et al*., 2010). Reads were assigned to operational taxonomic units (OTUs, cut-off 97 % sequence identity) using a closed-reference OTU picking protocol with QIIME-implemented scripts (Caporaso *et al*., 2010). Reads were taxonomically assigned using the Green Genes database, release 13_5, as reference. The number of SSU genes in different bacterial species varies from one to 15 copies per genome. We therefore normalized the number of SSU gene copies according to the protocol described by Langille *et al*. (2013) to reflect the relative abundance of bacterial taxa. Those reads which were assigned as 'chloroplast' and 'mitochondrion' were excluded from further analyses.

To assess the actively transcribed microbial gene pool, total trap RNA was extracted using the Plant RNeasy Midi Kit (Qiagen, the Netherlands) according to the manufacturer's instructions. Approximately 250 mg of fresh plant biomass was used, with traps pooled from a single plant considered as a replicate; three biological replicates were collected in total. DNA was removed from the extracts using the TurboDNAFree kit (Ambion, Austin, TX, USA). Transcriptomic eukaryotic libraries were created at the Institute of Genomics and Systems Biology, Argonne National Laboratory, Argonne (Chicago, IL, USA) using standard Illumina TruSeq RNA library prep kits. DynaBeads (Invitrogen) were used to remove the eukaryotic (plant) RNA fraction in the samples for the bacterial transcriptomic libraries, which were created from the same aliquots of RNA as the eukaryotic libraries. The Apollo 324 Platform (IntegenX, Pleasanton, CA, USA) was used to complete rRNA subtraction (RiboZero Plant and RiboZero Bacteria, 1:1 ratio of host and bacterial rRNA removal solution). Enriched mRNA was reverse transcribed to create bacterial metatranscriptomic libraries using the ScriptSeq V2 kit (Epicentre, Madison, MI, USA) and sequenced using an Illumina HiSeq platform (100 × 100 cycle paired-end run). We obtained approx. 40 million paired-end reads per sample. Reads were quality checked, and low-quality reads and reads with ambiguous bases were trimmed. The overlapping paired-end reads were joined using PANDAseq software (Masella *et al*., 2012). Approximately 80 % of reads were assembled and used for further analyses. First, the eukaryotic RNA with polyA tail was removed with trim_fasta.pl script (Frias-Lopez *et al*., 2008). Then, 1 million sequences were randomly selected using the perl script extract_fasta_records.pl (Meneghin, 2010). Reads were blasted against SILVA database release 111 to identify rRNAs. Those sequences (approx. 5·2 % of the total sequences remained after rRNA subtraction) that had a BLAST bit score greater than 80 were marked as potential rRNAs and extracted from the dataset. To identify potential prokaryotic functional gene transcripts, the remaining sequences without rRNAs were blasted against the non-redundant database with e-value of 0·001. A Unix *grep* script was then used to extract blast hits related to N_2 fixation.

$^{15}N_2$ fixation assay

Before the experiment, the plants were deprived of all branches. Shoot segments were dissected under water in a Petri dish, according to the position of adult leaf nodes, which is a precise measure of segment age (cf. Friday, 1989; Adamec, 2008). In *U. vulgaris*, they were: shoot growth tips including immature leaf nodes, very young shoots (3rd–6th adult nodes from the apex), middle shoots (11th–13th nodes) and old shoots (31st–33rd nodes); in *U. australis*: very young shoots (3rd–6th nodes) and old shoots (31st–33rd nodes); in *U. reflexa*: apical shoots (shoot apex with ten leaf nodes 8–12 cm long), basal shoots (11th–20th nodes 5–10 cm long), apical shoots without traps and 25–30 traps 2–6 mm long excised from the apical shoots; in *U. intermedia*: submerged green photosynthetic shoots (without traps, 12–15 cm long) and pale carnivorous shoots (with traps, 8–15 cm long); and in *A. vesiculosa*: apical shoots (shoot apex with ten nodes). As a control for estimation of the influence of periphyton and trap commensals on N_2 fixation, apical parts of *U. vulgaris* shoots (approx. 12–15 cm long) growing in an aseptic meristem tissue culture (see Adamec and Pásek, 2009) were also used. Two to three shoot segments from different plants represented one sample, the dry mass of which amounted to 5–41 mg.

The washed sample was gently blotted dry using a soft paper tissue and, according to the size of the segments, placed into a 125-mL glass bottle (Schott, Germany) filled with 20–40 mL of the filtered (25-μm mesh size) culture water (from the outdoor container with *U. vulgaris* and *A. vesiculosa*). The pH of this medium was 7·05, total alkalinity 1·04 meq L^{-1}, concentration of free CO_2 0·21 mmol L^{-1}, conductivity 19·2 mS m^{-1}, NH_4-N 48 μg L^{-1} and PO_4-P 12·3 μg L^{-1}. The bottles were

closed with a gas-tight rubber stopper fastened by a plastic screw lid with a central hole allowing gas exchange through a syringe needle. Half of the gas headspace volume (42–52 mL depending on the volume of the water) was removed by a syringe and replaced by the same volume of gas mixture comprising $^{15}N_2$ (99·8 at.%) and CO_2 in a molar fraction of 4000 μmol (CO_2) moL^{-1} ($^{15}N_2$). CO_2 was added to support photosynthesis of the shoot segments. Bottles with the shoot segments or excised traps in the culture water and $^{15}N_2$ in the headspace were placed in the inverted position into a growth chamber (Sanyo, UK) where the incubation continued at 23 °C and about 400 μmol (PAR) $m^{-2}\,s^{-1}$ for 24 h (15/9 h light/dark). Five independent replicates of each plant material type were used. Three additional control replicates without the $^{15}N_2$ addition were prepared in the same way.

^{15}N isotope analyses

After 24 h, each bottle was opened, plant segments washed under tap water and dried 48 h at 60 °C. The samples were homogenized to fine powder using a ball mill (Retsch MM200, Haan, Germany) and 10–15 mg of DM (shoots with sufficient mass quantity) or 0·7–0·8 mg (traps and apices with low amount of DM) were taken for isotopic and total N analyses. Tissue N content and the $^{15}N/^{14}N$ ratio were analysed with an isotope ratio mass spectrometer (IRMS; Thermo Finnigan DELTAplus, Brehmen, Germany) coupled to an elemental analyser (Carlo Erba, NA1110, Milan, Italy, or Elementar, vario MICRO cube, Hanau, Germany).

The abundance of ^{15}N in plant dry mass was expressed in the δ notation as the $^{15}N/^{14}N$ isotopic ratio of plant biomass (R_p) normalized to the isotopic ratio of N_2 in the free atmosphere (R_a = 0·0036765), $\delta^{15}N_p = [(R_p/R_a) - 1] \times 1000$ in permil (‰). We used IAEA-N-1 for calibration of the laboratory gas standard. Repeated measurements of IAEA-N-1 had a standard deviation of 0·11 ‰. As the large and small samples were analysed in two different IRMS laboratories (České Budějovice, Czech Republic, and Freising, Germany), an inter-laboratory cross check test was performed on nine selected samples. The $\delta^{15}N_p$ values obtained by both laboratories and plotted against each other formed a regression line with slope of 0·892 deviating slightly from the 1:1 line ($R^2 = 0{\cdot}991$).

Calculation of N_2 fixation rate

To follow the mass balance concept, we converted the $\delta^{15}N$ values to molar fractions defined as $^{15}N/(^{15}N + {}^{14}N)$ and expressed in atom-percent (*at%*). $\delta^{15}N$ is related directly to *at%* as follows (Montoya *et al*., 1996):

$$at\% = \frac{(1000 + \delta^{15}N)}{1000/R_a + (1000 + \delta^{15}N)} \times 100 \qquad (1)$$

To calculate the N_2 fixation rate by the experimental plants on the DM basis, we needed to know (1) ^{15}N abundance in the atmosphere above the water with experimental plants (headspace atmosphere enrichment in ^{15}N), $at\%_{ea}$, which ranged here from 47 to 58 *at%* depending on the volume of added $^{15}N_2$; (2) ^{15}N abundance in the atmosphere above the water with control plants (non-enriched headspace atmosphere), $at\%_{ca}$, assumed to be identical to free atmosphere $at\%_a = 0{\cdot}3676$; (3) ^{15}N abundance in control plants and in plants exposed to enriched ^{15}N atmosphere, $at\%_{cp}$ and $at\%_{ep}$, respectively; and (4) total N content in plants per unit dry mass, c (mg g^{-1}). It is useful to convert c to molar content of N per plant dry mass, N, in μmol ($^{14}N + {}^{15}N$) g^{-1}:

$$N_x = \left[\frac{c_x}{14}\left(1 - \frac{at\%_{xp}}{100}\right) + \frac{c_x}{15}\left(\frac{at\%_{xp}}{100}\right)\right] \times 1000 \qquad (2)$$

where the subscript x stands for c in control plants or e in plants exposed to ^{15}N-enriched atmosphere.

If the N_2 dissolved in water is fixed in the course of the experiment, ^{15}N accumulates in the experimental plants in excess compared with controls, N_{ep}:

$$N_{ep} = N_{cp} + N_\Delta \qquad (3)$$

As the only source creating the excess is the headspace $^{15}N_2$ (assumed to be in equilibrium with the plant water medium), the ^{15}N mass balance can be written as follows:

$$N_{ep}at\%_{ep} = N_{cp}at\%_{cp} + N_\Delta at\%_{ea} \qquad (4)$$

It is possible to find a fraction of the $^{15}N_2$ tracer fixed in experimental plants, f, by combining eqns (3) and (4):

$$f = \frac{N_\Delta}{N_{ep}} = \frac{at\%_{ep} - at\%_{cp}}{at\%_{ea} - at\%_{cp}} \qquad (5)$$

The tracer fraction f has built up during the course of the experiment. When divided by the duration of the experiment, time t, the rate of N_2 fixation relative to total N content (f/t in d^{-1}) is obtained. (The inverse value of the relative N_2 fixation rate, t/f, shows the time of N turnover expected when the N_2 assimilation is the only source of N gain.) f/t multiplied by total tissue N content in dry mass of the experimental plant, N_{ep}, gives the absolute rate of N_2 fixation, F_{N2} in μmol N g^{-1} (DM) d^{-1}:

$$F_{N2} = (f/t)N_{ep} \qquad (6)$$

After a particular time of cultivation, the plants reach a steady state when their dry matter peaks and stays constant. Should the $^{15}N_2$ label still be supplied at this stage, N isotopic composition of the plants would not change, i.e. the plant would reach an isotopic steady state. Let this phase be reached after T days. The fraction of N in the steady-state plant formed by N_2 fixation, f_{N2}, and expressed in per cent is:

$$f_{N2} = (f/t)T \times 100 \qquad (7)$$

Statistics and data deposition

Where possible, data sets were tested with either Student's *t*-test or one-way analysis of variance (ANOVA; Tukey post-hoc test) using the Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Raw reads were deposited in the NCBI Sequence Read Archive (SRA) under the submission ID SUB378555 and BioProject ID PRJNA227401.

RESULTS

Mean trap fluid NH_4-N concentrations in *Utricularia vulgaris* and *U. reflexa* usually varied slightly and non-significantly both between species and within traps of different age, ranging between 2·0 and 4·3 mg L^{-1} (Fig. 1), i.e. about 50–80 times higher than those in the ambient culture media. Although no clear pattern related to the age or species could be seen, the single values were high in all samples and always exceeded 0·5 mg L^{-1}.

Analyses of bacterial 16S rRNA amplicons from the trap fluid and periphyton of *U. vulgaris* confirmed the presence of microbial taxa with previously known ability to fix N_2 (Fig. 2). We identified a total of 66 bacterial OTUs in 22 families and five orders in this group. Members of Rhizobiales were the most abundant potentially diazotrophic bacteria in most samples and constituted between approx. 0·1 and 8·5 % of the total recovered bacterial sequences, depending on the treatment. Members of seven cyanobacterial families from three classes were also detected; several OTUs were assigned to the basal 4C0d-2 Cyanobacteria-like lineage. When comparing traps of various age, or the trap fluid and periphyton, no statistically significant differences in the relative abundance or composition of the diazotrophic species were found. The sequences that could be assigned to genes associated with N_2 fixation constituted less than 0·01 % of the active gene transcript pool. Most N_2 fixation-associated transcripts were attributed to *Chitinophaga* sp. from the phylum Bacteroidetes, followed by sequences attributed to uncultured, unclassified organisms, and *Anabaena* sp. (Cyanobacteria).

Dry matter of plants exposed to $^{15}N_2$ atmosphere was significantly enriched in ^{15}N compared with controls (Table 1). In *U. vulgaris*, for instance, the $\delta^{15}N$ increased from an average of 0·37‰ in controls to 3·71‰ in $^{15}N_2$-exposed apices and to 33·8‰ in $^{15}N_2$-exposed basal shoot parts. The ^{15}N enrichment increased gradually from young apical parts of the shoots toward their base in all investigated *Utricularia* species and was up to 15 times higher in the carnivorous shoots with traps than in the photosynthetic trap-free shoots in *U. intermedia.* The tissue N content in experimental plants ranged between 1·1 and 2·7 % DM and gradually declined from shoot apices towards shoot bases in single species.

Differences in $\delta^{15}N$ between controls and $^{15}N_2$-treated plants were converted into the linearly related N_2-fixation rates, F_{N2}, using eqn (6). The data clearly show gradients of N_2 uptake with F_{N2} values increasing from the youngest apical tissue toward the oldest basal shoot segments in *U. vulgaris*, *U. reflexa* and *U. australis* (Fig. 3A–C) and indicate the dominant role of traps in *U. reflexa* and *U. intermedia* (Fig. 3B and D, respectively).

Despite low F_{N2} values, all below 1 μmol fixed N g^{-1} (DM) d^{-1}, the N_2 assimilation rate was detectable and significant. The fraction of N fixed from the atmosphere, calculated using eqn (7), did not exceed 1·5 % and mostly formed less than 1 % of the total plant N (Fig. 3). Again, basal shoot segments and traps were confirmed to be the dominant organs to fix and accumulate atmospheric N. Interestingly, the entire *Aldrovanda* shoots with snapping traps exhibited a minimum N_2 fixation activity (Fig. 3C).

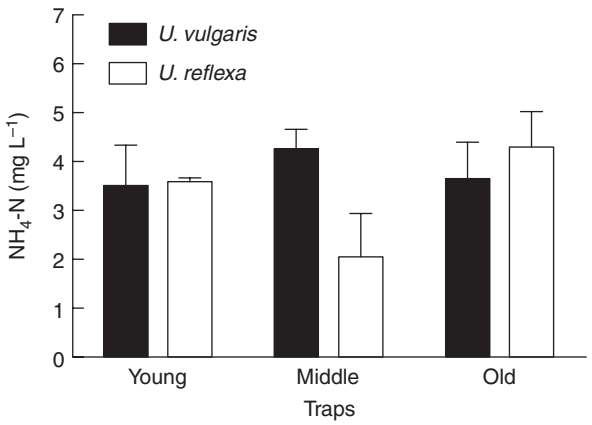

FIG. 1. Ammonium nitrogen concentrations in the *U. vulgaris* and *U. reflexa* trap fluid from traps of different age. Means ± s.e. are shown ($n = 4$). The differences between different trap ages within one species or between both species within one trap age were not statistically significant at $P < 0·05$ (one-way ANOVA).

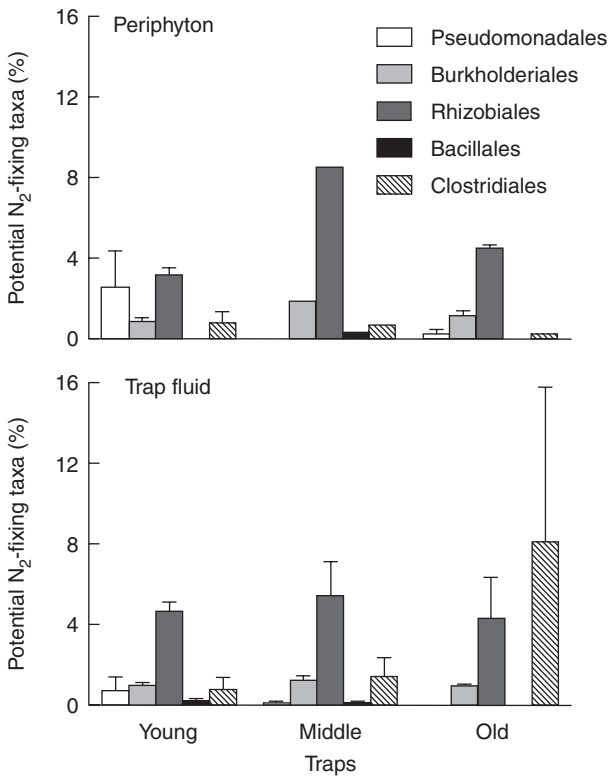

FIG. 2. The relative abundance of potential N_2-fixing taxa (% of total recovered sequences) identified in the periphyton and the trap fluid from *U. vulgaris* shoot segments of different age (means ± s.e. are shown, $n = 3$, except for middle trap fluid, where no replicates were available).

DISCUSSION

Although low, N_2 fixation was detectable in all our experimental aquatic carnivorous plants. The comparison of trap-free shoots and traps in *U. reflexa* and *U. intermedia* has shown that the trap lumen is the dominant site of N_2 fixation. When comparing the N_2 fixation rates for *U. reflexa* shoots and traps (Fig. 3B) and

TABLE 1. *Total tissue N content (% dry mass, DM) and $\delta^{15}N$ values in control plant tissues and plants exposed to ^{15}N-enriched atmosphere ($\delta^{15}N_c$ and $\delta^{15}N_e$, respectively) for all experimental plants and treatments; means ± s.e. are shown* (n = 5)

Species, treatment	N (% DM)	$\delta^{15}N_c$ (‰)	$\delta^{15}N_e$ (‰)
U. vulgaris, growth tips	2·68 ± 0·36	2·89 ± 1·10	3·71 ± 0·99
U. vulgaris, apical shoot segments	1·62 ± 0·22	1·76 ± 0·28	9·43 ± 5·60
U. vulgaris, middle shoot segments	1·35 ± 0·21	2·36 ± 0·68	18·7 ± 7·96
U. vulgaris, basal shoot segments	1·08 ± 0·10	2·43 ± 0·64	33·8 ± 15·8
U. vulgaris, sterile shoots	1·98 ± 0·04	−18·1 ± 0·62	−21·8 ± 0·36
U. reflexa, apical shoot segments	1·85 ± 0·16	6·55 ± 0·36	98·0 ± 71·3
U. reflexa, basal shoot segments	1·59 ± 0·27	5·70 ± 0·32	162 ± 36·8
U. reflexa, trap-free shoots	2·23 ± 0·15	6·06 ± 0·41	37·3 ± 34·8
U. reflexa, traps (excised)	1·66 ± 0·09	5·8 ± 0·33	81·8 ± 54·5
U. australis, apical shoot segments	2·40 ± 0·32	5·65 ± 0·38	11·8 ± 3·02
U. australis, basal shoot segments	2·11 ± 0·20	4·43 ± 1·48	49·4 ± 29·5
U. intermedia, photosynthetic shoots	2·37 ± 0·07	3·11 ± 0·10	6·08 ± 2·21
U. intermedia, carnivorous shoots	1·46 ± 0·12	2·84 ± 0·05	92·7 ± 28·3
A. vesiculosa, entire shoots	1·89 ± 0·18	2·00 ± 0·16	6·68 ± 0·90

assuming a 60 % proportion of traps to total shoot DM (L. Adamec, unpubl. res.), then the proportion of trap-associated N_2 fixation to the total shoot N_2 fixation is approx. 80 %. The only exceptions among our experimental plants without any detected ^{15}N enrichment and no positive N_2 fixation rate were the sterile *U. vulgaris* plants: the $^{15}N_2$-exposed plants were even slightly more depleted than controls (Table 1). Moreover, both treated and control sterile plants had markedly lower $\delta^{15}N$ than all other non-sterile plants, probably caused by inorganic N salts depleted in ^{15}N in the sterile mineral nutrient solution.

The absence of $^{15}N_2$ fixation in sterile plants clearly points to the involvement of microbial diazotrophs. Indeed, our results showed that there was a significant fraction of bacteria associated with *U. vulgaris* traps that are potentially capable of fixing atmospheric N_2 (Fig. 2). The most abundant group – the Rhizobiales – are well known for their symbiotic association with leguminous plants. Others, such as members of the Pseudomonadales and Burkholderiales, are ubiquitous in soil and many are known to have plant growth-promoting activity (Vessey, 2003; Drogue *et al.*, 2012). These groups were also present in the plant periphyton and no significant differences were found between the traps and shoots in the composition and abundance of the diazotrophic community members at the level of microbial order. This is consistent with previous results, which showed that the trap community is highly similar to that of the periphyton on the same plant, where it most probably originates (Sirová *et al.*, 2009). We attribute the N_2 fixation associated with trap-free shoots to periphytic microorganisms, but endophytes could also play a role. It has been shown that endophytic diazotrophs such as *Azospirillum*, *Burkholderia*, *Enterobacter* and *Pseudomonas* could supply up to 40 % of N to field-grown sweet potato plants (Yoneyama *et al.*, 1998). These genera were also present in our *U. vulgaris* samples.

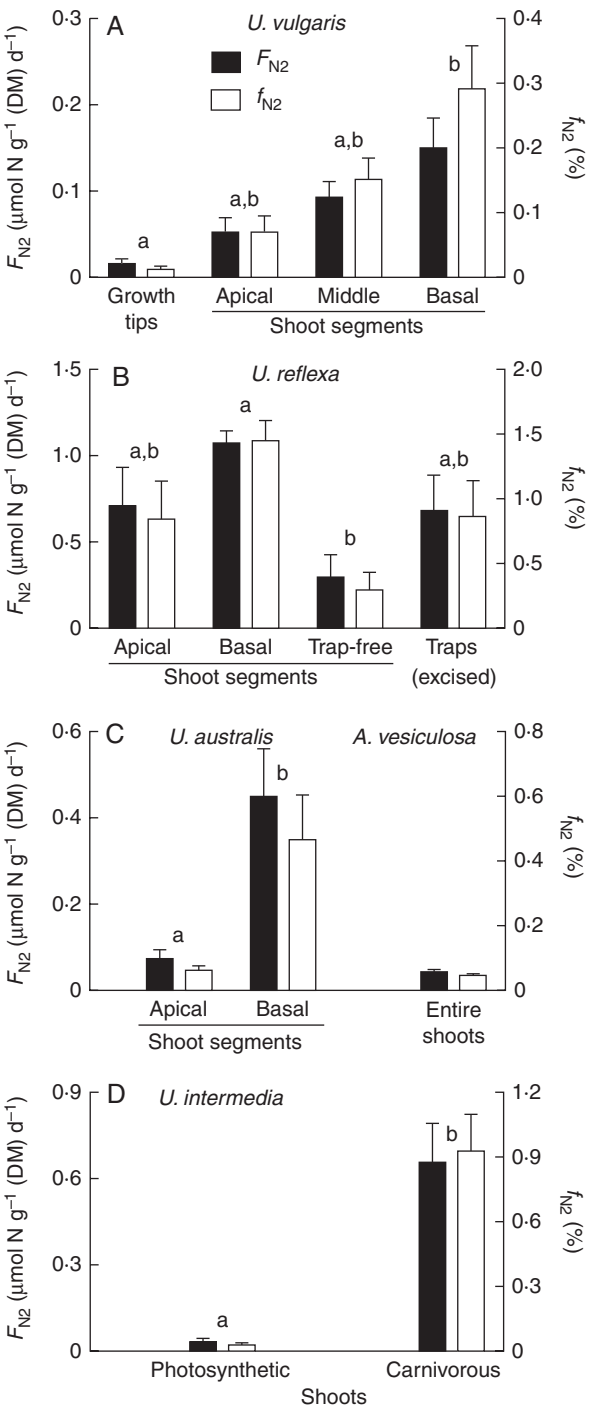

FIG. 3. Fixation rates of atmospheric N_2 (F_{N2}, left vertical axes) and its fraction in the total plant N (f_{N2}, right vertical axes) in different shoot segments or organs of four *Utricularia* species and in *Aldrovanda.* Means ± s.e. are shown; $n = 5$; distinct letters indicate statistically significant differences ($P < 0{\cdot}05$). 'Traps' denotes traps excised from shoots in *U. reflexa*, while 'trap-free' denotes samples consisting of apical shoots with excised traps; 'photosynthetic' and 'carnivorous' denote photosynthetic trap-free shoots and pale carnivorous shoots with traps of *U. intermedia*, respectively.

Although for technical reasons we have only applied molecular tools to study the proportion of diazotrophs in the traps of one *Utricularia* species, we expect similar patterns at the lower

taxonomical resolution to occur in others, as the trap lumen of different aquatic *Utricularia* is known to have similar physico-chemical properties. These include stable pH, low dissolved oxygen concentrations, high concentration of easily degradable dissolved organic carbon and high P content (Sirová *et al*., 2003, 2009, 2010, 2011; Adamec, 2007; Borovec *et al*., 2012). All of these factors are known to be favourable for the highly energetically demanding N_2 fixation process (Martinez-Argudo *et al*., 2005). However, neither the analysis of the actively transcribed N_2 fixation-associated genes nor the ^{15}N labelling results have confirmed that N_2 fixation represents a significant ecological benefit for the plants. The nitrogen fixation rates shown in Fig. 3 for the different species do not exceed 1·3 μmol N g^{-1} (DM) d^{-1}, while N_2 fixation rates in root nodules (López *et al*., 2008), in soil rhizosphere (Černá *et al*., 2009) or those associated with aquatic macrophytes such as *Lemna* (Zuberer, 1982) or *Azolla* (Reddy, 1987) are commonly one to two orders of magnitude higher. The fixation rates in *A. vesiculosa* were comparable with those observed in *U. intermedia* trap-free photosynthetic shoots, and can be attributed to the peri/endophytic communities.

In *U. vulgaris*, *U. reflexa* and *U. australis*, there was a marked increase of the N_2 fixation rates with increasing shoot age. This is in agreement with previously published results which show that the total microbial biomass is significantly larger in older traps (Sirová *et al*., 2009). This pattern is also reflected in the fraction of N_2-originating N in the total plant tissue N pool, although it does not exceed 1·5 % in any of the studied species, not even in the oldest tissues, and is therefore unlikely to be of significant benefit to plant N acquisition. Because plant tissues used in the analysis were contaminated by the presence of micro-organisms labelled by ^{15}N, the 1·5 % plant uptake is probably an overestimate, and represents a theoretical maximum for this study. This is confirmed by a model based on exponential plant growth [doubling time of biomass of 15 d and/or the daily DM increase of 4·73 %, 2 % N in plant DM, daily N gain of 68 μmol N g^{-1} (DM), 1 μmol of fixed N g^{-1} (DM) d^{-1}; see Table 1, this study; Adamec, 2011*a*] and on the assumption that, due to the fast turnover or organic matter in traps, most of the N_2 fixed within the lumen will be utilized by the plant. It similarly suggests that only approx. 1·5 % of the daily *Utricularia* N gain could theoretically be covered by this N_2 fixation rate.

It has been well documented previously that microbial N_2 fixation is inversely related to the concentration of available mineral N (Howarth *et al*., 1988; Vitousek *et al*., 2002). Surprisingly little is known about its concentration in the trap fluid of terrestrial carnivorous species with pitcher traps, although these are important model organisms in ecological studies (e.g. Butler *et al*., 2008; Mouquet *et al*., 2008; Baiser *et al*., 2013). Judging from the response of these plants to N addition, however, it can be assumed that these species tend to be N-limited (Ellison and Gotelli, 2002). Aquatic *Utricularia* therefore stands apart in the carnivorous plants group: we hypothesize that the reason for the limited associated N_2 fixation is the high NH_4-N concentration (2·0–4·3 mg L^{-1}) in their traps (Fig. 1), which probably inhibits N_2 fixation in most micro-organisms present.

The subunits of the main enzyme of the N_2 fixation process – nitrogenase – are encoded by *nif* genes, and the *nifH* gene encodes dinitrogen reductase, one of the nitrogenase components in N_2-fixing bacteria. In many diazotrophs, nitrogenase activities correspond well with the levels of *nifH* transcription (Terakado-Tonooka *et al*., 2013), which were very low in all our samples. The trap internal environment is rather heterogeneous, with numerous organic detritus particles of various sizes and shapes in different stages of decomposition (Richards, 2001; Peroutka *et al*., 2008; Sirová *et al*., 2009; Płachno *et al*., 2012); this probably results in numerous microhabitats or niches colonized by different microbes, as is the case in the water column, sediments or sedimenting organic flocks (Long and Azam, 2001). Although the concentration of NH_4-N in most traps is comparable with that in hypertrophic waters or waste waters, and probably inhibits N_2 fixation, certain trap microhabitats may experience reduced substrate diffusion and hence harbour microorganisms actively fixing N_2. This may be the case of *Chitinophaga* spp., which were found to be the most active diazotrophs in *U. vulgaris* traps.

It has been suggested previously that *Utricularia* traps are primarily used to enhance the acquisition of P rather than N (Sirová *et al*., 2003, 2009; Ibarra-Laclette *et al*., 2011). Ibarra-Laclette *et al*. (2011), in their analysis of the *U. gibba* transcriptome, have proposed that a significant proportion of N uptake may be channelled via the vegetative parts. This could explain why trap N concentrations (both NH_4-N and organic dissolved N) are consistently high, even in species growing in highly oligotrophic waters with low prey-capture rates (this study; Sirová *et al*., 2009, 2011).

In conclusion, the high trap NH_4-N concentrations, most probably resulting from rapid organic detritus turnover and low affinity for ammonium uptake of trap tissues, probably inhibit the expression of microbial *nif* genes. Although diazotrophs associated with trap lumen constitute a significant part of the microbial community, the possibility of a significant contribution by the N_2 fixation process to the nutrition of aquatic *Utricularia* under their typical growth conditions is unlikely. Despite this, when considering, for instance, typical *U. australis* stands (dozens of g DM m^{-2}), from which the plants for this study were collected, and the average N_2 fixation rate for this species of approx. 0·3 μmol N g^{-1} (DM) d^{-1} (Fig. 3C), it is apparent that hundreds of mg N m^{-2} can be supplied by the plant–microbe system yearly. This may not seem a lot, but it may represent an important N source in the nutrient-limited littoral zone at *Utricularia* sites.

ACKNOWLEDGEMENTS

This study was supported by the Czech Scientific Foundation project No. P504/11/0783 and partly by the long-term research development project No. RVO 67985939 (L.A.). Sincere thanks are due to Brian G. McMillan for correction of the English text. Special thanks are due to two anonymous referees for valuable comments that helped to improve the manuscript. Access to computing and storage facilities owned by parties and projects contributing to the National Grid Infrastructure MetaCentrum, provided under the programme 'Projects of Large Infrastructure for Research, Development, and Innovations' (LM2010005), is greatly acknowledged. We are grateful to the IGSB-NGS Sequencing Core at Argonne National Laboratory for preparing the metatranscriptomic libraries and completing the sequencing.

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