

## *Bromeliothrix metopoides*, a boom and bust ciliate (Ciliophora, Colpodea) from tank bromeliads

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### Abstract

We investigated the recently described colpoid ciliate *Bromeliothrix metopoides* in a series of laboratory experiments to reveal the environmental factors that constrain this species to its peculiar habitat, i.e. the tanks of bromeliads. Our results demonstrated that the various life stages of this ciliate (bacterivorous theronts and microstome trophonts, flagellate-feeding macrostomes) have specific demands in terms of food quality and quantity. *Bromeliothrix* required a high food threshold ( $>1.4 \text{ mg CL}^{-1}$ ) in order to thrive. Food quality also affected resting cyst formation of *B. metopoides* when the experimental containers dried out. Its maximum growth rates ( $\mu_{\text{max}} = 4.71 \text{ d}^{-1}$ , i.e. 6.8 doublings  $\text{d}^{-1}$ ) belong to the highest ones recorded thus far for free-living ciliates. The pH niche of *B. metopoides* was relatively wide (pH  $\sim 4$  to  $>9$ ) under optimal food conditions. However, its high sensitivity to unfavourable environmental conditions let the population collapse within several hours. We conclude that *B. metopoides* is a boom and bust ciliate that is specifically adapted to its peculiar habitat but virtually unviable in other environments.

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**Keywords:** *Bromeliothrix metopoides*; Feeding threshold; Growth rates; pH response; *Polytomella*; Tank bromeliads

### Introduction

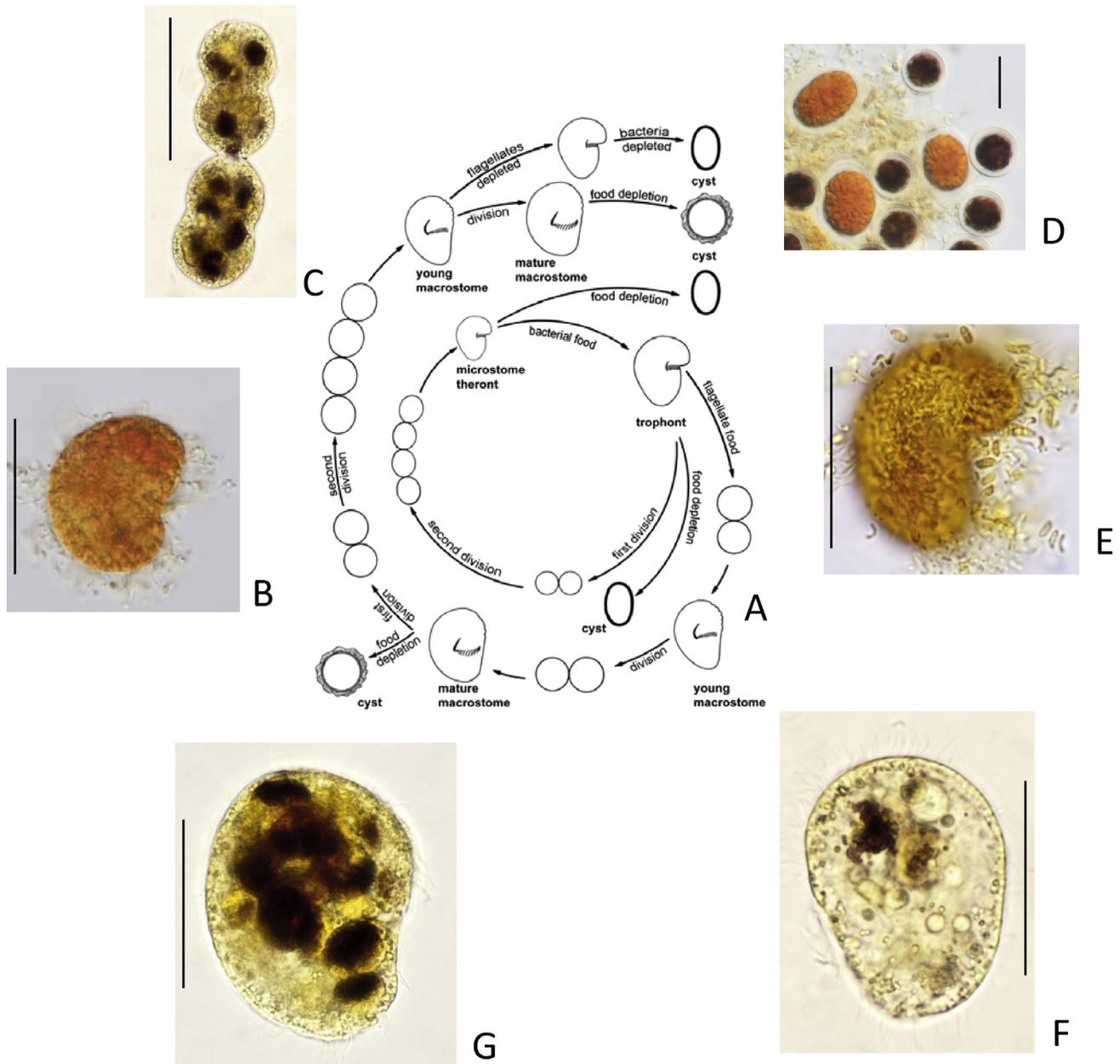
Epiphytic plants of the family Bromeliaceae are widespread and species-rich in Central and South America. Many bromeliads are able to store water in a reservoir formed by the tightly overlapping bases of their rosette leaves. Such phytotelmata (Varga 1928), which are also known as tanks or cisterns, may contain up to 30 L of water; they represent a highly specialized aquatic habitat (Foissner et al. 2003; Schönborn 2003). Although the fauna and flora of those tanks is poorly explored and biased towards macroorganisms, many

species, including over 400 metazoans, have already been recorded (summarized by Carrias et al. 2001; Kitching 2000; Schönborn 2003).

Recently, the ciliate fauna from bromeliad reservoirs has been studied in detail by Foissner and co-workers (Foissner 2003a,b, 2010, 2013; Foissner et al. 2003, 2011; Foissner and Wolf 2009; Omar and Foissner 2011, 2012). One of the most remarkable species is *Bromeliothrix metopoides* Foissner, 2010. This species has a complex life cycle (Fig. 1), with bacterivorous microstome theronts (dividing cells) and trophonts (usually 20–40  $\mu\text{m}$  in length) and large mouthed, flagellate-feeding macrostomes (usually  $>45 \mu\text{m}$  in length). All morphs form resting cysts; another characteristic feature of this ciliate is the formation of division chains. This ciliate is wide-spread, occurring in bromeliads from Mexico to southern Chile

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**Fig. 1.** The life cycle of *Bromeliothrix metopoides*: (A) with its various stages after Lugol's fixation; (B) microstome theront; (C) macrostome division chain; (D) resting cysts of theronts (large and light brown) and the flagellate *Polytomella* sp. (small and dark brown); (E) trophont; (F) young macrostome with few food vacuoles; (G) mature macrostome packed with food vacuoles containing *Polytomella* sp. Scale bars: 30  $\mu\text{m}$  (B, E, F), 40  $\mu\text{m}$  (C, G), and 20  $\mu\text{m}$  (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 1A reproduced from Foissner (2010).

(W. Foissner, unpubl. observations) and can be easily cultivated in an array of freshwater and soil media. The species was portrayed as an r-strategist, with doubling time  $\leq 5$  h. Food depletion induced cyst formation in both microstomes and macrostomes (Foissner 2010). However, the previous field and laboratory investigations could not explain why this ciliate is restricted to tank bromeliads and does not seem to occur in any other freshwater or soil environment (Foissner 2010). Foissner (2010) did not find *B. metopoides*

in about 2000 soil and freshwater samples investigated globally, including several hundred samples from Central and South America. The only other habitat where it sporadically occurred was the water and mud accumulating in bamboo stumps (Foissner 2010). Similarly, Foissner et al. (2003) noted that several other new ciliate species occurring in bromelian tanks are endemics, although they are morphologically similar to those found in more common freshwater habitats.

The goal of the present study was to investigate why *B. metopoides* appears to be limited to such a peculiar habitat. Water volume (typically ~0.01–3 L), light, pH (usually ranging from 4.0 to 7.0), nutrients and allochthonous organic input from leaf litter are the most important abiotic factors characterizing phytotelmata of tank bromeliads (Armbruster et al. 2002; Brouard et al. 2011, 2012; Laessle 1961; Lopez et al. 2009; Marino et al. 2011). However, detailed studies measuring environmental factors in tank bromeliads are rare. In the state of Rio de Janeiro, Brazil, temperature varies annually from 22 °C in July to 30 °C in January (Marino et al. 2011). These authors reported typical temperature variation in the tanks of bromeliads in this area in the range of 10 °C; pH was relatively variable, usually exceeding 2 pH units. A similar range of temperature (~10 °C) and pH (2–3 units) is known from the tanks of Jamaican bromeliads (Laessle 1961). However, habitat size (i.e. water volume) appears to be the primary abiotic variable affecting biomass of protists and small metazoans in tank bromeliads (Armbruster et al. 2002; Marino et al. 2011). In accordance with these sparse data, we focused our investigation on the formation of macrostomes and cysts in response to food, pH, and desiccation. Together with competition, predation and parasitism, these are likely the environmental key variables determining the survival of ciliate populations. For logistic constraints, we could not investigate the effect of predation and parasitism on the life cycle of *B. metopoides*. Competition experiments with *B. metopoides* were performed in our laboratory together with *Glaucomides bromelicola* Foissner 2013, another new ciliate species common in tank bromeliads (Foissner 2013). Because the competition experiments required a different experimental design as used in the present study, results will be reported elsewhere (Weisse et al. submitted).

Since the goal of our study stated above does not represent a falsifiable hypothesis, we hypothesized more specifically that

- larger food items would induce the formation of macrostomes,
- adverse conditions other than food depletion (e.g. low pH) may also induce the formation of cysts,
- the combination of several environmental factors narrows the realized ecological niche of *B. metopoides*.

Concerning the last hypothesis, it has recently been demonstrated for freshwater ciliates, flagellates and microeukaryotes that a combination of several environmental key variables such as food, temperature and pH may confine the ecological niche in situ to a relatively small range (Moser and Weisse 2011; Weisse 2006; Weisse et al. 2002, 2013a,b). By analogy, we assumed that the combination of food (quantity and quality), pH, and specific requirements related to cyst formation may restrict the ecological niche of *B. metopoides* to tank bromeliads.

## Material and Methods

### Origin and maintenance of stock cultures

All organisms used in this study were collected from tank water of small bromeliads growing on trees in subtropical forests of Brazil. Details of the origin of our study organisms have been reported by Foissner (2010). Cultures were established in Eau de Volvic (French table water) enriched with some sterilized crushed wheat grains to promote growth of indigenous bacteria and bacterivorous flagellates. Pure cultures were obtained by repeated dilution with Volvic and pipetting of individual target cells, respectively by removing unwanted predators or competitors. Stock cultures were maintained in ‘filter caps’ culture flasks (Biomedica, Vienna, Austria) with 50 mL of Eau de Volvic enriched with 1–2 wheat grains. The stock cultures were kept in an incubator at 22.5 °C under 14:10 h light:dark cycle; pH was ~7.5. New cultures were inoculated once per week by transferring 25 mL of the aged culture to a new flask containing 25 mL Eau de Volvic and one new wheat grain.

*Bromeliothrix metopoides* Foissner, 2010 is a flexible (cell size <math>20\text{--}55 \times 15\text{--}36 \mu\text{m}</math>) ciliate of the order Colpodida (Fig. 1). We kept this ciliate in stock cultures with and without the flagellate *Polytomella* sp.; this flagellate, which was isolated together with *B. metopoides*, has four flagella at the basis of a distinct papilla, four contractile vacuoles, lacks an eyespot, and has the nucleus in the anterior body half (Foissner 2010). The genus *Polytomella* comprises several colourless species of nutritional versatile, auxotrophic flagellates that thrive on acetate and other organic acids, peptone, and yeast extract (de la Cruz and Gittleson 1981; Pringsheim 1955). The species used in this investigation is possibly undescribed and will be described elsewhere (Foissner et al. in preparation); this species occurs in two differently sized morphs (see section “Results”). We added two wheat grains to 50 mL of Eau de Volvic to provide a nutritious medium for our *Polytomella* sp. cultures.

Bacteria were always present in stock cultures. Due to ciliate grazing, bacterial abundance was usually lower in flagellate-free cultures ( $0.2\text{--}1.0 \times 10^7$  cells mL<sup>-1</sup>) than in cultures with *Polytomella* sp. ( $0.7\text{--}1.8 \times 10^7$  cells mL<sup>-1</sup>). This is because if *Polytomella* are present, macrostomes of *B. metopoides* ingest the flagellates (see section “Results”). Abundance of the latter ranged from  $1\text{--}5 \times 10^4$  cells mL<sup>-1</sup>. Ciliate cell numbers in stock cultures usually ranged from  $0.1\text{--}2.2 \times 10^3$  cells mL<sup>-1</sup>.

### Growth experiments with different food organisms

We measured specific growth rate ( $\mu$ ) of *Bromeliothrix metopoides* in response to different food quality and quantity. If not specified, cell numbers of *B. metopoides* include microstomes and macrostomes. In asexually reproducing

ciliates,  $\mu$  is a direct proxy of their fitness (Weisse 2006). The ciliate was inoculated together with the respective food organism(s) into 50 mL culture flasks. Except for the first growth experiment (see legend of Fig. 2 for details), treatments with food organisms but without ciliates served as controls. Target food levels were obtained by diluting strongly growing cultures with Eau de Volvic. The initial ciliate abundance was 50–100 cells mL<sup>-1</sup>. All growth experiments were performed in the dark to prevent photoautotrophic food (the flagellate *Cryptomonas* sp.) from growing. Similarly, we removed remnants from wheat grains from the experimental containers to limit bacterial growth. Experimental duration lasted from several days to several weeks.

Samples (5 mL) were taken from the experimental containers at 24-h intervals and fixed with acid Lugol's iodine (final concentration 2%, vol/vol). Cell numbers of motile ciliates and their resting cysts were determined microscopically either in counting chambers of 3 mL volume or in Sedgewick Rafter chambers of 1 mL volume. Flagellate cell numbers were also counted microscopically, either together with the ciliates or separately in a 1-mL Sedgewick chamber. At higher abundance (>10<sup>4</sup> cells mL<sup>-1</sup>), flagellates were also counted and sized electronically by means of an automatic particle counter (CASY 1-model TTC, Schärfe System, Reutlingen, Germany; Weisse and Kirchoff 1997). Bacterial levels were measured in Formalin-fixed (2%, vol/vol) samples (2 mL) taken together with the ciliate samples. Bacterial cell numbers were assessed by flow cytometry after staining with the green fluorescent nucleic acid stain SYTO-13 (Molecular Probes), using a FACSCalibur flow cytometer (Becton Dickinson, BD Biosciences, San Jose, USA) equipped with an argon ion laser emitting light at 488 nm.

Cell size of ciliates and flagellates was measured with Lugol's fixed material using an inverted microscope and a semi-automatic image analysis system (LUCIA version 4.51, Laboratory Imaging, Prague, Czech Republic). Flagellates were also sized in unfixed material. The automatic particle counter yielded an independent estimate of flagellate cell size, which was used mainly to determine the cell volume of living cells. Both methods yielded similar results.

The cell volume of the prey flagellates used in this study (*Polytomella* sp. and *Cryptomonas* sp.) was converted to carbon units assuming the allometric equation provided by Menden-Deuer and Lessard (2000), i.e. pg C cell<sup>-1</sup> = 0.216 × cell volume<sup>0.939</sup>. To calculate carbon biomass of bacteria, we assumed a conservative estimate of 26 fg C cell<sup>-1</sup> for our cultured bacteria (Troussellier et al. 1997).

Cell volume ( $V$ ) of the ciliates was determined from length ( $l$ ) and width ( $w$ ) measurements, assuming a prolate spheroid shape:

$$V(\mu\text{m}^3) = \frac{\pi}{6} \times l \times w \times b \quad (1)$$

where  $b$  is cell breadth (in  $\mu\text{m}$ , as  $l$  and  $w$ ). Measurements were made on 50 ciliates each at the end of several

experiments. Since we could not measure the third dimension, we assumed that  $b$  is equal to  $0.7 \times w$ . Cell size was highly variable in the different experiments, mainly depending on food and the percentage of macrostomes in the population. Assuming typical average dimensions of  $l = 34 \mu\text{m}$  and  $w = 25 \mu\text{m}$  and accounting for 10% shrinkage due to fixation, we calculated an average cell volume of *B. metopoides* of 8570  $\mu\text{m}^3$ . The assumption of 10% shrinkage results from comparing results obtained in the present study with in vivo measurements of *B. metopoides* reported by Foissner (2010).

## Desiccation experiments

We studied the response of *Bromeliothrix metopoides* to desiccation by exposing ciliate cultures (50 mL) in open wide neck Erlenmeyer bottles to an experimental temperature of 25 °C and a 16:8 h light:dark cycle for several days. We used three different treatments: (A) *B. metopoides* with bacteria as sole food source and one wheat grain in each of the three replicates; (B) *B. metopoides* plus bacteria and *Polytomella* sp. with feeding 2 d after the beginning of the experiment; (C) *B. metopoides* plus bacteria and *Polytomella* sp. without feeding during the experiment. Bacteria and flagellates were provided in satiating amounts at the beginning of these experiments. The difference between treatments (B) and (C) was that 2 mL of a dense ( $4.3 \times 10^4$  cells mL<sup>-1</sup>) *Polytomella* sp. culture were added to the experimental flasks B to prevent the ciliates from becoming food limited during the experiment. Samples for the measurement of protist and bacterial cell numbers were taken once per day, as reported above. The experimental volume remaining in each container was measured on each sampling occasion. The volume removed at each subsampling was replaced with medium containing the respective target food concentrations.

To test for the viability of ciliate cysts produced in the course of the desiccation process, we repeated the desiccation experiment using a 12-well culture tissue plate. Four millilitres of a strongly growing *B. metopoides* culture were dispensed in each of the wells and incubated with open lid at 22.5 °C for 5 d. The wells were monitored several times per day over a period of five days, when all wells were completely dry. Three days thereafter, we added fresh Volvic medium with and without *Cryptomonas* sp. as food to each well. We used nine replicates with *Cryptomonas* sp. added and three without. Ciliates were monitored at least once per day during the first week and occasionally thereafter.

## Response to pH

We investigated the growth and survival of *Bromeliothrix metopoides* over pH ranging from 4.0 to 9.0; pH was measured using a microprocessor pH-mV metre (WTW, Weilheim, Germany, model pH 526) to the nearest 0.01 unit. The pH sensor was 2-point calibrated with standard buffer solutions of pH=6.87 and pH=9.18 before each series of

measurement. Ciliates and their prey (provided at satiating amounts) were acclimated to the experimental conditions in steps of 0.5 pH unit change  $\text{d}^{-1}$  for 2–5 d. We measured and adjusted the pH in each experimental container twice per day, i.e. if the pH differed by more than 0.2 from the target pH, it was adjusted by addition of small amounts (15–35  $\mu\text{L}$ ) of 0.1  $\text{mol L}^{-1}$  NaOH or HCl (Weisse et al. 2007, 2013b; Weisse and Stadler 2006). The general experimental design followed that of the growth experiments.

Except for the experimental series with small and large *Polytomella* sp. as food organisms (results reported in Fig. 4), all experiments described above were performed in triplicate. Results reported in the text and figures are mean values  $\pm 1$  standard deviation (SD).

## Data analysis

Ciliate growth rate ( $\mu$ ) was calculated from changes in cell numbers, assuming exponential growth over the experimental period according to

$$\mu = \frac{\ln(N_t/N_0)}{t} \quad (2)$$

where  $N_0$  and  $N_t$  are ciliate numbers at the beginning and end of the experimental period, respectively. Please note that ‘experimental period’ does not denote the total duration of an experiment, but specific periods of 1–4 d each during which the ciliate population increased exponentially. Details are reported in Results.

Ciliate growth rates were related to the geometric mean prey concentration ( $P$ ) during the experimental period (Frost 1972; Heinbokel 1978) according to Eq. (3):

$$P = \frac{P_t - P_0}{\ln(P_t/P_0)} \quad (3)$$

where  $P_0$  and  $P_t$  are the initial and final prey concentrations (cells  $\text{L}^{-1}$ ) during incubations.

Ciliate growth rates were fit to Eq. (4), which includes a positive  $x$ -axis intercept, using the Marquardt–Levenberg algorithm (SigmaPlot, SPSS Inc., Chicago, USA).

$$\mu = \frac{\mu_{\max}(P - x')}{(k + P - x')} \quad (4)$$

where  $\mu$  = growth rate,  $\mu_{\max}$  = maximum growth rate,  $P$  = prey concentration (Eq. (3)),  $k$  = a constant,  $x'$  = the  $x$ -axis intercept (i.e. threshold concentration, where  $\mu = 0$ ). This equation is similar to the Michaelis–Menten model and Holling’s type II functional response (Holling 1959), but assumes a positive  $x$ -axis intercept where population growth equals mortality (Weisse et al. 2002). This assumption was based upon our observations from maintenance cultures with *B. metopoides* that the ciliate needs relatively high food levels to thrive. The numerical response curve has the shape of a saturation curve, i.e. is characterized by an initial linear or nearly linear increase at low food concentrations (initial slope) that levels off asymptotically to the constant maximum

growth rate. Our data mainly fell into the linear part of the curve; accordingly, we also calculated least-squares linear regression (Eq. (5)) to calculate the  $x$ -axis intercept:

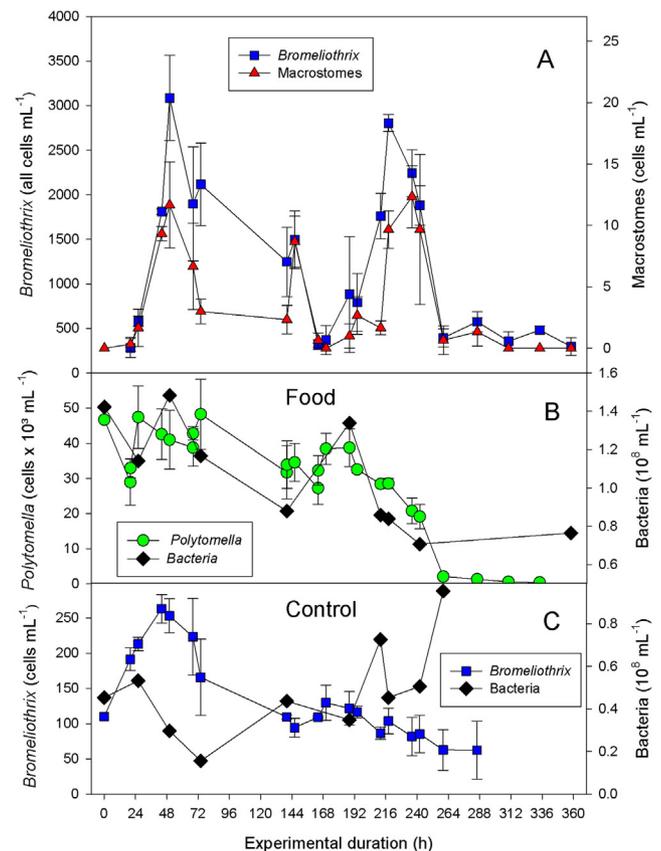
$$\mu = a + b \times P \quad (5)$$

where  $a$  is a constant and  $b$  is the slope of the regression.

## Results

### Response to flagellate and bacterial food – induction of macrostomes

The formation of macrostomes of *Bromeliothrix metopoides* can be induced by feeding with *Polytomella* sp. (Foissner 2010). Preliminary experiments had shown that the ciliate population (i) increases rapidly when small *Polytomella* sp. is offered as food in sufficient quantity ( $>2 \times 10^4$  cells  $\text{mL}^{-1}$ ) and (ii) *B. metopoides* cannot thrive if bacteria are the sole food and bacterial levels fall below



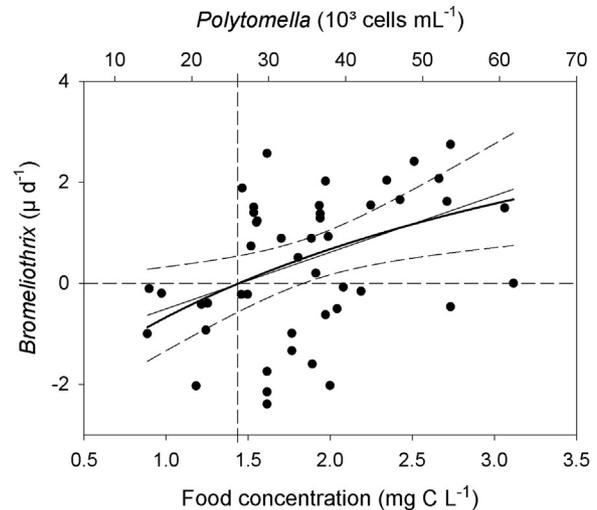
**Fig. 2.** Population development of the ciliate *Bromeliothrix metopoides*; (A) total ciliate cell number and abundance of macrostomes fed with the flagellate *Polytomella* sp. and undefined heterotrophic bacteria (B); the bottom panel (C) shows the abundance of *B. metopoides* in control treatments without *Polytomella*, together with the bacterial abundance. Symbols denote mean values, error bars one standard deviation.

$\sim 10^8$  cells  $\text{mL}^{-1}$  (data not shown). Microstome theronts did not feed on *Polytomella*; we observed trophonts or young macrostomes ( $\sim 30$ – $35$   $\mu\text{m}$  in length when Lugol's fixed) with single or, in rare cases, two flagellates ingested. Mature macrostomes ( $>45$   $\mu\text{m}$ ), in contrast, contained up to  $\sim 20$  food vacuoles filled with flagellate cells (Fig. 1G).

The first detailed growth experiment confirmed these preliminary results. We measured total cell numbers of *B. metopoides*, the formation of macrostomes, and the bacterial concentrations in the experimental flasks with *Polytomella* sp. as food and controls without flagellates (Fig. 2). The formation of macrostomes was significantly positively and linearly correlated ( $r^2 = 0.648$ ,  $n = 64$ ) to the total *Bromeliothrix* population, i.e. macrostomes were always present in low numbers, corresponding to  $<1\%$  of the total (Fig. 2A). We observed two peaks of *Bromeliothrix* at 48 h and 220 h after the beginning of the experiment. Growth rates calculated for the periods of maximum increase ( $\mu_{\text{max}}$ ) were  $2.4 \text{ d}^{-1}$  (between 20 and 44 h), respectively  $2.1 \text{ d}^{-1}$  (between 194 and 218 h). Growth rates were positively related to *Polytomella* sp. biomass (Fig. 3). The non-linear fit (Eq. (4)) yielded a slightly better, but still poor overall curve fit than the least-squares linear regression calculated from Eq. (5) ( $r^2 = 0.148$ , respectively  $r^2 = 0.143$ ). Both curve fits indicated a significant ( $P < 0.0001$ ) threshold food level (where  $\mu = 0$ ) of  $26,700$  cells  $\text{mL}^{-1}$ , corresponding to  $\sim 1.4$   $\text{mg C L}^{-1}$ ; parameter estimates of the regression equations are reported in the supplementary Table S1.

The course of the bacterial cell numbers observed in the experimental bottles (Fig. 2B) and controls without flagellates (Fig. 2C) suggests that *Bromeliothrix* microstomes ingested bacteria and macrostomes fed on *Polytomella* (Fig. 2B). However, *B. metopoides* declined both in the experimental bottles (Fig. 2A) and in the controls without *Polytomella* (Fig. 2C) if bacterial levels fell below  $\sim 0.9 \times 10^8$  cells  $\text{mL}^{-1}$  (Fig. 2B, C). This bacterial abundance corresponds to carbon levels of  $\sim 2.3$   $\text{mg C L}^{-1}$ , i.e. the threshold food level appeared to be higher when bacteria were used as the primary food. We did not observe macrostomes in the controls without flagellates, but cannot rule out that they were present in low numbers below the detection limit ( $1 \text{ cell mL}^{-1}$ ).

We had observed in our routine cultures that the flagellate *Polytomella* sp. occurs in two morphs; a dominant small form ( $\sim 10 \times 8$   $\mu\text{m}$  in length and width) used in the previous experiments and a distinctly larger form (up to  $\sim 40 \times 32$   $\mu\text{m}$ , Foissner 2010). The ratio between these two forms was variable in different treatments, but it also seemed to fluctuate with time in a single treatment. Accordingly, we monitored the abundance of *B. metopoides* in relation to the occurrence of both *Polytomella* sp. morphs in several experimental flasks over a period of two weeks (Fig. 4). In the first experimental treatment, the abundance of both *Polytomella* sp. morphs (Fig. 4A) and the ciliates (Fig. 4B) were relatively low,  $<2 \times 10^3$  cells  $\text{mL}^{-1}$ , and food was kept at low levels due to the ciliate grazing. The average

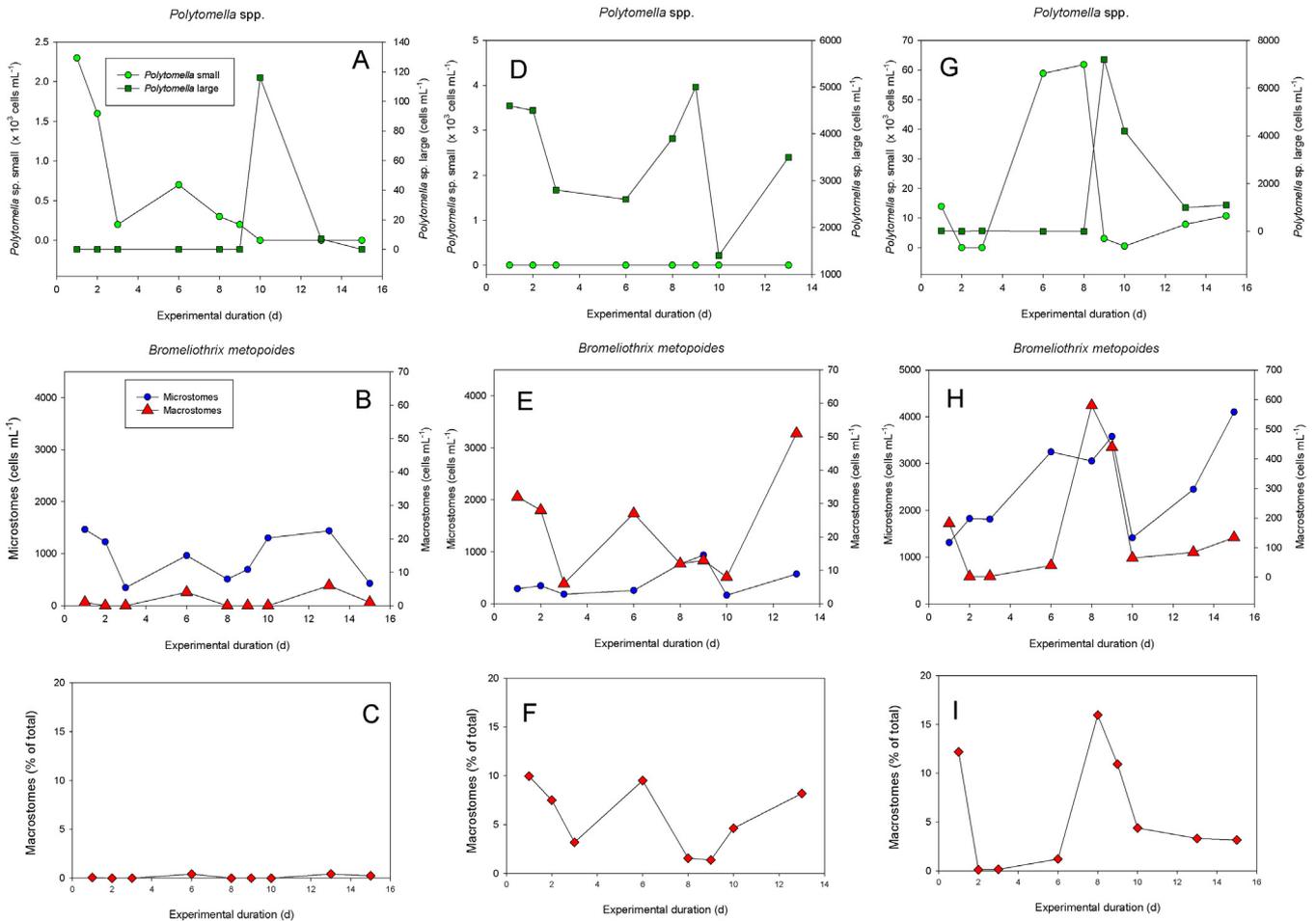


**Fig. 3.** Growth rate ( $\mu$ ) of *Bromeliothrix metopoides* vs. abundance (top x-axis), respectively biomass (bottom x-axis) of the prey flagellate *Polytomella* sp. Ciliate growth rates were fit to the non-linear Eq. (4) (thick solid curve) and to a linear regression (thin solid curve and 95% confidence intervals represented by long dashes). The horizontal dashed line indicates zero population growth ( $\mu = 0$ ); the intercept of the vertical dashed line with the x-axes indicates prey biomass (bottom), respectively prey abundance (top) at  $\mu = 0$ .

flagellate food level (i.e. without bacteria) was  $\sim 0.1$   $\text{mg C L}^{-1}$  in this series. The *Bromeliothrix* population declined from initially  $1464$  cells  $\text{mL}^{-1}$  to  $429$  cells  $\text{mL}^{-1}$  recorded at the end of the experiment (Fig. 4B). The large *Polytomella* sp. morph was recorded only once, at 10 d after the beginning of the experiment (Fig. 4A). Macrostomes were present in low numbers ( $\sim 1\%$  of the total ciliate population) throughout the experiment (Fig. 4C).

In the next experiment, *Polytomella* sp. was almost exclusively composed of the large form (Fig. 4D). Due to its larger cell size, the initial flagellate food concentration was  $\sim 1.0$   $\text{mg C L}^{-1}$ ; except for the low level measured on the 10<sup>th</sup> day of the experiment, food remained  $>0.6$   $\text{mg C L}^{-1}$  throughout this experiment. Both the absolute number (Fig. 4E) and the ratio of macrostomes to microstomes of *B. metopoides* (Fig. 4F) were higher in this experiment than in the previous one. On average, macrostomes accounted for  $5.7 \pm 3.4$  (SD) % of the total *Bromeliothrix* population in this experiment.

In a third experiment, the initial numerical ratio between small and large *Polytomella* sp. was approximately 10:1. We observed a rapid increase in the abundance of the small morph up to  $\sim 60,000$  cells  $\text{mL}^{-1}$  ( $\sim 3.2$   $\text{mg C L}^{-1}$ ), followed by a sudden shift from small to large *Polytomella* sp. (Fig. 4G); concurrently, cell numbers of *B. metopoides* macrostomes increased (Fig. 4H). Macrostomes reached 16% of the total *B. metopoides* population (Fig. 4I) during this period, the highest proportion of macrostomes that we recorded during all our experiments.

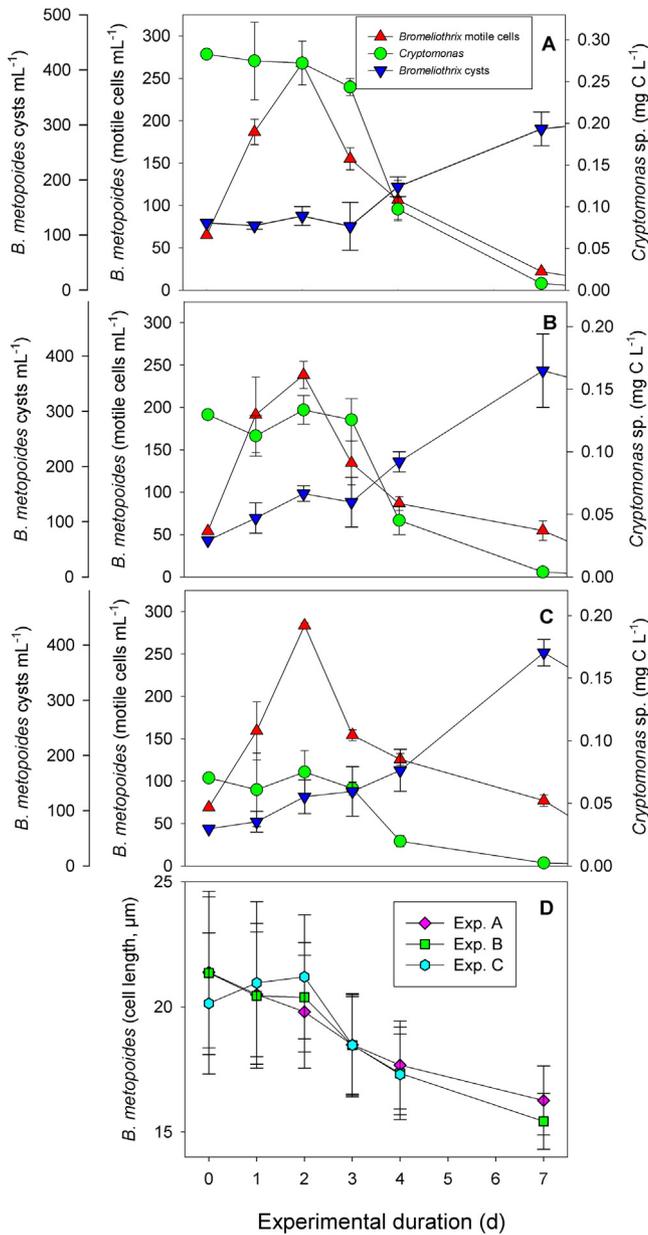


**Fig. 4.** Population development of the ciliate *Bromeliothrix metopoides* fed with small and large forms of the flagellate *Polytomella* sp. in three different treatments (A–C, D–F, G–I); the treatments differed in prey concentration and the relative contribution of the two flagellate morphotypes. Panels A, D and G show the abundance of the two flagellate forms; graphs B, E and H show cell numbers of the ciliate with its two different sizes (microstomes and macrostomes). The bottom panels (C, F and I) report the occurrence of macrostomes as percentage of the total ciliate abundance.

Some other food sources tested in our laboratories did not support sustainable growth of *B. metopoides*. Small (*Colpoda steinii*, *Cyrtolophosis mucicola*) and medium-sized (*Colpoda maupasi*) ciliates from bromeliads and the flagellate *Chilomonas* sp. were not ingested (Foissner 2010). Our initial attempts to rear *B. metopoides* with the common flagellate *Cryptomonas* sp., which is the preferred food of many freshwater ciliates (Weisse 2006), and with the small ciliate *Urotricha furcata* failed (this study; data not shown). In those experiments, we had tried to mimic dispersal of individual *B. metopoides* into new freshwater habitats. Individual cells of *B. metopoides* were transferred into wells of tissue culture plates and provided with flagellate or ciliate food at different levels ranging from  $\sim 10^2$  cells mL<sup>-1</sup> to  $5 \times 10^4$  cells mL<sup>-1</sup>. The latter corresponds to  $\sim 1.4$  mg CL<sup>-1</sup>, i.e. it is close to the threshold food level obtained in this study with *Polytomella* sp. as food. In a second step, we investigated the growth performance of *B. metopoides* with *Cryptomonas* sp.

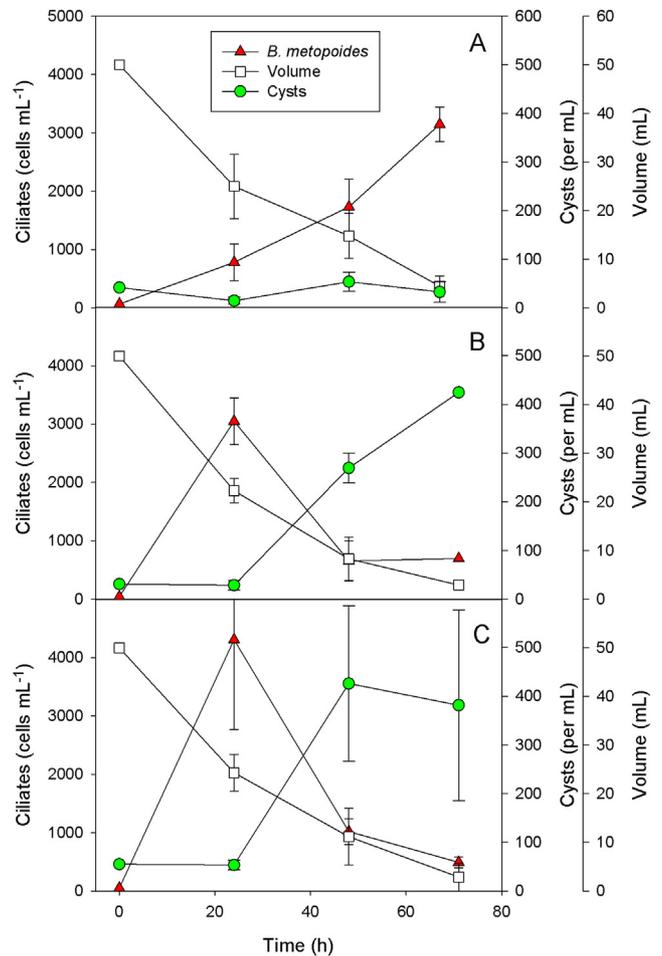
as food in the same culture flasks as used with *Polytomella* sp., using an inoculum of several mL (i.e. several hundred cells) of an exponentially growing *Bromeliothrix* culture. Bacterial abundance was relatively low ( $3.5\text{--}4.6 \times 10^7$  cells mL<sup>-1</sup>) in the experiments with *Cryptomonas* sp. and did not increase because Eau de Volvic is a poor medium for heterotrophic bacteria.

We observed the same pattern over a wide range of flagellate food levels (0.05–2.0 mg CL<sup>-1</sup>): cell numbers of *B. metopoides* increased during the first 2 d, then mass encystment began and motile cell numbers declined to zero levels within two weeks. At the lower food levels (0.05–0.3 mg CL<sup>-1</sup>, corresponding to  $0.3\text{--}1.2 \times 10^4$  cells mL<sup>-1</sup>), which is typical for most oligotrophic to moderately eutrophic freshwater bodies, *Cryptomonas* sp. did not support survival of motile *B. metopoides* cells (Fig. 5A–C). Virtually all motile cells had encysted after one week, i.e. low to moderate *Cryptomonas* sp. food supply triggered mass encystment of the



**Fig. 5.** Population development and cell length of the ciliate *Bromeliothrix metopoides* fed with the flagellate *Cryptomonas* sp. at three different food levels (A–C). Graphs A–C show the abundance of motile ciliate cells and the occurrence of ciliate cysts, together with the flagellate cell numbers. Cell length of the ciliate in these three treatments is shown in the bottom panel (D).

ciliate. The *Cryptomonas* sp. levels declined in all experimental containers and in the controls (data not shown) after three days because the experiments were conducted in the dark. Different from the experiments with *Polytomella* sp., macrostomes were not formed if algae were offered as prey. Another indication that *Cryptomonas* sp. was not a suitable food organism for *B. metopoides* is that the ciliate cell size significantly declined in the treatments with *Cryptomonas* sp. (Fig. 5D).



**Fig. 6.** Population development of the ciliate *Bromeliothrix metopoides* fed with heterotrophic bacteria (A), respectively bacteria plus the flagellate *Polytomella* sp. (B, C) at continuously reduced culture volumes (mimicking desiccation) in three different treatments. Cell numbers of motile ciliates (triangles, left y-axis) and cysts (circles, right y-axis), and the experimental volume (squares, right y-axis offset) are reported in each graph; bacterial and flagellate levels are not shown. The difference between treatments B and C is that only in B the ciliate was fed with the flagellate in the course of the experiments.

## Response to desiccation

The small tanks of tree bromeliads may dry up occasionally. We, therefore, investigated the response to desiccation of *Bromeliothrix metopoides* in another experimental series. The initial experimental volume of 50 mL was rapidly reduced to <5 mL over a period of 72 h (Fig. 6). When bacteria were the sole food (treatment A), the ciliate population increased but did not respond with augmented cyst formation (Fig. 6A). Average bacterial concentrations in this experiment increased steadily from  $0.22\text{--}2.01 \times 10^8$  cells mL<sup>-1</sup> and were equivalent to  $>2.0$  mg C L<sup>-1</sup> during the final 2 d of the experiment (data not shown).

With *Polytomella* sp. as additional food source (treatments B and C), ciliate cell numbers composed of theronts,

**Table 1.** Total number and percentage of motile *Bromeliothrix metopoides* cells in 9 containers (wells) with medium and *Cryptomonas* sp. as food and 3 containers with medium only.

Time after addition of medium (h)	With <i>Cryptomonas</i> sp.	Without <i>Cryptomonas</i> sp.
2.75	0 (0%)	0 (0%)
5.1	15 (78%)	0 (0%)
20	(100%) <sup>a</sup>	24 (100%)
24	(100%) <sup>a</sup>	30 (100%)
264	(100%) <sup>a</sup>	(100%) <sup>b</sup>

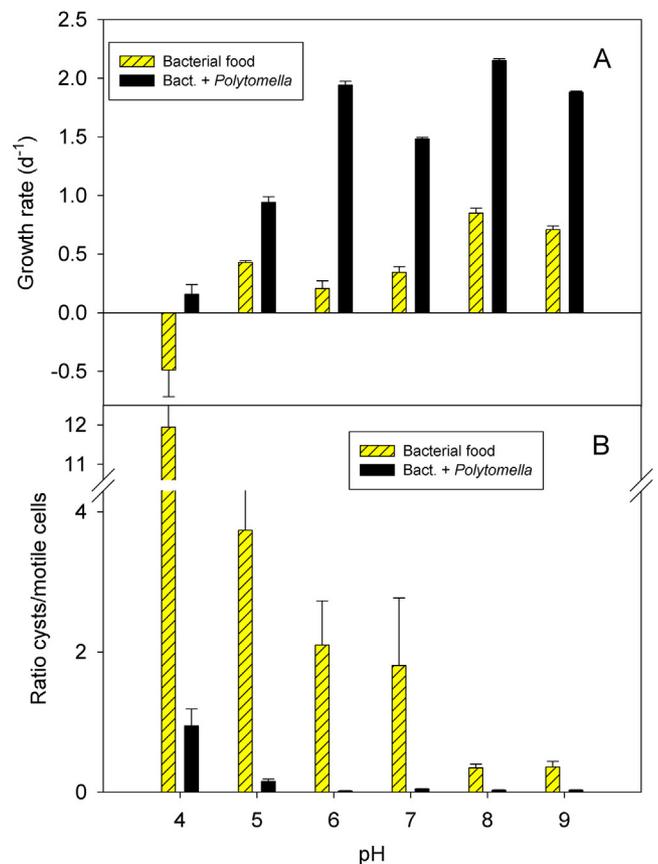
<sup>a</sup>Denotes >20 cells in each well.

<sup>b</sup>Denotes >10 cells in each well.

trophonts and macrostomes increased rapidly during the first 24 h, but then declined (Fig. 6B, C) although food was present in satiating amounts (>2.0 mg C L<sup>-1</sup>). The treatments reported in Fig. 6B and C differed because only to the three replicates of the treatment shown in Fig. 6B small amounts of fresh *Polytomella* sp. were added during the experiments. However, because the flagellates grew at high rates and macrostomes were present in low numbers, ciliate feeding had little effect on the flagellate cell numbers. The initial flagellate cell numbers were  $3.5 \times 10^4$  cells mL<sup>-1</sup>, final average cell numbers were  $>5 \times 10^5$  cells mL<sup>-1</sup> in both experimental series. In contrast to the experimental series with only bacterial food (Fig. 6A), encystment was strongly stimulated, starting 24 h after the beginning of the experiment. The final average cyst number was close to 400 mL<sup>-1</sup> (Fig. 6B, C), i.e. almost 50% of the ciliate population were encysted at the end of the experiment.

The ciliates reached high growth rates in the desiccation experiments. The average maximum growth rates (i.e.  $\mu_{\max}$  averaged over the three replicates of each experimental series) recorded for *B. metopoides* were 2.44 d<sup>-1</sup> with bacterial food only (Fig. 6A), respectively 4.26 d<sup>-1</sup> and 4.48 d<sup>-1</sup> with additional flagellate food. The maximum growth rate recorded in a single treatment was 4.71 d<sup>-1</sup>, corresponding to one cell division every 3.5 h, respectively 6.8 doublings d<sup>-1</sup>. Maximum growth rates of *Polytomella* sp. were lower, reaching 1.84 d<sup>-1</sup> at the end of the experiment.

To test for the viability of the cysts, we repeated the desiccation experiment using a 12-well culture tissue plate. On the fourth day of the experiment, small drops remained in most wells. On day 5, all wells were dry. Three days thereafter, we added fresh medium with and without *Cryptomonas* sp. as food to each well. No ciliates excysted during the first 3 h after the addition of medium (Table 1). Two hours later, motile cells occurred in seven of the nine containers (78%) with food algae, whereas no ciliates had excysted in the treatments without food. Twenty hours after the beginning of this experiment, numerous ciliates swam in the containers with food algae, and motile cells were also encountered in each of the wells without flagellate food. We found the same pattern 24 h and 11 d after the beginning of the experiment. Since



**Fig. 7.** Specific growth rate (A) and relative occurrence of cysts (B) of the ciliate *Bromeliothrix metopoides* vs. pH when fed with bacteria only or with a combination of flagellate (*Polytomella* sp.) and bacterial food.

the tissue plates were not axenic, bacterial growth may have supported survival of excysted cells.

### Response to pH

The experiments reported above were all conducted under circumneutral to moderately basic conditions (pH 7.1–8.3). To account for the effect of pH, we investigated the ciliate response to pH over a range of pH from 4 to 9 under satiating food conditions. Average growth rates of *Bromeliothrix metopoides* were highest at circumneutral conditions (Fig. 7A). Similar to the previous experiments, growth rates measured in the treatments with *Polytomella* sp. as food were higher than those recorded with bacteria as the sole food (Fig. 7A). However, in contrast to the desiccation experiment reported above, bacteria stimulated encystment (Fig. 7B). Under the most adverse conditions, i.e. a combination of low pH (pH 4) and suboptimal food (bacteria only), >90% of the ciliate population encysted in the course of the experiment. Similar to the previous experiments, the proportion of cysts was low if *Polytomella* sp. was present and pH was >5 (Fig. 7B).

## Discussion

### *Bromeliothrix metopoides* is an extreme r-strategist

Taken together, our experiments characterize *Bromeliothrix metopoides* as an extreme r-strategist, doubling more than six times per day under favourable conditions. Doubling time  $\leq 5$  h has already been recorded by Foissner (2010) for this species. Its growth rates belong to the fastest ones reported for free-living ciliates in the literature (reviewed by Fenchel 1987; Lynn 2008). The ciliate can increase its numerical population size more than tenfold within 24 h because of its peculiar life cycle: rapid cell divisions by microstome theronts, coupled with the formation of division chains, and the switch of bacterial feeding theronts to flagellate-feeding trophonts and macrostomes. We did not observe that *B. metopoides* divides in cysts, as it is known from other colpodid ciliates (Foissner 1993; Gutiérrez et al. 2001).

Growth rates presented in Fig. 3 were derived from time course experiments, i.e. they do not represent typical numerical response experiments where the predator was acclimated to prey for several generations (Weisse et al. 2002). Accordingly, nutritional history may have influenced the growth rate: if the ciliate had plenty of food the day before it might have grown faster than if exposed to the same mean prey concentration after acclimation. This bias may explain the high scatter around the threshold level (Fig. 3). Our attempts to acclimate *B. metopoides* to sub-saturating food levels below  $\mu_{\max}$  failed because we could not keep the flagellate prey levels sufficiently constant. Accordingly, the lower growth rates obtained in the pH experiments at saturating food levels (Fig. 7) may more realistically represent sustainable  $\mu_{\max}$  comparable to those obtained in numerical response experiments. On the other hand, the latter underestimate the ability of *B. metopoides* to rapidly increase its population size; the  $>5$  cell divisions per day that we observed repeatedly are ecologically important. Shortest generation times recorded for the small terrestrial colpodeans *Colpoda aspera* and *Grossglockneria acuta* were 4.1 h, respectively 3.8 h (Lüftenegger et al. 1985), i.e. close to the shortest generation time presented in this study for *B. metopoides* (3.5 h).

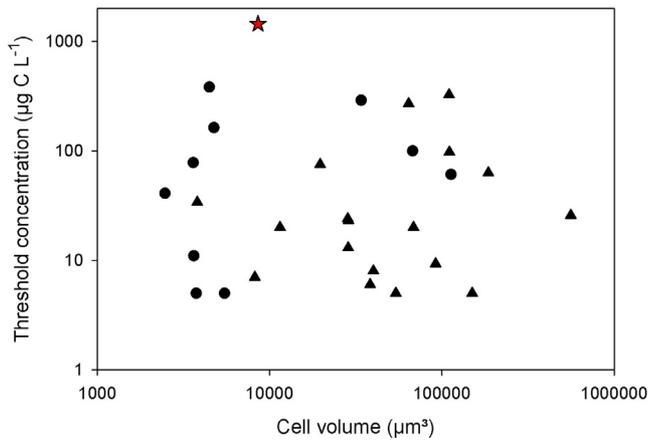
Concerning the induction of macrostomes, our experimental results do not provide an unequivocal answer. The experimental series reported in Fig. 4 revealed that, in accordance with our initial hypothesis, the proportion of macrostomes is higher if larger food items (i.e., *Polytomella* sp., in particular the large morph) prevail. The results shown in Fig. 4G–I and our unpublished observations made during the routine culturing of *B. metopoides* with *Polytomella* sp. suggest that it may also be the flagellate that responds to the ciliate grazing pressure by forming large cells, which are inedible to the majority of the *B. metopoides* population. Accordingly, the presence of *B. metopoides* macrostomes and of the large *Polytomella* morph may both be indicative of heavy grazing pressure on the small *Polytomella* morph.

We observed repeatedly that the population size of *B. metopoides* may not only increase rapidly, but can also drastically decline within 24 h. For instance, in the experiment reported in Fig. 2A, the ciliate population was reduced twice by a factor of 5 within 24 h, equivalent to negative population growth rates of  $2.1 \text{ d}^{-1}$ . This was not compensated by a switch to mass encystment. The desiccation experiments revealed that up to  $\sim 40\%$  of the total ciliate population may encyst within 24 h at circumneutral pH (Fig. 6B, C). Considering that we observed rapid declining of *B. metopoides* under food-replete conditions, reduction in its population size may be even stronger if food becomes limiting and/or other environmental stressors such as a shift to low pH deteriorate its habitat. We conclude that *B. metopoides* leads a boom and bust existence in its natural habitat.

### *Bromeliothrix metopoides* has high food requirements typical of edaphic ciliates

Irrespective of the food source and its feeding mode (carnivorous macrostomes vs. bacterivorous microstomes), our experimental results showed that *B. metopoides* has high threshold food concentrations for positive population growth. The results shown in Fig. 2 and similar results obtained in a preliminary experiment (data not shown) demonstrate that in the presence of moderate bacterial levels ( $<10^8 \text{ cells mL}^{-1}$ ), the ciliate population needs  $>10^4$  *Polytomella* sp. cells to increase in numbers. Based upon the measured size of the flagellate, we calculated an average cellular carbon content of 54 pg C for the small *Polytomella* (see section “Material and Methods”). Accordingly, the threshold food concentration is  $\sim 1.4 \text{ mg CL}^{-1}$  if the flagellates were the sole food. The question is, if large trophonts and macrostomes, which are the only stages in the life cycle of *B. metopoides* that feed upon *Polytomella* sp. (Foissner 2010; this study), can cause the decline in prey abundance observed in our experiments. If we assume that each macrostome contained, on average, eight flagellate cells in its food vacuoles and that the mean food vacuole life time is 2 h (Kenter et al. 1996; Verni and Gualtieri 1997; Verni and Rosati 1992), 500 macrostome *B. metopoides*  $\text{mL}^{-1}$  would ingest 48,000 *Polytomella* cells  $\text{mL}^{-1} \text{ d}^{-1}$ . Apparently, this calculation is in agreement with our experimental results (Fig. 4G, H).

When bacteria were the only food, the threshold was close to  $10^8 \text{ cells mL}^{-1}$  (Fig. 2C). The bacterial threshold biomass of  $\sim 2.3 \text{ mg CL}^{-1}$  for *B. metopoides* microstomes feeding on bacteria is even higher than the threshold estimated above for macrostomes. Although the calculation of carbon levels from measured cellular abundances may provide only a crude approximation of the actual carbon concentration, these estimates are distinctly higher than any other food thresholds calculated for various aquatic ciliates under comparable experimental conditions (reviewed by Weisse 2006). Most threshold levels reported in the literature fall into the range  $<0.01\text{--}0.1 \text{ mg CL}^{-1}$ , and saturation is typically reached in



**Fig. 8.** Threshold food concentrations (i.e., where population growth rate is zero) of various freshwater (circles) and marine (triangles) ciliates (modified from Weisse 2006) and *Bromeliothrix metopoides* (star) used in this study.

the range 0.4–0.8 mg C L<sup>-1</sup>. None of the 28 case studies with marine and freshwater ciliates summarized by Weisse (2006) reported threshold food levels >1 mg C L<sup>-1</sup> (Fig. 8). Notably, the threshold level of *Glaucoides bromelicola*, which is sympatric with *B. metopoides* (Dunthorn et al. 2012), falls into the ‘normal’ range reported for small and medium sized ciliates (Weisse et al. submitted).

We cannot rule out that there may be other bacteria than those used in our experiments that are more suitable food; however, the bacterial flora was enriched from the original habitat. Similarly, other flagellates than *Polytomella* sp. may sustain populations of *B. metopoides* in the tank bromeliads. We have demonstrated that, although *Cryptomonas* sp. may stimulate excystment (Tab. 1), it is not a suitable food source enabling sustainable growth of *B. metopoides*. The same applies to the flagellates *Chilomonas* sp. (Foissner 2010) and *Goniomonas truncata* (W. Foissner, unpubl.).

It remains to be investigated if *B. metopoides* is dependent on specific chemical properties that acetate flagellates such as *Polytomella* sp. (de la Cruz and Gittleson 1981) may possess and phototrophic flagellates such as *Cryptomonas* sp. do not.

The Neotropical phytotelmata ciliate fauna is characterized by the lack of typical and common freshwater genera such as *Coleps*, *Colpidium*, *Frontonia*, *Glaucoma*, *Nassula*, *Paramecium*, *Stylonychia* and *Trithigmastoma* (Dunthorn et al. 2012). Similarly, typical soil ciliates are rare in bromeliad tanks (Foissner et al. 2003). These authors concluded that the ciliate fauna in bromeliads evolved from cosmopolitan pond and moss biota. Several euryoecious species of the genus *Colpoda* were regularly found in bromeliad phytotelmata (Foissner et al. 2003). Colpodeans typically live in astatic humid environments associated with mosses, forest litters, soil and meadow puddles, and tree holes (reviewed by Lynn 2008), i.e. they are considered ‘terrestrial’ or ‘edaphic’ ciliates that may thrive in small water bodies. The smaller colpodeans are primary

bacterivorous, fast growing r-strategists with doubling times <6 h (Chessa et al. 1983; Drake and Tsuchiya 1977; Lüftenegger et al. 1985). *Colpoda steinii* fed with *Escherichia coli* achieved maximum growth rates when bacterial abundance reached 1 × 10<sup>8</sup> cells mL<sup>-1</sup> (Drake and Tsuchiya 1977); Kracht (1982, University of Hamburg, Germany, unpublished dissertation) measured maximum division rates of *C. cucullus* fed with *E. coli* at bacterial levels ranging from 4 × 10<sup>8</sup> cells mL<sup>-1</sup> to 1.3 × 10<sup>9</sup> cells mL<sup>-1</sup>. Investigating patterns of colonization experimentally as a function of distance from a pond, Maguire (1963) observed that the genus *Colpoda* was quickly eliminated and prevented from reinvading the environment by *Paramecium*. Summarizing the scarce literature in his dissertation, Kracht concluded that the competitive ability of *C. cucullus* is low, relative to other common ciliates. Obviously, the growth and feeding characteristics that we described in this investigation for *B. metopoides* fits into this general scenario known from colpodean ciliates.

### Survival in its natural habitat and competitive ability in other environments

The interpretation of the results presented in this study is hampered by the fact that the specific environmental conditions encountered in the tank bromeliads remain largely unknown. For instance, in the state of Rio de Janeiro, Brazil, Marino et al. (2011) reported typical seasonal temperature variation in the tanks of bromeliads of 10 °C; however, maximum temperature recorded was as high as 54.9 °C. These authors also concluded that each bromeliad species represents a set of physical and chemical characteristics. Considering bromeliad diversity in the vast area in Central and South America where *Bromeliothrix metopoides* occurred (Dunthorn et al. 2012; W. Foissner, unpubl. observations), a detailed survey of the environment was impossible during this investigation.

Although we have investigated the response of *B. metopoides* to only a limited number of the major environmental variables, severe constraints for the survival of this species became obvious. First, the earlier observation that this a ‘weed species’ that grows in all media containing bacteria and medium-sized flagellates (Foissner 2010), must be interpreted with some caution. Bacterial abundance ≥10<sup>8</sup> cells mL<sup>-1</sup> is common in media rich in nutrients and organics, but is rarely found in natural freshwater habitats. The scarce data currently available point to mean bacterial levels ranging from 1 to 10 × 10<sup>6</sup> cells mL<sup>-1</sup> in bromeliad phytotelmata (Brouard et al. 2011, 2012). However, Janetzky (1997, University of Oldenburg, Germany, unpublished dissertation) reported a bacterial peak abundance of 1.3 × 10<sup>8</sup> cells mL<sup>-1</sup> from bromeliads in Jamaica. A burst in bacterial numbers may, for instance, follow the input of organic-rich material (due to decomposition of leaf litter, carcasses of insects and other small metazoans). Although

the water in the reservoirs is primarily acidic and the nutrient supply indicative of impoverished rather than nutrient-replete conditions (Laessle 1961; Lopez et al. 2009; Marino et al. 2011), chlorophyll *a* maxima close to  $40 \mu\text{g L}^{-1}$  have been reported (Marino et al. 2011). Accordingly, occasional inputs of large amounts of organic matter and nutrients leading to short-term eutrophic conditions appear likely (Lopez et al. 2009).

The rapid formation of cysts enables *B. metopoides*, similar to many other ciliates (Lynn 2008), to survive unfavourable environmental conditions. We tested its response to low pH and fast desiccation. Our results showed that 10–50% of the population may potentially survive as cysts if the reservoir dries up and pH is close to neutral conditions. Its pH response (Fig. 7A) characterizes *B. metopoides* as a neutrophil or acidotolerant species, which reaches high growth rates over the pH range commonly encountered (mostly moderately acidic; Laessle 1961) in the phytotelmata of bromeliads. Our results measured at circumneutral and basic (pH 8–9, Fig. 7A) conditions demonstrated that pH alone does not restrict the occurrence of *B. metopoides* to acidic conditions. Mass encystment, when >70% of the population encysted within 24 h, was triggered by unpalatable food (*Cryptomonas* sp., Fig. 5A–C) and by a combination of suboptimal food (bacteria) and low pH (Fig. 7B). This supports our initial hypothesis that, although food may be the most important trigger of (mass) encystment, other environmental clues contribute to the formation of cysts.

We observed regularly spontaneous excystment in our cultures and could stimulate excystment by adding growth medium to freshly produced cysts (Table 1). However, we can only speculate about how many of the cysts may be able to excyst *in situ* and build a new population once the original habitat returns to more favourable conditions or if the cysts are dispersed into a new, suitable environment. To mimic the latter situation, we offered culture medium with and without non-axenic *Cryptomonas* sp. as food to *B. metopoides* cysts that were formed when the culture dried up. Our observations on excystment of fresh (3–4 d old) cysts formed upon desiccation revealed that (i) the majority of cysts remained viable and (ii) *B. metopoides* may excyst even if in the presence of little food. Food supply is important to enable the excysted cells to proliferate. Recent experimental work with another colpodid ciliate, *Colpoda inflata*, yielded that 2–68% of the cysts may excyst after a period of six weeks of desiccation at a residual soil moisture of 9% (Müller et al. 2010). However, we did not try to stimulate excystment more systematically, because (i) too little is known about the rapidly changing environmental conditions in the tank bromeliads and (ii) laboratory investigations on the encystment/excystment cycle are generally of limited value to predict the situation *in situ* (Foissner 2006; Müller 2007; Müller et al. 2010).

Cannibalism, which may also be a successful strategy to survive shorter periods of food depletion, was never observed in *B. metopoides* (Foissner 2010; this study). Our results demonstrate that viability of motile cells is sensitive to

changing environmental conditions. We conclude that cyst formation is the only option for this ciliate to survive in a given habitat with fluctuating food supply and to disperse to new habitats.

## Conclusions

We conclude that the competitive ability of *B. metopoides* is heavily compromised in most natural environments because it needs threshold food concentrations that are rarely met in most habitats. Secondly, specific demands in terms of food quality may further restrict the occurrence of this species to its peculiar habitat. Thirdly, competition experiments performed in parallel to the present study (Weisse et al. submitted) suggest that *B. metopoides* is sensitive to competition at or above its food threshold. Even though vulnerability of this species to predation and parasitism needs to be explored, the results presented in this study support our third hypothesis: the ecological niche of this ciliate is unusually narrow. The ability of *B. metopoides* to form cysts enabled its survival only under some adverse conditions (poor food and low pH), while other unfavourable combinations (desiccation and poor food) did not trigger enhanced cyst formation. This species has reached a narrow peak along its fitness landscape (Gavrilets 2004; Wright 1932); any deviation from the optimum conditions will lead to lower fitness. Corollaries of our findings are that (i) *B. metopoides* is not viable in the vast majority of aquatic habitats and that (ii) the exceptionally high food concentrations needed by *B. metopoides* would be regularly met in the reservoir of tree bromeliads. More research is needed to test the latter conclusion.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejop.2013.02.001>.

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