RESEARCH ARTICLE
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INTERNATIONAL MICROBIOLOGY
OPEN ACCESS
INTERNATIONAL MICROBIOLOGY (2014) 17:31-40
doi:10.2436/20.1501.01.205 ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

Local ciliate communities associated with aquatic macrophytes

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Received 9 January 2014 · Accepted 30 March 2014

Summary. This study, based within the catchment area of the River Frome, an important chalk stream in the south of England, compared ciliated protozoan communities associated with three species of aquatic macrophyte common to lotic habitats: *Ranunculus penicillatus* subsp. *pseudofluitans*, *Nasturtium officinale* and *Sparganium emersum.* A total of 77 ciliate species were counted. No species-specific ciliate assemblage was found to be typical of any one plant species. Ciliate abundance between plant species was determined to be significantly different. The ciliate communities from each plant species were unique in that the number of species increased with ciliate abundance. The community associated with *R. penicillatus* subsp. *pseudofluitans* showed the highest consistency and species richness whereas *S. emersum* ciliate communities were unstable. Most notably, *N. officinale* was associated with low ciliate abundances and an apparent reduction in biofilm formation, discussed herein in relation to the plant's production of the microbial toxin phenethyl isothiocyanate. We propose that the results reflect differences in the quantity and quality of biofilm present on the plants, which could be determined by the different plant morphologies, patterns of plant decay and herbivore defense systems, all of which suppress or promote the various conditions for biofilm growth. [**Int Microbiol** 2014; 17(1):31-40]

Keywords: *Ranunculus* · *Nasturtium* · toxin phenethyl isothiocyanate (PEITC) · biofilms · macrophytes · ciliates · microbial biodiversity

Introduction

The vast majority of eukaryotes live as single cells [37]; of these, ciliated protozoa are the most complex. They are ubiquitous in aquatic environments, where macrophytes that oxygenate the water and act as a substrate and refuge provide a key habitat for them and for other microorganisms and invertebrates [2,17,30,35]. Ciliates are major consumers of bacteria, algae and other protozoa in aquatic food webs [16]. The protozoa collectively form a critical intermediate link between microbial and metazoan trophic levels [38]. They are also a fundamental component of the microbial loop [18,21], which describes the introduction of dissolved and particulate organic carbon into the food web through phytoplankton production and its consumption by microbes such that it becomes accessible to higher trophic levels [18,40]. There is evidence that without microbial reclamation of organic matter the levels of biomass in the food web could not be sustained by autotrophs alone [41].

Protozoa are now widely believed to play a major role in ecosystem processes [19], for example in soil ecology [9], freshwater ecology [16,20], biogeochemical cycling [29]

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such as carbon-fixation, and in the control of bacterial populations [19]. Despite their important ecological roles and their value as indicators of environmental health in ecosystem assessment [24], ciliate ecology remains underinvestigated. Studies of protozoan communities are scarce [23] and only a few of them have compared local biotopes [4,7,40], especially those in flowing waters [38]. For example, it is not known whether different aquatic macrophyte species in a stream support distinguishable protozoan communities.

The aim of this study was to address this deficiency by comparing assemblages of ciliated protozoa associated with three different species of aquatic macrophytes in a Dorset (UK) chalk stream, and then characterising each ciliate community according to the ciliate species present, ciliate abundance and species richness.

Materials and methods

Sampling site. Samples were taken from the East Stoke Millstream, a 1.2-km [10] diversion of the River Frome, Dorset, UK, 50°40′42″ N, 2°10′48″ W (Fig. 1). The River Frome is a designated UK Site of Special Scientific Interest (SSSI) and has been implemented as a priority habitat in the government's UK Biodiversity Action Plan [6].

The stream study site was 2.5–4 m wide and 0.15–0.8 m deep. The substrate was a mixture of gravel, sand and silt on which a variety of submerged aquatic macrophytes grew in small patches, dominated by *Ranunculus* sp. Sampling took place within a 200-m reach of the stream (Fig. 1C) in a section typified by a "pool and riffle sequence" [10], i.e. alternations of deep and shallow zones along the straight course of a river (Fig. 1D). Sample collections were made where the stream had a depth between 0.45 and 0.20 m and water flow was between 0.20 and 0.40 m/s.

Sampling methods. Samples were collected twice a week in August–September 2009 from the East Stoke Millstream. Three species of aquatic macrophytes were chosen for investigation; *Ranunculus penicillatus* (Dumort.) Bab. subsp. *pseudofluitans* (Syme) S.D.Webster (water crowsfoot), *Nasturtium officinale.* R.Br. (watercress) and *Sparganium emersum* Rehmann (unbranched bur reed) [29]. From these three species, six, five and five plants were sampled, respectively. The samples were picked by hand from plants as far from the bank as possible, to minimise effects of bank proximity. Handling and movement through the water were kept to a minimum. Plant samples of similar surface area, ca. 43 cm^2 (see below), were taken from a branch fully submerged from at least mid-way in the water column or deeper and stored with ca. 45 ml of stream water in separate, sterile 50-ml Falcon tubes.

Laboratory methods. The sampling tubes were shaken for 30 s to dislodge the ciliated protozoa from the plants and left to settle for 30 min before subsampling. Live ciliate subsamples (1 ml) were taken from the bottom of the tube using a sterile pipette and transferred to a Sedgewick Rafter counting chamber. The subsample was viewed under a light microscope until the entire 1-ml chamber had been inspected. The observed ciliates were measured and identified to species or genus level. For identification, several taxonomic guides were used in conjunction: [11,12,13,24 and references therein, 31]. Ciliates were measured either using an eyepiece graticule calibrated to the microscope or by photographing and measuring them using a MicroPublisher 3.3 RTV High Resolution IEEE 1394 FireWire Digital CCD Colour Camera with real-time viewing. Three subsamples were analysed from each sample within three days of collection. The samples were refrigerated at 4 ºC between subsampling.

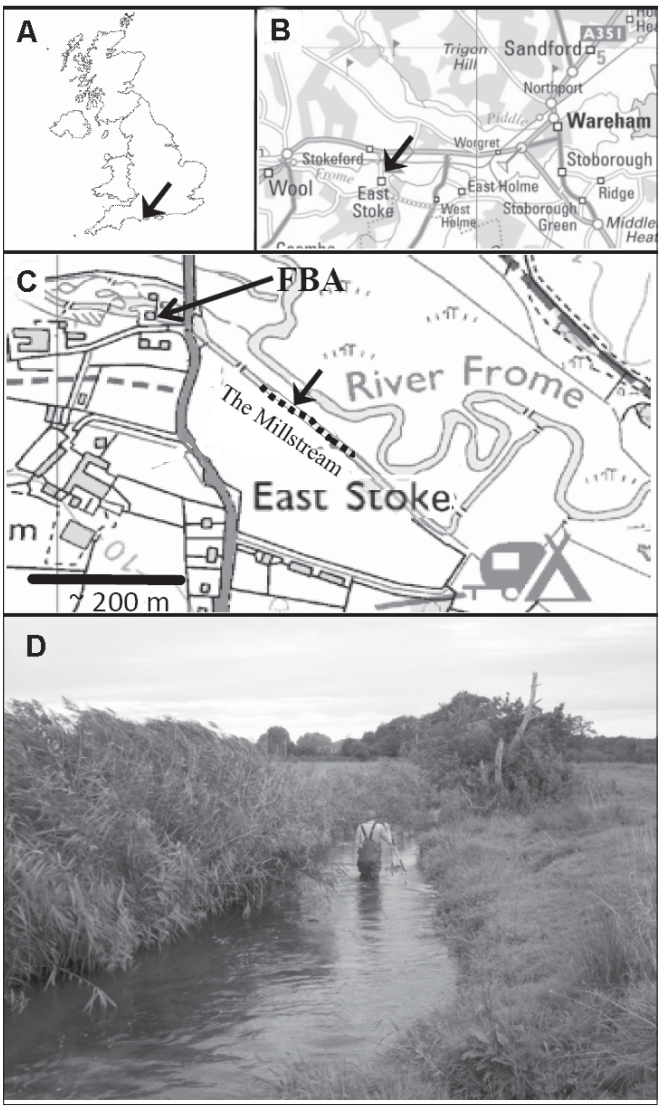

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Fig. 1. The study site. (**A**) Map of the UK locating the study site in Dorset, S. England (taken from http://www.worldatlas.com). (**B**) The sampling site is marked by an arrow (map adapted from http://streetmap.co.uk/map). (**C**) The Millstream in East Stoke, located with an arrow. The sampling area in the Millstream is marked with a dotted line. The Freshwater Biological Association (FBA) is also located on the map (adapted from [http://streetmap.co.uk/map]). (**D**) Photograph of the Millstream at the time of sampling.

Plant surface area calculation. For each plant species, three to six drawings representing the collected plant samples were made on graph paper from photographs of the original plant samples and from the plant morphology depicted in various taxonomic guides and herbarium catalogues (see supplementary material in the Bournemouth University repository). Total

surface area (cm^2), including both leaf sides and stem surface, was calculated from the squared graph paper. The average results of several drawings were 43 cm^2, 43 cm^2 and 46 cm^2, for *S. emersum*, *N. officinale* and *R. penicillatus* subsp. *pseudofluitans,* respectively. Thus, the sample surface areas of the plant species were very similar (see supplementary material in the BU repository for surface area calculations). The original count format, that is the number of ciliates/ml, was retained for analysis of the data. Thus, each 1-ml subsample was taken from a community of ciliates that derived from a plant sample with an average total surface area of about 43 cm^2.

Statistical analyses. To test whether species-specific ciliate assemblages were associated with the different macrophyte species, multivariate analyses in the form of a principle components analysis (PCA) and a cluster analysis were performed using the MultiVariate Statistical Package (MVSP). These are similar statistical tests that present the data in different ways to enable improved interpretation.

Differences in ciliate abundances between plant species were tested with a one-way ANOVA. Ciliate species evenness was compared between plant species using ciliate species rank abundance graphs. The relationship between species richness and abundance was investigated using cumulative species-abundance curves (CSAC), which were constructed for each plant species by comparing the number of additional ciliate species found in each subsequent 1-ml subsample with the abundance of ciliates found in each subsample. These graphs also indicate the amount of sampling effort required to reveal species diversity in a given community.

To examine the relationship between ciliate abundances and cell sizes, the observed ciliates were classified into cell size categories (≤50, 50–99, 100–149, 150–199, and ≥ 200 μm). These were used to plot ciliate species richness and abundance as a function of cell size for each plant species [22].

Results

The collection as a whole. The total sampled plant surface area was estimated to be 688 cm^2. We examined 48 1-ml subsamples and found 508 ciliates (on average, 10.6 ciliates per ml) from 77 species, 18 of which were not identified beyond being recognised as separate species and were thus classifieed as unidentified. A list of the ciliate species grouped by class [32], an indication of which plant species they were associated with and the total number of individuals of each species is presented in Table 1. In terms of ciliate species composition, about half (52 %) of the total number of ciliates collected accounted for only seven species while a large proportion of ciliates (46 %) was observed only once (Table 1).

Ciliate abundance and species richness associated with host plant. Within five samples of *R. penicillatus* subsp. *pseudofluitans*, 134 ciliates, comprising 42 species, were counted. Within the same number of samples of *S. emersum*, the number of species (46) was similar but the number of ciliates was more than two-fold higher (295). The samples of *N. officinale*, however, contained only 62 ciliates (62) and 20 species (20). The mean average number of ciliates per sample differed significantly between the plant species (data transformed using log (x+1); ANOVA, $F_{(2,42)} = 11.25$, $P < 0.001$; Fig. 2). In addition, there were differences in the number of ciliates and the number of ciliate species between samples from the same host plant, reflected in the large standard deviations (Fig. 2). This was most obvious for *S. emersum* and *N. officinale.* By contrast, the standard deviations obtained for *R. penicillatus* subsp. *pseudofluitans* were comparatively smaller, denoting relative evenness in both ciliate abundance and species number between samples (Fig. 2).

Species evenness within the ciliate communities associated with the three host-plant species was revealed by rank abundance graphs (Fig. 3). The greatest evenness was that of ciliate communities from *R. penicillatus* subsp. *pseudofluitans*, in which half of the abundance was represented by 17 % of the ciliate species (8 species), while, in *S. emersum* and *N. officinale,* 50 % of the ciliates accounted for only 13 % (5.5 species) and 14 % (4 species) of the ciliate species, respectively. The four most abundant species within each ciliate community are listed in Fig. 3. Among the five species in the lists, *Aspidisca* sp. appeared within all top ranks, largely dominating *S. emersum* and *N. officinale* at 22.4 % and 20 % respectively and ranking third (8.2 %) in *R. penicillatus* subsp. *pseudofluitans* samples. *Trochilia minuta* also appeared within the five top ranks of all plant species. *Acineria uncinata, Holosticha* sp. and *Chilodonella* sp. filled the remaining ranks.

As shown in the CSACs (Fig. 4), the rate of discovery of further ciliate species as a function of cumulative abundance differed for each plant species. *Sparganium emersum* CSAC had a long shallow curve with occasional large gaps between data points, while *R. penicillatus* subsp. *pseudofluitans* CSAC had a deeper curve that reached the same number of ciliate species much more rapidly, indicating that these samples were more species-rich and more consistent in their numbers of species. Indeed, on average, there was one new (i.e. not previously observed in the countings) species for every 6.3 ciliates in the *S. emersum* samples, while, in *R. penicillatus* subsp. *pseudofluitans* and *N. officinale,* additional species occurred at a rate of one in 3.2 and 3.1 ciliates respectively, about double that of *S. emersum*. Although the average ratio of ciliate species/ciliates observed was similar for *R. penicillatus* subsp. *pseudofluitans* and *N. officinale,* the rate of discovery per ml of sample was much lower for *N. officinale*, as represented by the shortness of the *N. officinale* graph along both axes compared to the curve for *R. penicillatus* subsp. *pseudofluitans*, illustrating again that the *N. officinale* ciliate community was comparatively depauperate in ciliate numbers.

The hyperbolic shape of the CSACs of *S. emersum* and *R.*

Table 1. Ciliate species comprising the α-diversity of the East Stoke Millstream, grouped by class [32]. Class abbreviations: Armophorea (Arm); Colpodea (Col); Heterotrichea (Het); Karyorelictea (Kar); Litostomatea (Lit); Nassophorea (Nas); Oligohymenophorea (Oli); Phyllopharyngea (Phy); Plagioplylea (Pla); Spirotrichea (Spi). The ciliate species found on three plant species, *Sparganium emersum* (S) *Ranunculus penicillatus pseudofluitans* (R) and *Nasturtium officinale* (N), were examined. The 'unidentified' ciliate species are presented with respect to the macrophyte. In every case there was one individual only, indicated, for example, as '7*1' (7 species, each with 1 individual ciliate).

	Class	Ciliate species	S	R	N	Total no.		Class	Ciliate species	S	R	N	Total no.
1	Spi	*Aspidisca* sp. (unridged)	●	●	●	86	32	Lit	*Acineria uncinata*	●	●	●	33
2	Spi	*Aspidisca costata*	●			6	33	Phy	*Thigmogaster oppositevacuolatus*		●	●	1
3	Spi	*Euplotes* sp1.	●			1	34	Phy	*Trithigmostoma cucullulus*	●	●		10
4	Spi	*Euplotes* sp2.	●	●		2	35	Phy	*Chilodonella* sp1.	●	●		8
5	Spi	*Euplotes* sp3. (unridged)	●		●	4	36	Phy	*Chilodonella* sp2.	●	●	●	11
6	Spi	*Euplotes* sp4.	●			1	37	Phy	*Chilodonella* sp3.	●	●		33
7	Spi	*Oxytricha fallax*		●		2	38	Phy	*Chilodonella uncinata*	●	●	●	14
8	Spi	*Oxytricha* sp1.	●	●		8	39	Phy	*Gastronauta clatratus*			●	1
9	Spi	*Oxytricha* sp2.		●		1	40	Phy	*Trochilioides recta*		●		1
10	Spi	*Oxytricha* sp3.	●	●	●	10	41	Phy	*Trochilia* sp.	●	●	●	14
11	Spi	*Holosticha* sp1.	●	●	●	32	42	Phy	*Trochilia minuta*	●	●	●	38
12	Spi	*Holosticha* sp2.	●	●	●	10	43	Nas	*Microthorax* sp.	●	●		15
13	Spi	*Holosticha* sp3.	●	●	●	14	44	Nas	*Pseudomicrothorax* sp.	●			1
14	Spi	*Tachysoma* sp.		●		5	45	Oli	*Vorticella* sp.	●			2
15	Arm	*Metopus* sp.		●		1	46	Oli	*Campanella umbellaria*		●		1
16	Arm	*Metopus* sp.	●			3	47	Oli	*Epistylis digitalis*			●	1
17	Arm	*Metopus laminarius minor*		●		1	48	Oli	*Epistylis* sp.			●	1
18	Col	*Bryometopus pseudochilodon*		●		1	49	Oli	*Zoothamnium* sp.		●	●	2
19	Het	*Stentor* sp.		●		2	50	Oli	Unidentified peritrich			●	1
20	Pla	*Trimyema compressum*		●		7	51	Oli	*Lagenophrys vaginicola*	●			1
21	Kar	*Loxodes striatus.*	●			5	52	Oli	*Lembadion lucens*	●	●		6
22	Kar	*Loxodes* sp.	●	●		8	53	Oli	*Paramecium* sp.		●		2
23	Kar	*Loxodes magnus*		●		1	54	Oli	*Frontonia accuminata*	●	●	●	23
24	Lit	*Dileptus tenuis*	●			3	55	Oli	*Tetrahymena pyriformis*	●	●	●	2
25	Lit	*Dileptus* sp.	●			3	56	Oli	*Cinetochilum margaritaceum*	●	●	●	8
26	Lit	*Dileptus margaritifer*	●			2	57	Oli	Unidentified hym.			●	1
27	Lit	*Litonotus* sp.	●			4	58	Nas	*Nassula* sp.	●			1
28	Lit	*Litonotus anguilla*	●			1	59	Nas	*Leptopharynx costatus*	●			1
29	Lit	*Litonotus fasciola*	●	●	●	6	60-66	-	Unidentified 1-7		●		7*1
30	Lit	*Amphileptus procerus*		●		1	67-71	-	Unidentified 8 -12			●	4*1
31	Lit	*Loxophyllum sp.*	●	●		14	72-77	-	Unidentified 13-18	●			5*1

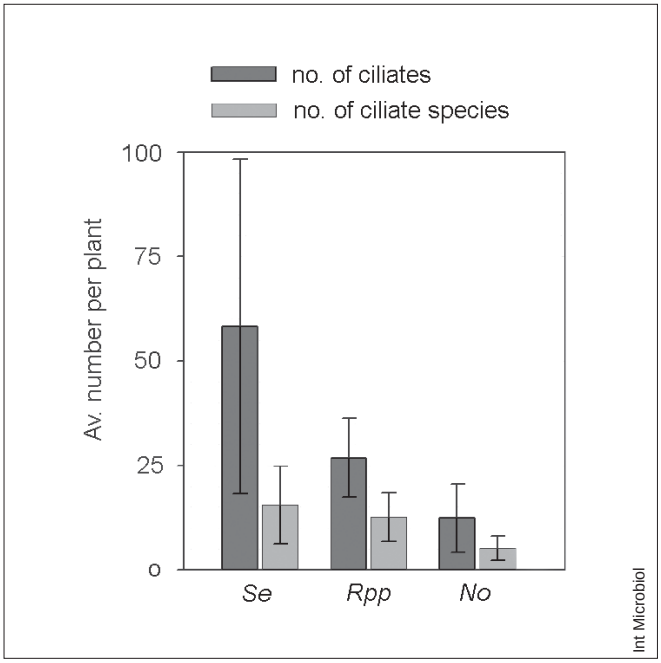

Fig. 2. Average number of ciliates and number of ciliate species per sample. Bars show the standard deviation. Se, *Sparganium emersum*; Rpp, *Ranunculus penicillatus* subsp. *pseudofluitans*; No, *Nasturtium officinale*.

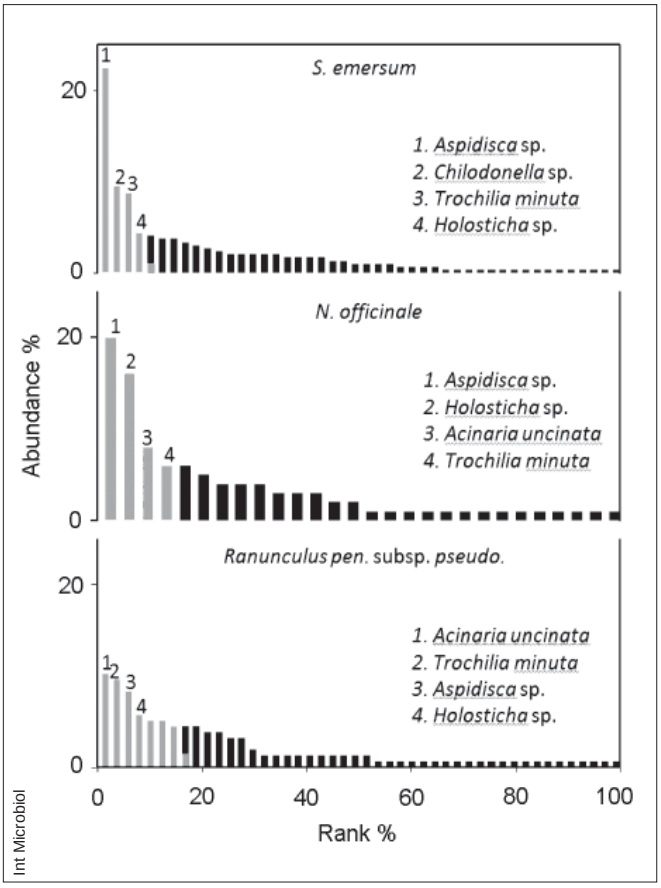

Fig. 3. Rank abundance graphs for ciliate species associated with each host-plant. For each plant, each bar represents a ciliate species, ordered from the largest (position 1) to the smallest abundance. The grey areas of the bars depict the first 50 % of the total number of ciliates. The four most abundant ciliate species are listed for each plant.

penicillatus subsp. *pseudofluitans* illustrates the decrease in species yield per ciliate, indicated as the cumulative number of observed ciliates (Fig. 4). The *S. emersum* CSAC plateaus at the 11th ml (46 species, 266 ciliates) indicated the inefficiency of further sampling; indeed, from 7–14 ml the rate of species discovery dropped to one in 9.1 ciliates species from one in every 4.9 in the first 7 ml. The rate of species discovery per number of ciliates also decreased for *R. penicillatus* subsp. *pseudofluitans*; in the first 7 ml another ciliate species was found on average in every 2.3 ciliates, dropping to one in every 4.6 ciliates in the next seven subsamples. This was still relatively frequent and implied that further sampling may have revealed more species. The *N. officinale* CSAC showed no signs of flattening; instead, a fairly steady, linear and shallow trajectory revealed a slow but fairly constant rate of about three additional ciliate species per ml, again indicating that more species might have been observed with further sampling.

Specific ciliate assemblages. No specific ciliate assemblages were found to be distinctive of any plant species. In Fig. 5A the cluster analysis provides an index of similarity between individual plant samples. The similarity of the ciliate species composition between samples was low in all cases (<59 %). Between samples of the same plant species most samples fell below 30 % similarity. Maximum similarity values reached only 53 % in *R. penicillatus* subsp. *pseudofluitans*, 40 % in *S. emersum* and 28 % in *N. officinale* samples. In fact the largest similarities were between samples of different plant species (Fig. 5, entries in bold) but these were also low. A PCA supported these findings in that the ordination showed no clustering of within-plant species samples and no discernible overall pattern was apparent between the plots, demonstrating that ciliate species did not characterise the communities associated with the host plant species (data not shown).

Ciliate cell-size frequencies. Trends in ciliate abundance/cell size frequencies were similar between plant species (Fig. 6A). In general, the relationship was inversely related so that the smallest ciliates (<50 μm) were most abundant, comprising nearly 50 % of the ciliate associations for all three host plant species, whereas the largest ciliates (>200 μm)

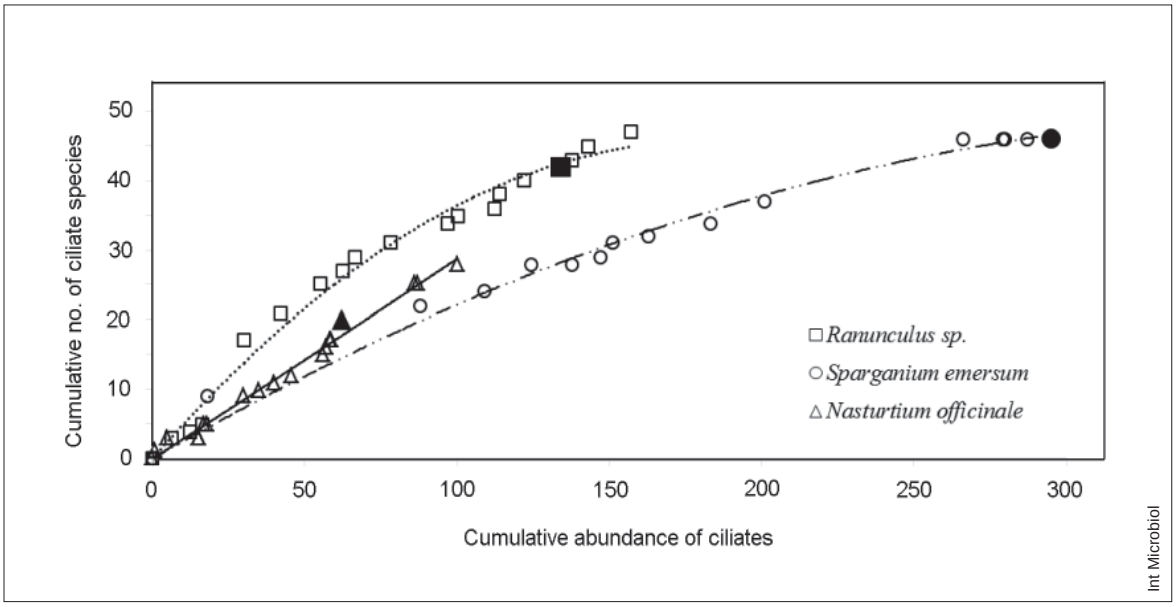

Fig. 4. Cumulative abundance–species curves. The 15th 1-ml subsample plot has been enlarged and filled black as an indicator of sample number vs. no. of ciliate species and ciliate abundance.

were least abundant in all cases, totaling 4.3 % of the abundance in *S. emersum* samples and a somewhat higher abundance in *R. penicillatus* subsp. *pseudofluitans* samples (8.7 %), while in the samples of *N. officinale* they were entirely absent. There was a single exception to the 'inverse trend' in which ciliates of 150–199 μm had a slightly higher abundance (14.9 %) than those of smaller size (100–149 μm, 9.8 %) within the *S. emersum* samples. The number of ciliate species

	S1	S2	S3	S4	S5	R1	R2	R3	R4	R5	R6	N1	N2	N3	N4	N5	N6
S1	*																
S2	31	*															
S3	33	40	*														
S4	36	22	32	*													
S5	29	14	33	32	*												
R1	7	0	0	0	0	*											
R2	47	43	37	29	19	3	*										
R3	**58**	39	46	35	38	0	**53**	*									
R4	28	9	26	32	**48**	15	21	32	*								
R5	17	7	32	24	26	0	16	26	24	*							
R6	31	10	34	30	**53**	4	21	35	35	31	*						
N1	9	5	3	1	19	0	3	4	3	5	30	*					
N2	16	5	0	0	0	0	18	9	0	0	0	0	*				
N3	36	39	28	16	20	0	**51**	39	6	5	18	14	27	*			
N4	21	23	29	30	43	6	32	31	35	9	22	0	0	24	*		
N5	0	3	11	13	19	0	10	9	20	23	26	0	0	0	20	*	
N6	28	37	42	34	19	0	36	30	13	12	27	8	0	25	28	11	*

S. emersum
N. officinale
R. penicillatus subsp. *pseudo.*
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Fig. 5. An index of similarity produced from a cluster analysis of the plant samples. Each column and row is headed with a letter and number signifying a particular plant sample. S1–S5 represent the five samples taken from *Sparganium emersum*, R1–R6, the six samples from *Ranunculus penicillatus* subsp. *pseudofluitans* and N1–N6 the six samples from *N. officinale*. Entries in the table show the percentage similarity between two samples; 100 % = identical; 0 % = no common species. The entries against a grey background show similarity levels between the samples of a given plant species. The five highest values are indicated in bold. Asterisks signify entries of 100 %.

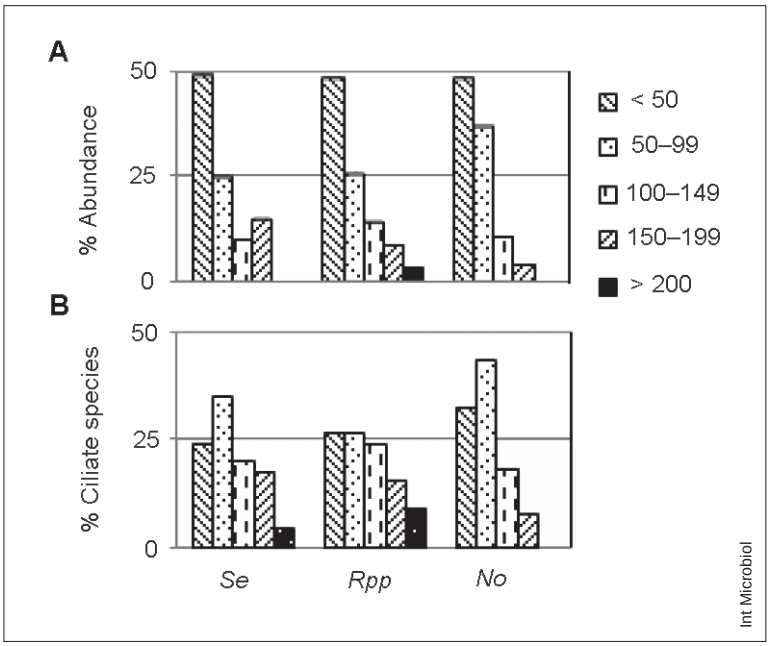

Fig. 6. Ciliate cell-size frequencies. Bars show the percentage ciliate abundance for each size category (**A**) and the percentage of ciliate species for each size category (**B**). Se, *Sparganium emersum*; Rpp, *Ranunculus penicillatus* subsp. *pseudofluitans*; No, *Nasturtium officinale*. Size units are μm.

in each size category also decreased somewhat as cell size increased; however, the trend was much weaker and category 50–99 μm was an exception in each case, containing the highest numbers of ciliate species in *S. emersum* and *N. officinale* and the same number of ciliate species as the <50 μm category in *R. penicillatus* subsp. *pseudofluitans* (Fig. 6B).

Discussion

The East Stoke Millstream ciliate community. Altogether 77 ciliate species were found, an indication of the active α-diversity of the ciliated protozoa associated with the aquatic macrophytes in the East Stoke Millstream. However, this is probably an underestimate because subsampling was not exhaustive, as shown by the CSAC, and rare or more elusive species might have remained undetected. Furthermore, due to the inherent difficulty in identifying smaller ciliates to the species level, this category was prone to misidentification and underestimation.

Comparing our findings with those of previous studies is difficult because of the differences in sampling methods and quantification. Foissner et al. [25] have focused on different continents and their countings include non-freshwater species. Moreover, neither the sizes of the sample areas nor the sample volumes are reported. Finlay and Esteban [19] have stated that a freshwater lake was likely to contain 15 ciliates per ml, although this figure is an estimate of the number of ciliates in the water column rather than associated with a substrate; consequently, comparison of our findings with those reported in that study is also limited. According to Schmid-Araya [39] the total ciliate abundances in the sediments of an Austrian brook range from about 20 to 40 individuals per ml. The samples were taken in August, however, when ciliates are less abundant than at other times of the year. In addition, ciliate abundances in sediments may be quite different from those in plants. Gray [26], using a methodology similar to our own, has reported that the abundances of ciliates and ciliate species found on plant samples of *R. penicillatus* are 'generally low' (0–10 per ml). The data in that study, expressed per ml, reflect the ciliate abundance found in the present work when expressed in the same way, 10.6 ciliates/ml on average. Overall, the densities of ciliates detected in the current study were of the same order of magnitude as those previously reported [19,25,39]. Comparisons, however, should be viewed with caution given the disparity of the sampling methodologies, the habitats sampled, and the measurement techniques.

The aim of this study was to determine whether in a local area distinguishable communities of ciliates are associated with distinct mesohabitats [1]. Specifically, we asked whether three different species of aquatic macrophytes, *Sparganium emersum*, *R. penicillatus* subsp. *pseudofluitans* and *N. officinale*, living adjacently in the East Stoke Millstream, support differentiable communities of ciliated protozoa. Although the plant species did not host ciliate communities that were plant-species-distinguishable, we found differences in terms of cili-

ate abundances, species richness, community evenness and the relationship between species richness and abundance.

We assumed that the potential for ciliate recruitment by each plant species was the same; in fact, each plant received ciliates from the same sources, originating from the seed bank in the soil, which had distributed them evenly along the stream, from re-inoculation from other macrophyte ciliate communities and from the running water itself [22,26]. Since the plants were selected from as far from the bank as possible, biases in the data relating to the proximity of the plants to the banks should be minimal, if any. Differences in the abundances and species composition of the ciliate communities on different host plants could thus be attributed to the different environments provided by the host plant species.

Plant species–ciliate associations. The ciliate community associated with *S. emersum* was characterised by large variations between samples with respect to both the numbers of ciliates and the number of ciliate species, while species composition was skewed by just one or two species. Despite the large total number of ciliates, there were comparatively few species and very few large ciliates. The CSAC clearly indicated a maximum species richness of 46 species. We discuss these findings based on our view that they are symptomatic of an unstable habitat.

Ranunculus penicillatus subsp. *pseudofluitans* hosted the most consistent ciliate community, given the relative reproducibility between samples both in ciliate species and numbers. This plant harboured as many species as *S. emersum*, with higher species richness per number of ciliates and the highest species evenness. In addition, *R. penicillatus* subsp. *pseudofluitans* was colonised by the greatest number of large ciliates (> 200 μm) and had the most even spread of ciliates between size categories. These findings are suggestive of a more developed community [9], which in our opinion is an effect of the habitat permanence and complexity provided by the host plant, as discussed below.

Overall, *N. officinale* samples were depauperate in ciliate numbers, with correspondingly low numbers of species and an absence of large ciliates (> 200 μm). A determinant factor stronger than the stability or complexity of the environment offered by *N. officinale* might have influenced its associative ciliate community. One such potential factor is the plant's use of phenethyl isothiocyanate (PEITC), a secondary metabolite produced as a defence against herbivory [34,36] (see below).

The host-plant environment. It is a well-established tenet of biology that environmental complexity strongly determines community complexity and this in turn is characteristic of community stability [9,33]. To apply this theory in terms of macrophyte-associated microbial communities, we might consider the complexity of the environment of the host plant by way of its topology, such as leaf, branch and nodal patterns, together with patterns of plant growth and decay.

The structure of the studied macrophytes in the stream habitat showed that *R. penicillatus* subsp. *pseudofluitans* was the most multifaceted in structure, based on its iteratively branching stems creating many internodal spaces and its varying branch lengths that bear thin, thread-like capillary leaves [43]. *Nasturtium officinale* morphology was also relatively multipart although it consisted of broad, pinnately compound leaves, less branching and fewer leaf nodes. *S. emersum* had a simple structure of long, flat, trailing, strap-like leaves from small stands. The contrasting morphologies and life cycles of these macrophytes may have translated into differing protozoan abundances, as discussed below.

Work by Sleigh et al. [40] illustrates how environmental complexity and stability can directly produce community complexity. Those authors found that the internodal spaces of *R. penicillatus* subsp. *pseudofluitans* harbour a large protozoan diversity, in which the ciliates show little seasonal variation. The plant's nodes were suggested to offer refuge from the water current and, for attached and for swimming ciliates, to provide habitats not apparently found elsewhere on the plant. The numerous leaf nodes of the *R. penicillatus* subsp. *pseudofluitans* samples collected for this study presumably offered similar refuge from seasonal variation and water currents, allowing certain species to reside on the plant, which otherwise would not have been suitable, and therefore time for communities to develop in complexity, thus accounting for both the high species richness and the comparatively low variation in ciliate abundance.

Environmental constancy can also be ascribed to the perennial, semi-deciduousness of *R. penicillatus* subsp. *pseudofluitans*, which has capillary leaves all year round [43]. Furthermore, the small, multiple and iterated plant parts meant that disturbances of one area have small proportional effects on the ciliate community.

Sparganium emersum is also a perennial species but with hard die-back in winter and growth peaks in summer [28]; throughout the year its leaves show various levels of decay. During the collection for this study, we noticed that the leaves of *S. emersum* often had brown patches of various sizes, indicating biodegradation. The leaves of *S. emersum* are much larger than those of *R. penicillatus* subsp. *pseudofluitans*, implying a higher potential for the total quantity of decay across the leaf. Decay entails bacterial colonisation, which in turn

provides rich feeding grounds for protozoans and the establishment of an abundant protozoan community. However, its progression leads to eventual leaf drop-off or disappearance from the plant. Accordingly, while *S. emersum* provides rich feeding grounds that support ciliate communities of large abundance, they are temporary. Temporary environments are usually characterised by unevenness and relative species poorness in the communities associated with them [33]. In a microbial community, a temporary environment might restrict species establishment to those of rapid growth and high turnover, namely, smaller species. The results of our study were consistent with these explanations; in fact we found that the ciliate communities of *S. emersum* had large variations in ciliate abundances between samples: they were dominated by one or two species (Fig. 3) and inhabited mainly by smaller ciliates while larger ciliates were inhibited. It is thus suggested that the habitat provided by *S. emersum* is unstable in that it undergoes constant change in terms of decay, with the potential at times to recruit large microbial communities but also to cause a sudden and dramatic collapse of the community through the loss of a single leaf.

The depauperate ciliate community associated with *N. officinale* is difficult to explain in terms of plant morphology or life-cycle. An additional source of environmental complexity, not yet fully discussed but perhaps the most important, is the presence of a microbial biofilm that typically grows upon the plant surface, providing shelter and food for its resident and constituent microorganisms [3]. Biofilm removal or reduction thus greatly impacts the number of ciliates. During sampling of *N. officinale* in the Millstream we noticed that it was relatively easy to detect in its submerged location from the stream bank, due to its bright green colour. By contrast, the other two plant species, and particularly *S. emersum*, were less visible partly because their outline and colour had been masked by an accumulation of biofilm and other particulate matter that seemed to be more or less absent from *N. officinale*. Microscopy revealed a thinner biofilm on *N. officinale* than on the two other plant species; also, there was a remarkable absence of general particulate organic matter, flora and fauna, including the microbes (bacteria, diatoms, algal filaments, flagellates, amoeba, and ciliates) that typically have high abundances in and are the main constituents of biofilms [16]. The usual associated meiofauna, such as nematodes, copepods, rotifers and small insect larvae, were also all but absent. Indeed, the water was particularly clear and the plants from which the samples were collected could be easily seen. *Sparganium emersum* and *R. penicillatus* subsp. *pseudofluitans* samples contained variable, often relatively large quantities of additional matter such that flora and fauna, including ciliates, were much more difficult to observe as many of these organisms would have been obscured.

These observations are most likely explained by *N. officinale*'s production of PEITC, a compound that protects against herbivory [34,36]. The antimicrobial effects of isothiocyanates in general have been reported in the scientific literature since the 19th century [14]. Walker et al. [in 41] reported the phenomenon in 1937 and Foter and Golick [in 42] found that PEITC, when extracted from the roots of turnips, acts like a "natural insecticide". More recent research by Beevi et al. [5] confirmed these findings, in a study that also recounted the historical usage of cruciferous plants for food preservation. Less well studied is the effect of PEITC on aquatic fauna. In 2011, Dixon and Shaw [15] reported a negative impact of PEITC on *Gammarus pulex,* a freshwater shrimp known for its ecological robustness (Schmid-Araya, personal communication). In their study, Dixon and Shaw [15] found that gammarid mortality increases with high concentrations of PEITC and that gammarids elude water containing PEITC.

Our findings suggest that the ciliate community associated with *N. officinale* is in a state of constant renewal. In other words, each ciliate is only temporarily associated with *N. officinale*, through a process of constant loss and replenishment. The high and steady turnover of species suggested by the CSACs (Fig. 4) can be explained by a process of continual emigration and immigration of the ciliates, because the drift of the water column prevents them from settling on *N. officinale* or the conditions are too unfavourable for them to settle for long on the plant's surfaces such that they move on whilst the continual supply of ciliates in the drift replaces them. The low numbers of ciliated protozoa associated with *N. officinale* might then have been an indirect result of the impedance of bacterial colonisation and thus of the microbial fauna it supports both as a food source and as a structural component of the biofilm; alternatively, the paucity of ciliates may have reflected direct chemical inhibition of the ciliates themselves; or perhaps both mechanisms were involved. Thus, whether the perceptible lack of biofilm and the lower ciliate numbers were related to PEITC and its release from by *N. officinale* remains a question for future research.

Acknowledgements. The authors acknowledge funding support from the Esmée Fairbairn Foundation (UK).

Competing interests. None declared.

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