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FEATURE ARTICLE

Most ciliated protozoa in extreme environments are cryptic in the 'seed bank'

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ABSTRACT: This study focuses on the species richness of ciliated protozoa in inland saltpans. A low number of ciliates were found thriving under extreme high salinity; however, a diverse assemblage of ciliate species was revealed by gradually diluting the salt concentration, meaning that most ciliate species in these environments are hidden in the seed bank awaiting favourable growth conditions. In some saltpan samples, up to 100% of the ciliate species found were retrieved from the seed bank. Our results highlight the importance of ciliate seed banks as repositories of microbial diversity in the natural environment, enabling ecosystems to react to environmental change. The ability of microbial communities to respond to a changing environment depends on a large local diversity of rare and encysted species.

KEY WORDS: Ciliates · Cryptic biodiversity · Hypersaline · Saltpans

Inland saltpans in Andalucía, Spain. Insets show ciliated protozoa found in hypersaline aquatic habitats: *Fabrea salina* (top); *Euplotes harpa* (bottom).

Images: F. Guerrero (saltpans); A. Galotti (protozoa)

INTRODUCTION

Extreme environments refer to habitats with environmental conditions considered so harsh that they would eradicate or prevent growth of most forms of life on Earth. Hypersaline habitats are considered extreme ecosystems (Madigan & Marrs 1997, Horikoshi & Grant 1998, Rothschild & Mancinelli 2001, Mancinelli & Rothschild 2002). Biological diversity in these extreme environments is reduced; however, major groups of prokaryotes (Cho 2005, Hauer & Rogerson 2005), microbial eukaryotes (Esteban & Finlay 2003, Park et al. 2006) and even some metazoa (Elloumi et al. 2009) tolerate extreme salinity conditions. The cosmopolitan ciliate *Fabrea salina*, rotifers, copepods, ostracods and the anostracod *Artemia salina* are amongst the best known examples. Osmoregulation is the main mechanism underpinning endurance under high salinity concentrations; however, several biochemical processes, e.g. accumulation of amino acids, glycerol and other

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polyols, are also involved (see e.g. Yancey et al. 1982, Stock et al. 2002). Microbial (and other) eukaryotes can also adapt to changing salinities, as shown in protistan clones isolated from hypersaline habitats and successfully grown in freshwater laboratory cultures (Finlay et al. 2006), and in experiments aimed at detecting environmental transitions in salinity ranges (Lee & Bell 1999, Logares et al. 2009).

Numerous microbes thrive in hypersaline environments, but ciliates rarely develop large populations. Many microorganisms can also withstand hostile habitats in the form of inactive cysts, spores or other dormant stages (also known as microbial dormancy) waiting for the development of favourable growth conditions (Esteban & Finlay 2003, Olmo et al. 2011). This cryptic biodiversity is the hidden seed bank of species that also includes inactive forms (dormant microbes) and trophic organisms present in very low numbers. The seed bank is essential in the functioning of ecosystems as it allows them to respond to and recover after environmental change; it is also responsible for species turnover (Stevenson 1977, Cole 1999, Esteban & Finlay 2010). Ciliate seed banks can successfully be revealed by experimental manipulation of samples in the laboratory using cultivation techniques (Fenchel et al. 1997); these studies have revealed the role of microbial seed banks as the foundations of local microbial biodiversity in fresh waters and other habitats (Finlay et al. 1996, Esteban & Finlay 2003, Jones & Lennon 2010, Lennon & Jones 2011). As far as cryptic and dormant microbial richness are concerned, the expansion and development of environmental molecular tools have contributed significantly to our understanding of the hidden microbial richness in aquatic environments (e.g. Stock et al. 2002, Pawlowski et al. 2011; see Lennon & Jones 2011 for a review on microbial dormancy). However, microbial seed banks contain much more than resting microbes (Fenchel et al. 1997) and still remain an unexplored field in environmental microbial ecology research. The aim of this investigation was to assess and reveal the ciliate cryptic diversity as defined above in inland saltpans.

MATERIALS AND METHODS

Study area

The inland evaporation saltpans investigated are located in the Alto Guadalquivir region, northeast Andalusia (southern Spain), on the Subbetic and Olistostromes units of the Betic Cordillera (Rodríguez-Rodríguez 2007). These units are characterised by the presence of large proportions of clays, marls and evaporites of the Triassic period (Keuper facies; de Galdeano & Vera 1992), responsible for the salinity of the water.

Sediment samples (see below) were collected from 6 saltpans between the end of May and mid June 2009: Barranco Hondo, Brujuelo, Don Benito, San José, Peñaflor and Los Vélez.

Sample collection and processing

The top 2 cm of superficial sediment and the sediment–water interface were collected with sterile plastic tubes (50 ml volume), leaving a 2 cm headspace (Esteban & Finlay 2004). The tubes were kept in a cool box for transport to the laboratory. Salinity was measured as total dissolved solids (TDS) estimated from the conductivity data (mS cm^{-1}) taken *in situ* with a YSI multiparameter probe field model 556 MPS (Table 1). The estimation was calculated using the regression line equation $y = 1.3912x - 0.9557$, $p \leq 0.05$, obtained from 36 saltpans with salinities between 99 and 316 g l^{-1} TDS (A. Galotti unpubl. data).

Table 1. Salinity concentration (measured as total dissolved solids; TDS, g l^{-1}) in the gradual-dilution and the non-gradual-dilution experiments (see 'Materials and methods')

Saltpan	Initial salinity	Salinity concentration (TDS, g l^{-1})							
		Gradual dilutions			Non-gradual dilutions				
		1st dilution (50%)	2nd dilution (25%)	3rd dilution (12.5%)	80%	60%	50%	40%	20%
Brujuelo	136	68	34	17	109	82	68	54	27
Don Benito	174	87	44	22	139	104	87	70	35
Los Vélez	193	97	48	24.1	154	116	97	77	38.7
San José	195	98	49	24.4	156	117	98	78	39.1
Peñaflor	205	103	51	26	164	123	103	82	41
Barranco Hondo	249	125	62	31	199	149	125	100	50

In the laboratory, original samples were examined for the presence of ciliated protozoa. These samples were monitored for 2 d for emergence of further ciliate species; if none was detected (as was the case), subsamples were taken and their salt concentration was diluted following 2 methods: gradual (slow) and non-gradual (fast) dilution. Original samples were kept as controls at 20°C, and a sterile wheat grain was added as food.

Ciliate species were identified following established methods (Esteban et al. 1998), identification keys and specialised literature (Kahl 1935, Ruinen 1938, Tucolesco 1962, Dragesco & Dragesco-Kernéis 1986, Foissner 1993, Lee et al. 2002, Esteban & Finlay 2003, 2004). Some ciliates were identified to genus level due to the low number of individuals present and therefore the impracticality of applying microscopy techniques to discriminate species.

Gradual dilution

Progressive dilutions were carried out on a known volume of a subsample from the original sample. For this, dilution flasks were prepared by placing a 5 ml subsample from each original sample into a sterile culture flask, and adding filtered (0.2 µm pore size), 50% diluted seawater to acquire the required dilution (Table 1). Any one subsample was diluted once, e.g. down to 50% of original salt concentration, and monitored for emergence of ciliate species. Subsequently, this dilution was diluted 50% further in the same flask until reaching 12.5% of the original salt concentration. Dilution flasks were supplemented with food by adding 1 sterile wheat grain to maintain bacterial growth, and kept at 20°C. Ciliate communities were monitored over 5 to 7 wk for emergence of new species; further dilutions were set up when no further (i.e. not previously recorded) ciliate species were observed in any one dilution.

Non-gradual dilution

A set of dilutions on a gradient scale from 20 to 80% of the initial salt concentration (Table 1) was prepared for each original sample. To prepare this gradient, a known volume of subsample from each original sample (volume size taken according to original initial salinity and the final dilution to be achieved, i.e. 20, 40, 50, 60 or 80%) was placed in a culture flask and topped up to a final volume of 20 ml with filtered (0.2 µm pore size) Volvic® mineral water. This procedure was replicated for each dilution using separate flasks and up to 80% of the original salinity. One or 2 of the mid-range dilutions had to be skipped for a few of the original samples due to the small volume available. One sterile wheat grain was added as food to each dilution flask; flasks were incubated at 20°C. Dilution flasks were monitored for up to 3 wk for ciliate emergence.

Statistical analysis

A Spearman's correlation test was performed using Statistica 7.0 software to establish a relationship between salinity and species richness ($p \leq 0.05$).

RESULTS AND DISCUSSION

The species richness of active ciliated protozoa in sediment samples from 6 inland saltpans was assessed in the laboratory (see Appendix); the results showed that the number of active ciliate species found in these samples was unrelated to the salinity concentration ($p > 0.05$; Table 2). Original samples with the lowest salinity only showed 1 or 3 more species than samples with higher salinity (Table 2). The ciliate species richness encountered in diluted subsamples — that is, ciliate species not observed before dilution and that were undetectable active forms — was 2- to 4-fold higher in subsamples from Brujuelo saltpan, which had the lowest initial salinity (Table 2).

Table 2. Percentage of cryptic ciliate species retrieved from inland saltpans (southern Spain) after dilution of the original salt concentration. The final species number in the non-gradual dilutions corresponds to the total number of species retrieved considering all the dilutions. TDS: total dissolved solids

Saltpan	Initial salinity (TDS g l^{-1})	No. of species in original sample	Gradual dilutions		Non-gradual dilutions	
			Final species number	Cryptic species (%)	Final species number	Cryptic species (%)
Brujuelo	136	3	36	92	20	85
Don Benito	174	0	9	100	10	100
Los Vélez	193	2	12	83	9	78
San José	195	0	10	100	6	100
Peñaflor	205	2	19	89	14	86
Barranco Hondo	249	0	9	100	9	100

The number of ciliate species revealed after dilution was related to the dilution method applied (Table 2); hence, the gradual dilutions (Fig. 1) showed a steady increase of ciliate species over time with decreasing salinity, and also had a higher final number of species compared with the non-gradual dilutions (Table 2). These results indicate that ciliates can respond to environmental change provided the change is gradual (salinity concentration in this study) and if given enough acclimatization time (Finlay et al. 2006, Forster et al. 2013). This finding is particularly relevant to hidden individuals that, although they are present in the samples, are not detected in routine surveys.

Adaptation of protozoa to a changing environment has been extensively tested in relation to temperature, salinity, oxygen depletion, food availability and various other parameters (e.g. Montagnes & Lessard 1999, Hauer & Rogerson 2005, Fenchel 2010, Cometa et al. 2011). However, adaptation to changing environments can be reversible once the stimulus ceases. Finlay et al. (2006) gradually adapted clones of the ubiquitous ciliate *Cyclidium glaucoma* — a species able to thrive in fresh, brackish and sea water — to grow on a salinity gradient, whereby clones isolated from a marine habitat were successfully grown in freshwater cultures following a slow, gradual dilution process; the cultures were then slowly reverted back to sea water by progressively increasing the salt concentration. Microorganisms are able to adapt, especially when the change occurs slowly and over a long period of time (Esteban & Finlay 2010, Forster et al. 2013).

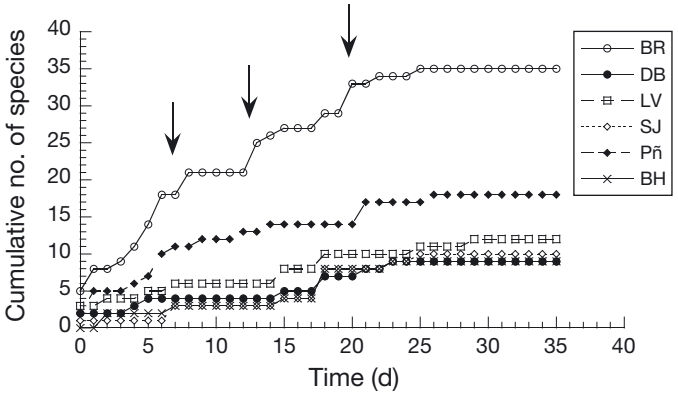

Fig. 1. Gradual dilution (see 'Materials and methods'): cumulative number of ciliate species found at a range of salinity gradients (from 100 to 12.5%) in laboratory dilutions of the original inland saltpan water samples. BR: Brujuelo; DB: Don Benito; LV: Los Vélez; SJ: San José; Pñ: Peñaflor; BH: Barranco Hondo. Arrows indicate when dilutions were applied

Most ciliate diversity is cryptic

Most microbial diversity at any one time is cryptic (Finlay et al. 1996, Fenchel et al. 1997, Esteban & Finlay 2003, Jones & Lennon 2010, Lennon & Jones 2011). Cryptic species biodiversity or hidden seed bank refers to microbial forms that are inactive (as dormant microbes) or, albeit active, are present in very low abundance, i.e. the rare species (Fenchel et al. 1997). In our study, freshly collected samples from 3 out of the 6 saltpans investigated had no active ciliates (Table 2). In fact, most of the ciliate diversity in the saltpans analysed was detected after dilution of the original salinity: 83 to 100% in the gradual-dilution experiments, and 78 to 100% in the non-gradual-dilution manipulations.

The pattern of species emergence was similar in the gradual-dilution and non-gradual-dilution experiments; also, the number of ciliate species increased at the point of further dilution, but with a few exceptions (gradual dilutions — Fig. 1). This pattern was obtained even if samples were monitored for up to 75 d (data not shown).

The cumulative number of species was always lower for the 20% dilutions except for Brujuelo saltpan, the habitat with lowest salinity concentration (Fig. 2). Dilutions in the range 40 to 60% rendered the highest species numbers in those saltpans that had higher original salinities. Below a 40% dilution, very few species developed population growth. Species emergence patterns were random at the 80% dilutions, which did not seem to be related to any initial salt concentration of the samples (Fig. 2). One ciliate species only, the hypotrich *Euplotes moebiusi*, was able to thrive at the whole range of salinities tested.

The newly created environmental conditions of lower salinity favour development of dormant species, which emerge and develop population growth, resulting in an increase in species numbers.

Importance of the seed bank

The species seed bank is essential in the functioning of ecosystems, as it allows them to respond to natural environmental changes and anthropogenic perturbations. Microbial seed banks are also responsible for species turnover (Stevenson 1977, Cole 1999, Esteban & Finlay 2010, Jones & Lennon 2010, Lennon & Jones 2011). This cryptic diversity is a fundamental part of the local pool of ciliate diversity (Esteban & Finlay 2003). The

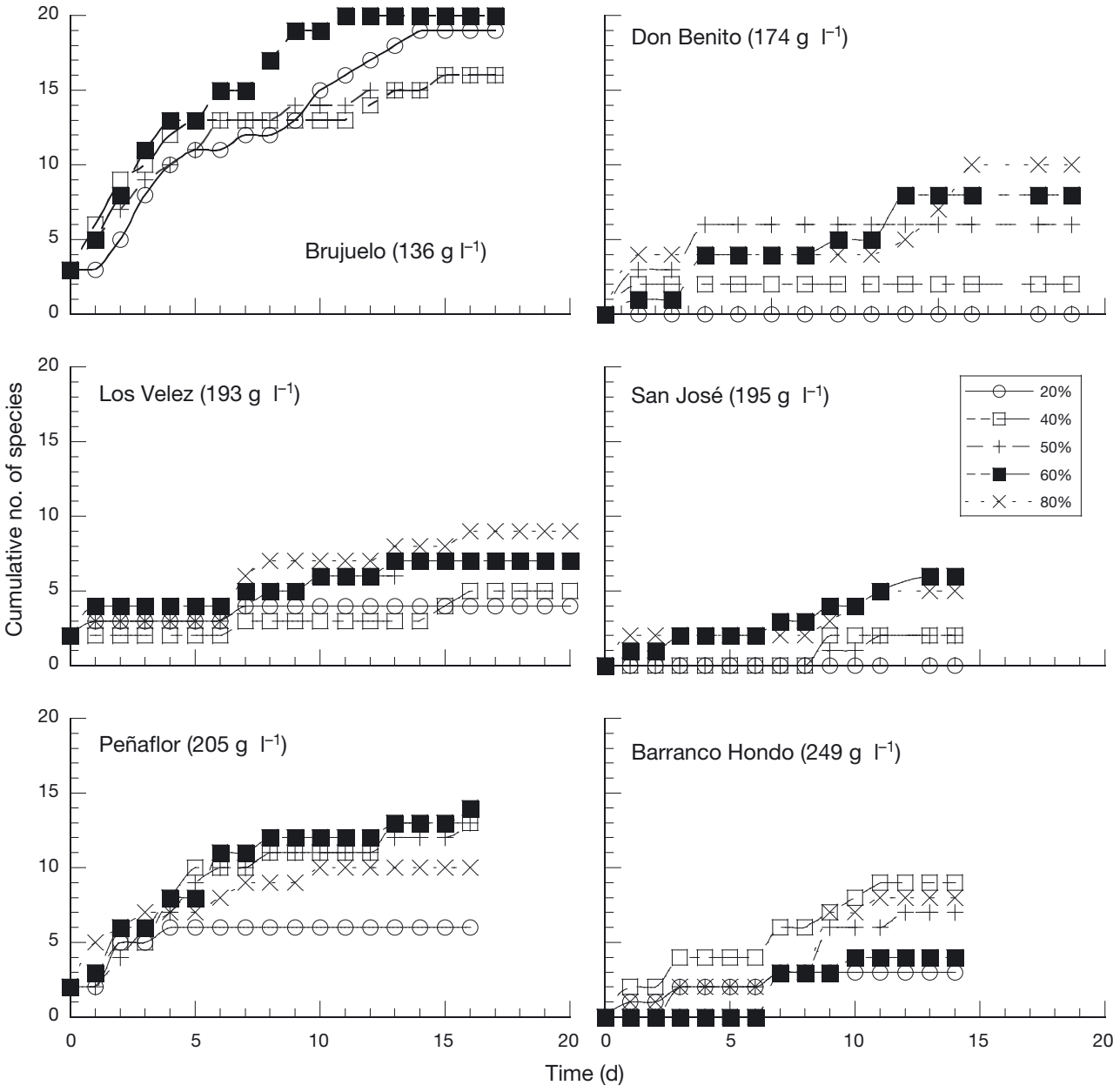

Fig. 2. Non-gradual dilution (see 'Materials and methods'): cumulative number of ciliate species retrieved at different dilutions (20, 40, 50, 60 and 80 %) of the original salt concentrations (shown in parentheses)

ability of microbial communities to respond to a changing environment relies on a large local diversity of rare and encysted species (Finlay & Esteban 1998).

The idea that most microbial eukaryotes at any one time are dormant is not new (Fenchel 1990, Moscatello & Belmonte 2009); nor is the remark on the importance of species seed banks — annual plants, for example, spend most of the year as seeds. However, few investigations deal with dormant eukaryotic diversity (Finlay et al. 1996, 2000, Esteban & Finlay 2003, 2007), making comparisons of our results somewhat difficult. These studies have shown the high proportion of cryptic species in the different habitat types investigated (Fig. 3).

Research into the hidden potential of microbial seed banks is needed at both morphological and molecular levels. Future research merging molecular and phenotypic approaches, targeting further microbial eukaryotes, will help to unravel the true richness of microbial life hiding in the seed banks of extreme and other environments.

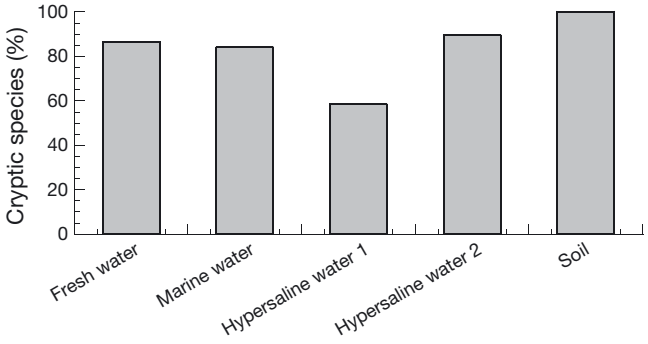

Fig. 3. Cryptic ciliate diversity (morphospecies) detected after laboratory manipulation of samples collected from different habitat types. Data taken from: Finlay et al. (1996) for fresh water; Esteban & Finlay (2007) for marine water (coastal rockpools); Esteban & Finlay (2003) for hypersaline water 1 (coastal saltpan); present study for hypersaline water 2 (inland saltpans); and Finlay et al. (2000) for soils

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Appendix. Species found in all samples

Species	Barranco Hondo	Brujuelo	Don Benito	Los Vélez	Peñaflor	San José
Aspidisca costata		x				
Blepharisma sp.		x		x	x	
Bothrostoma undularis		x			x	
Chilodonella sp.		x				
Chlamydodon mnemosyne		x	x			
Cinetochilum sp.		x	x		x	
Cladotricha elongata	x					
Cladotricha koltzowii	x	x		x	x	
Cristigera sp.			x	x	x	x
Cyclidium sp.	x	x	x		x	x
Euplotes charon				x		
Euplotes harpa		x				
Euplotes moebiusi	x	x	x	x		x
Fabrea salina		x		x		
Frontonia sp.		x				
Gonostomun sp.		x				
Litonotus sp.					x	
Mesodinium pulex		x				
Metacystis annulata	x		x	x	x	x
Metacystis tesselata		x				
Metacystis truncata	x	x		x	x	x
Opistroticha halophila		x			x	
Oxytricha sp.	x	x	x	x	x	
Plagiocampa rouxi		x			x	
Pleuronema crassum		x			x	
Pleuronema sp.		x			x	
Pseudoconilembus pusillus	x	x	x		x	
Spathidium sp.		x				
Strombidium sp.		x			x	
Stylonychia sp.		x				
Suctorian sp.		x				
Trachelocerca sagitta		x				
Trachelonema sp.		x				x
Trimyema kahli	x	x			x	x
Trimyema marinum		x		x		
Uronema sp.		x				
Vasicola sp.		x				
Unidentified 1		x	x	x	x	x
Unidentified 2		x		x		x
Unidentified 3		x				x
Unidentified 4		x				

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