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Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution

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Summary

1. Laboratory microcosm experiments using protists as model organisms have a long tradition and are widely used to investigate general concepts in population biology, community ecology and evolutionary biology. Many variables of interest are measured in order to study processes and patterns at different spatiotemporal scales and across all levels of biological organization. This includes measurements of body size, mobility or abundance, in order to understand population dynamics, dispersal behaviour and ecosystem processes. Also, a variety of manipulations are employed, such as temperature changes or varying connectivity in spatial microcosm networks.

2. Past studies, however, have used varying methods for maintenance, measurement, and manipulation, which hinders across-study comparisons and meta-analyses, and the added value they bring. Furthermore, application of techniques such as flow cytometry, image and video analyses, and *in situ* environmental probes provide novel and improved opportunities to quantify variables of interest at unprecedented precision and temporal resolution.

3. Here, we take the first step towards a standardization of well-established and novel methods and techniques within the field of protist microcosm experiments. We provide a comprehensive overview of maintenance, measurement and manipulation methods. An extensive supplement contains detailed protocols of all methods, and these protocols also exist in a community updateable online repository.

4. We envision that such a synthesis and standardization of methods will overcome shortcomings and challenges faced by past studies and also promote activities such as meta-analyses and distributed experiments conducted simultaneously across many different laboratories at a global scale.

Key-words: Ciliates, comparability, ecological theory, experimental ecology, methods, protists, protocols, protozoa, standardization

Introduction

A major contemporary challenge in ecology is to causally link processes and patterns across different levels of complexity (Fig. 1). This requires a causal understanding of (i) how traits and behaviour at the level of individuals affect fitness-relevant processes of birth, death and dispersal, (ii) trait distribution within populations, consequences on population dynamics and potential evolutionary changes, (iii) community assembly and structure and (iv) ecosystem functioning. A large body of theoretical work has been developed to conceptualize these processes. However, a major challenge is to link theoretical concepts with empirical data from natural study systems. In many cases, natural study systems do not allow the replication and control needed to validate model assumptions and to test model predictions, or experiments are logistically or ethically prohibitive.

Experimental microcosms, reflecting 'small worlds', offer a possibility to test concepts in ecology and evolution (see Table 1, Beyers & Odum 1993; Jessup *et al.* 2004; Srivastava *et al.* 2004; Cadotte, Drake & Fukami 2005; Benton *et al.* 2007), and various groups of organisms, including bacteria, algae and arthropods, have been used as model systems. Protist microcosms (Fig. 2, Lawler 1998; Petchey *et al.* 2002;

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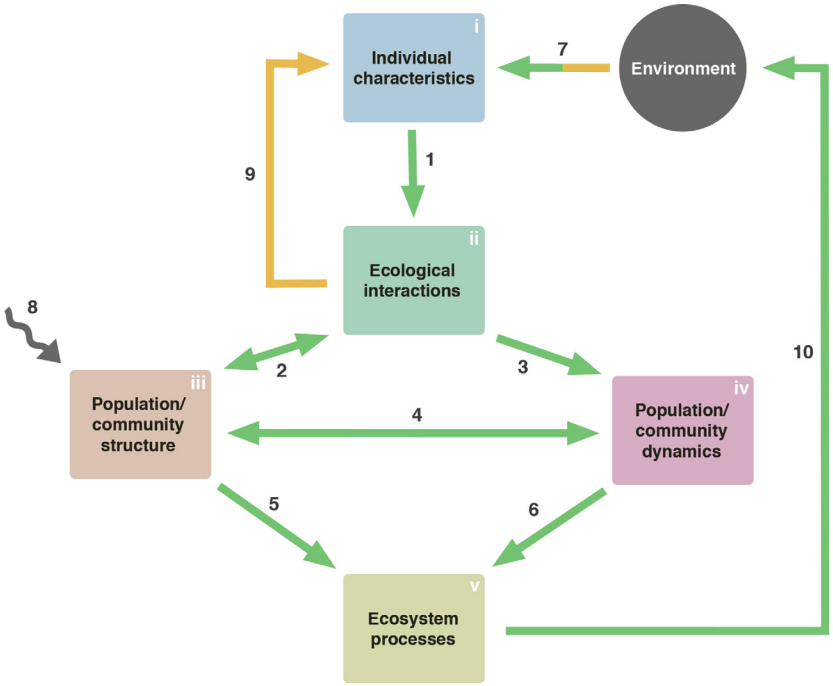

Fig. 1. Causalities between environmental factors, individuals and populations on structure and dynamics across different levels of biological organization (see also Table 1). Green and yellow arrows illustrate ecological and evolutionary causalities, respectively. Individual properties (i), such as traits, behaviour and physiology, dictate ecological interactions (ii), such as competition and predation. These ecological interactions in turn affect population and community structure (iii), population and community dynamics (iv) and ecosystem processes (v; arrows 1–6) on ecological timescales (arrows 1–6). Furthermore, the abiotic environment plays a major role in shaping the causalities. From an ecological point of view (green part of arrow 7), the environment influences the properties of individuals (i) through, for example, environmental filtering and plastic responses. Environmental effects also have the potential to change population and community structure through stochastic events that may cause, for example, abundance decline or extinctions (8). Environmental effects can induce evolutionary change (yellow part of 7) in traits due to selection. Such trait changes will change the ecological interactions, which in turn can induce further evolutionary change (9), which ultimately affects structure (iii), dynamics (iv) and ecosystem processes (v). Finally, ecosystem processes may feedback on to the environment (10).

Holyoak & Lawler 2005) have long been used to study ecological processes, based on pioneering work of Dallinger (1887), Gause (1934), Vandermeer (1969), Gill & Nelson (1972), Luckinbill (1973) and many others (for a more extensive literature overview, see Section 1.1 of Appendix S1 in the Supporting Information). Gause's study is exemplary of how protist microcosms can bridge empirical case studies and theoretical work. Indeed, Gause experimentally linked theoretical concepts of predator–prey dynamics (Lotka 1910; Volterra 1926) and fluctuations observed in natural populations, developing and using a protist microcosm system containing the ciliate *Paramecium aurelia* and its predator *Didinium nasutum*. Since then, several hundred studies have used such protist microcosm systems, with dozens of studies being published every year over the last decade. Research areas include the phylogenetic limiting similarity hypothesis (e.g. Violle, Pu & Jiang 2010), effects of disturbance and productivity on diversity (e.g. Haddad *et al.* 2008; Altermatt, Schreiber & Holyoak 2011b), the significance of trade-offs (e.g. Cadotte 2007; Violle, Pu & Jiang 2010), synchrony in population dynamics (e.g. Vasseur & Fox 2009), effects of environmental change on food web structure and species interactions (e.g. Petchey *et al.* 1999; Fox & Morin 2001), the study of predator–prey interactions and inducible defences (e.g. Kratina *et al.* 2009; Kratina, Hammill & Anholt 2010), the regulatory effects of biodiversity on ecosystem processes (e.g. McGrady-Steed, Harris & Morin 1997), invasion dynamics (e.g. Mächler & Altermatt 2012; Giometto *et al.* 2014), the significance of spatial dynamics on diversity and species interactions (e.g. Holyoak & Lawler 1996b; Carrara *et al.* 2012), scaling laws in ecology (e.g. Fenchel 1974; Giometto *et al.* 2013), epidemiological dynamics (e.g. Fellous *et al.* 2012b) and evolutionary and eco-evolutionary dynamics (e.g. Dallinger 1887; Schtickzelle *et al.* 2009; Hiltunen *et al.* 2014).

In almost all of the above-mentioned studies, variations of the basic methods developed and used by Gause (1934) were employed. This variation, however, is poorly documented and a standardization of methods is largely lacking. Furthermore, more sophisticated techniques are available nowadays and the range of study questions has broadened (Table 1). While the use of protists as model organisms in microbiology and cell biology (especially species of the genus *Paramecium* and *Tetrahymena*) can rely on a wider range of classic (e.g. Sonneborn 1950; Lee & Soldo 1992) and advanced methodological tools (e.g. Asai & Forney 2000; Cassidy-Hanley 2012), ecological and evolutionary research using these species is lagging behind. This calls for a common methodological toolbox, also covering recent technological advances.

Table 1. Overview of variables of interest ('What measured?'), organized along an increasing level of organization (from individuals to ecosystems). The variables of interest are used in different disciplines in ecology and evolution. Each method is described in detail in Sections 2 and 3. Some of the manipulation methods are also referring to general maintenance manipulations, which are described in Section 1. Dispersal and evolutionary changes (in yellow) are overarching processes that can be linked to all other variables

Level of organization	Variable of interest ('What measured?')	Examples of disciplines	Measurement methods (examples)
Individual	Morphology and species identity	Evolutionary Ecology, Ecology	Microscopy, image analysis
	Individual-level behaviour (e.g. movement)	Evolutionary Ecology, Behavioural Ecology	Image and video analysis
	Physiology (chemical composition)	Ecophysiology, Evolutionary Ecology	RAMAN microspectroscopy
	Genes/gene-expression	Ecological Genetics, Evolutionary Ecology	Genomics/transcriptomics
Population	Population density (number of individuals)	Population Ecology, Macroecology	Microscopy, image analysis
	Population dynamics (r/K)	Population Ecology	Microscopy, image analysis
	Size distribution/Biomass	Population Ecology, Macroecology	Particle counter, image analysis
	Use of resources (bacteria population)	Population Ecology, Behavioural Ecology	Plating, optical density, flow cytometer
	Intraspecific interactions	Behavioural Ecology	Microscopy, image analysis
	Extinctions/time to extinctions	Population Ecology, Viability analyses	Microscopy
	Dispersal	Metapopulation, Metacommunity and Spatial Ecology	Microscopy, image analysis
	Evolutionary change	Evolutionary Ecology	Microscopy, image analysis, respirometer
Community	Diversity (species identification)	Community Ecology, Macroecology	Microscopy, image analysis
	Types of species interactions	Community Ecology, Functional Ecology	Microscopy, image analysis
	Species-interaction strengths	Community Ecology	Microscopy, image analysis
	Resilience	Disturbance ecology	Microscopy, image analysis
	Invasion resistance	Invasion biology	Microscopy, image analysis
	Phylogenetics	Community Ecology, Community Phylogenetics	DNA Sequencing/Barcoding
Ecosystem	Nutrient/Carbon cycling, Decomposition rate	Ecosystem Ecology, Meta-ecosystem Ecology	Respirometer, litter bags
	Energy fluxes (O_2-consumption, CO_2 production)	Ecosystem Ecology, Meta-ecosystem Ecology	Respirometer
	Stoichiometry	Ecosystem Ecology, Meta-ecosystem Ecology	Nutrient analysis

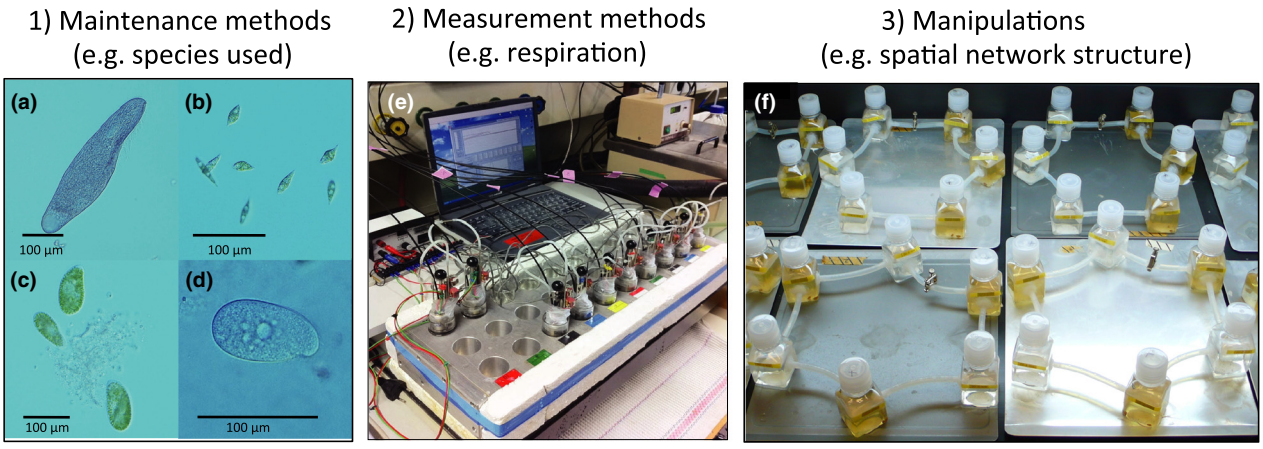

Fig. 2. Experiments with protist microcosms have the advantage that general maintenance methods can be highly standardized (e.g. a–d showing four species which have been commonly used and for which trait data are readily available; a: *Blepharisma* sp., b: *Euglena gracilis*, c: *Paramecium bursaria*, d: *Colpidium* sp.), that there exists a wide set of measurement tools, covering individuals to ecosystem processes (e: respirometer to measure ecosystem functioning) and that many types of experimental manipulation are possible (f: experiment in which the spatial connectivity of patches and availability of nutrients is manipulated simultaneously).

We provide a synthetic and comprehensive overview of methods (Table 1) for using protist microcosms as a model system in ecology and evolution. The scope of the methods covered includes experimentation with unicellular freshwater eukaryotes that are at least partly heterotrophic and often summarized under the term 'protists' (Adl *et al.* 2012) (note that some of the experiments can also include rotifers and algae). We focus on semi-continuous batch cultures, which can be highly replicated (hundreds of replicates). We highlight that experiments with protists can also be conducted under

semi-natural conditions in pitcher plant communities (or in other phytotelmata), and there is an extensive literature on experiments therewith (e.g. Addicott 1974; Kneitel & Miller 2003). Experimental systems including other micro-organisms, such as batch cultures of bacteria and phages (e.g. Buckling *et al.* 2000; Bell & Gonzalez 2011) or chemostats containing autotrophs, are only excluded here for reasons of space and certainly not because we believe them to be in any way less valuable experimental systems. Many of them share similarities with protist microcosms with respect to scientific questions addressed.

Methods overview in an eco-evolutionary framework

In the following, we use an eco-evolutionary framework of causalities between individual properties, environmental factors, eco-evolutionary processes, dynamics, structure and ecosystem processes (Fig. 1) to describe methods commonly used in protist microcosm experiments. Methods are given in three main sections (Fig. 2, see also table of content in Appendix S1): (i) maintenance, covering the set-up and handling of protist cultures; (ii) measurements, which allow the quantification of over 20 different categories of variables of interest (Table 1), covering behavioural ecology, ecophysiology, ecological genetics, population ecology, macroecology, spatial ecology, community ecology, ecosystem ecology and evolutionary ecology; and (iii) manipulations, which are necessary for determining causality among variables. Measurement methods are structured from more traditional to newer methods (e.g. Sections 2.1 to 2.5) at the individual level and thereafter follow a pattern of increasing complexity and derivation, looking at the individual and physiological level (Sections 2.6 to 2.8, all recent methods), then at processes (Sections 2.9 and 2.10) and finally at two important aspects of measurement commonly applied (though not exclusive) to protist microcosms: time series and species interactions (Sections 2.11 and 2.12).

Each method is shortly described and summarized in a section of the main text. Additionally, we provide standardized protocols in supplementary material and as a freely accessible online document (emeh-protocols.rtfd.org) that can be contributed to (see this webpage on how to contribute). Their focus is on describing detailed techniques and aspects often omitted in Method and Material sections, but which are crucial for the successful and standardized execution of experiments.

1 General maintenance methods

1.1 SPECIES USED

The choice of study species/lineages used in microcosm experiments is crucial, as it determines traits, behaviours and physiology (Fig. 1 [i]) as well as the resulting ecological interactions (Fig. 1 [ii]) and potential evolutionary dynamics (Fig. 1 [9]). When making this choice, one therefore has to take into account the specific topics (e.g. Do species cover different trophic levels or not?) as well as the variables of primary interest (e.g. diversity, species interactions). Species used for protist microcosm experiments cover several major domains of life and a large part of eukaryotic phylogenetic diversity (Adl *et al.* 2012). In the following, we use the term 'protist' to cover free-living, unicellular eukaryotes that are not purely autotrophic (sometimes interchangeably used with the term 'protozoa'). This includes species within the Cryptophyta, Foraminifera, Alveolata, Chloroplastida, Discoba and Amoebozoa (Adl *et al.* 2012). Very typical and commonly used representatives are species of the genera *Paramecium*, *Tetrahymena* or *Colpidium* (all Alveolates, used in >80 studies; for an extensive list of species used and an overview of representative protist microcosm studies, see Appendix S1, Section 1.1). The advantage of using a common set of species across studies and laboratories is the availability of prior information (such as species traits) and the possibility to link findings across studies (McGrady-Steed, Harris & Morin 1997; Altermatt, Schreiber & Holyoak 2011b; Carrara *et al.* 2012).

The selection of species is often a combination of practical reasons, such as morphological distinctness, cultivability or availability, and the respective question of interest (e.g. including different trophic levels or not). All species can in principle be collected directly from natural populations in ponds, tree holes or other aquatic habitats. This approach allows the use of co-evolved, potentially genetically diverse populations of natural co-occurring species. However, the difficulties faced during the isolation, cultivation and identification of naturally collected species often preclude this approach (see also Lee & Soldo 1992). A set of identification manuals (Foissner & Berger 1996; Lee, Leedale & Bradbury 2000; Patterson 2003) as well as genetic barcoding techniques (Section 2.9, Pawlowski *et al.* 2012) should allow identifications at least to the genus level even to non-taxonomists. Nevertheless, many studies used species either already available in laboratory stocks or species from culture collections.

1.2 CULTURE MEDIUM

The chemical composition of the nutrient medium is a major environmental feature (Fig. 1 [7]) affecting growth and reproduction of protists (next to temperature, for example). Therefore, the comparison of basic life-history traits (growth rate, carrying capacity, interspecific interaction coefficients) across species and studies depends on the use of a standardized medium. Protists are generally kept in a freshwater-based medium containing nutrients and sometimes bacteria (Section 1.3). Many types of medium have been used (e.g. Lee & Soldo 1992), which can be classified into chemically well-defined media (e.g. Bristol medium, Chalkley's solution, see Appendix S1), and media made out of suspensions of less-defined organic matter (e.g. proteose peptone medium, protozoan pellet medium, wheat or wheat-hay suspensions). Medium made of ground protozoan pellets (provided by Carolina™, Biological Supply Company, Burlington NC, USA), either suspended in well or tap water or in Chalkley's solution, is commonly used (for an overview of studies using different media types, see the Appendix S1, Sections 1.1 and 1.2, where we also refer to many more media types suited for specific species). We recommend using proteose peptone medium for axenic cultures (e.g. Schtickzelle *et al.* 2009; Pennekamp *et al.* 2014b) and using protozoa pellet medium otherwise (e.g. Petchey *et al.* 1999; Haddad *et al.* 2008; Altermatt, Schreiber & Holyoak 2011b). Often, autoclaved wheat seeds or other organic material are added to the standard media in order to provide slow release of nutrients, leading to larger and more stable populations of protists, but are less standardized. Many of the protists can still survive and reproduce in the above-mentioned media at 10- to 20-fold dilutions (e.g. Altermatt & Holyoak 2012).

1.3 BACTERIA

Many protists are primarily or exclusively bacterivorous, and thus, many experiments involve bacteria as a food source for the protists. Next to the chemical composition of the medium, the availability of a common set of bacteria as a food source is a critical step towards standardization. Bacteria may be a central component of protist experiments and can potentially affect ecological and evolutionary dynamics

as they are both part of the environment (Fig. 1 [7]) as well as involved in ecological interactions (predation; Fig. 1 [ii]). While different non-pathogenic bacteria species have been successfully added and used in protist microcosm experiments (e.g. *Bacillus subtilis*, *B. brevis*, *B. cereus*, *Enterobacter aerogenes, Serratia fonticola*, or *S. marcescens*), the bacterial community is often the least understood and controlled element of the microcosm due to the invasion and establishment of cryptic species in the community. A better control of the bacterial communities in protist microcosm experiments would be a desired improvement for future work. Bacteria can be stored frozen and added to cultures in known compositions and quantities and quantified using flow cytometry (Section 2.5).

An even higher level of standardization and reproducibility is reached by using axenic cultures (i.e. cultures containing no bacteria). To maintain axenic cultures, to transform non-axenic cultures into axenic ones, or to create mono-xenic cultures, the culture medium needs to be treated with antibiotics. Subsequently, strict sterile technique is required. Axenic cultures are often used for single-species experiments (especially *Tetrahymena* sp.) (e.g. Asai & Forney 2000; Pennekamp & Schtickzelle 2013; Pennekamp *et al.* 2014b), while almost all experiments containing multiple species of protists are done under non-axenic conditions as most species cannot be axenized (e.g. Petchey *et al.* 1999; Haddad *et al.* 2008; Altermatt, Schreiber & Holyoak 2011b).

1.4 APPARATUS

A laboratory equipped with general microbiological apparatus is required, including microbalances (precision 0·1 mg), an autoclave, incubators, pH meter, microscopes and a sterile bench (for working with axenic cultures). Protist cultures can be maintained and handled with general laboratory equipment, though this must be inert with respect to chemicals leaking into the medium (e.g. using silicon tubes or glass jars). Jars and pipettes should be rinsed with deionized water to remove detergents. Glass jars and polystyrene microwell plates are common experimental habitats. Care needs to be taken when making habitats, as for example silicone glue, even if recommended for aquaria use, often contains antifouling chemicals (e.g. Altermatt & Holyoak 2012).

1.5 LABORATORY PRACTICES

A clean and tidy laboratory can make the difference between success and failure of protist experiments. Thus, reproducible and standardized laboratory routines are highly recommended. Experiments with protists may or may not be carried out in sterile conditions (e.g. Fellous *et al.* 2012b; Pennekamp *et al.* 2014b), depending on the variables that need to be measured and/or kept under control. An important practice is to avoid the spread/escape of protists from laboratory cultures into natural ecosystems; thus, all material used in the laboratory should be disposed of appropriately (e.g. autoclaving or rinsing with bleach).

1.6 LONG-TERM MAINTENANCE OF STOCK CULTURES

Keeping stock cultures over long periods of time (e.g. years) is often desirable, as it allows using the same strains and species across different experiments (e.g. Section 3.8). A classical example are protist species isolated by the laboratory of Peter Morin (McGrady-Steed, Harris & Morin 1997), which have been subsequently used in dozens of studies over many years (e.g. Fox & Morin 2001; Petchey *et al.* 2002; Jiang & Morin 2005; Haddad *et al.* 2008; Altermatt, Schreiber & Holyoak 2011b). Depending on the species, stock cultures should be subcultured every 2 weeks to 2 months, especially predatory species (e.g. *Didinium*) need more frequent (daily or weekly) subculturing. Stock cultures of 100 mL medium in glass jars of approximately 250 mL volume are ideal for long-term maintenance (e.g. glass Erlenmeyer jars covered with a loose fitting lid).

To protect against accidental loss of species, 4–8 replicate cultures of each species should be kept in two separate incubators. Stock cultures should be maintained at large population sizes, including the transfer of cultures during the regular maintenance procedures, to avoid loss of genetic diversity, accumulation of mutations due to bottlenecks or increased drift processes in general. Environmental conditions should be controlled. Population density should be recorded at each subculture, to provide a long-term record of changes, such as impending extinction. Experimenters should document the origin and collection date of the study species and use a common nomenclature across studies. Cryopreservation as another long-term storage is described in Section 1.7.

Long-term stock cultures should not be used directly to start experiments for several reasons: (i) stock cultures often contain organisms other than those desired in experiments, for example stock cultures of predators are often kept with multiple prey species, as this can increase the persistence of the predator (Petchey 2000). Stocks must therefore be cleaned (i.e. isolating the target organism through serial transfers or dilution to eliminate unwanted contaminating organisms) prior to an experiment (see Section 1.1). (ii) Experiments may require larger numbers of individuals or volumes of media than are available in long-term stocks. (iii) Disturbances of stock cultures should be minimized. (iv) Experimental environmental conditions may be different from those of long-term maintenance, such that some acclimation is required. Hence, for experiments, it is usual to set up separate experimental stock cultures from the stock cultures devoted to long-term maintenance.

1.7 LONG-TERM PRESERVATION

Many of the described methods to quantify and measure protist cultures yield the best results with recently subcultured live protists. However, in many cases, long-term storage is desirable. This may be advantageous when a large number of samples are taken at a time, preventing processing all samples immediately. Dead protists can be stored in Lugol's solution for longer time periods and subsequent identification or counting (Section 1.1, Risse-Buhl *et al.* 2012; Lee & Soldo 1992), though note that individuals preserved in Lugol's solution can change their size and shape. For long-term preservation with respect to genetic analyses, see Section 2.7.

A second long-term storage is cryopreservation, whereby individuals are stored alive at very low temperatures, such that they can be revived at a later time point. This is also an alternative to maintaining liquid cultures using serial transfer (see Section 1.6). Cryopreservation allows the recreation of strains in case of loss in liquid cultures, preserves genotypes from evolutionary changes and allows the sampling of cultures at specific time points for later reference (e.g. for studies on experimental evolution, see Section 3.8 and Kawecki *et al.* 2012). The preferred storage for long-term cryopreservation is in liquid nitrogen (−196°C). Standard protocols for the cryopreservation of protists have been developed especially for *Tetrahymena* (Cassidy-Hanley 2012), but also many other protist species (Lee & Soldo 1992), and detailed protocols are given in Appendix S1, Section 1.7. This involves a phase of culturing cells under specific conditions before freezing to ensure a high recovery rate after thawing, the use of specific cryoprotectants, and a progressive and controlled cooling down before long-term storage in liquid nitrogen. Thawing requires specific precautions to limit the

thermic shock and allowing cells going back to normal reproduction. The basic principle of cryopreservation is 'slow freeze and quick thaw'.

2 Measurement methods

Many measurements of ecologically and evolutionarily relevant variables are possible at all levels of organization (individual to ecosystem, Fig. 1, Table 1). Measurements can be made in a local, non-spatial or in a spatial context (for examples of the latter, see figures given in the Appendix S1, Section 3.5). Furthermore, they can be made in a scenario that focuses on ecological dynamics only, or on (eco-) evolutionary dynamics. Some variables can be measured by eye and some by machine. Although measurements by machines have advantages, we strongly recommend that researchers spend considerable time directly observing the organisms they work with, in order to detect unexpected aberrations (e.g. contaminations).

Almost all the measurements discussed below are described at a per-sample level. While some methods can be automated and conducted by laboratory robots, which allows the processing of much larger numbers of samples/replicates, we do not cover such automation in detail.

2.1 SAMPLING

Observing properties of microbial microcosms, such as individual traits (Fig. 1 [i]) and population/community dynamics (Fig. 1 [iv]), often cannot be carried out *in situ* and usually cannot be performed for the entire microcosm or every individual therein. Hence, observation virtually always involves observing properties of a sample of the microcosm and removing this sample from the microcosm (though see below for exceptions).

How much volume to sample depends on what is being observed and on population density in the microcosm. Generally, when estimating population density, larger samples will give better estimates. Sampling greater volumes reduces the sampling error, but can be more time-demanding and also represents a larger disturbance if the sampling involves medium removal. How frequently to sample depends on the goals of the experiment and on the variables of interest.

It is often possible and desirable to make multiple measurements from the same sample, such as abundance of different protist species and bacteria and chlorophyll concentration (e.g. Sections 2.2, 2.3 and 2.5). For reasons of practicality and sterility, the volume sampled is discarded and replaced with the same volume of sterile culture medium. However, when larger volumes must be sampled (e.g. 5 mL from a 100 mL microcosm), they can be returned to the microcosms in order to minimize disturbance, provided that adequate steps are taken to avoid contamination. For some questions, such as those concerning extinction times or the detection of rare large protists in a community of abundant small protists (e.g. Carrara *et al.* 2012; Clements *et al.* 2014), sampling the entire microcosm is highly desirable. This can be achieved by using a vessel with a transparent bottom that can be placed directly under a dissecting microscope.

2.2 ESTIMATING ABUNDANCES BY EYE (MANUAL MICROSCOPY)

Protist ecology has used optical microscopes for estimating protist densities and for observing cell features since its very beginning (Gause 1934; Luckinbill 1973; Lee & Soldo 1992) (see also the extensive list of examples given in Appendix S1, Section 1.1). A dissecting microscope with dark field illumination, capable of low (10×) to high (~160×) magnification, is ideal for counting protists (size range approximately 10–500 μm, Giometto *et al.* 2013). Counting is either done in droplets of known volume or with the aid of counting chambers (e.g. hemocytometer or Sedgwick Rafter counting chamber) that contain known volumes of media. Compound microscopes, capable of higher magnification, are required for counting smaller organisms (e.g. microflagellates, individual bacteria) and observing cells in detail (e.g. for evidence of parasitism, Fellous *et al.* 2012b).

2.3 IMAGE AND VIDEO ANALYSIS

Direct microscopy by a trained experimenter provides accurate abundance measurements for single-species or complex communities and is unrivalled in terms of registering specific qualitative behaviours and morphology for species identification (Section 2.1). However, such population- or community-level properties (Fig. 1 [iv]) are insufficient in the light of recent trait-based approaches in ecology, requiring quantitative measurements of individual-level traits, such as morphology and behaviour, for large numbers of individuals (Fig. 1 [i]). Digital image and video analysis can provide this information (Pennekamp & Schtickzelle 2013; Dell *et al.* 2014).

Reliable and accurate image and video analysis relies on an optimized workflow regarding magnification, illumination, observation chamber, image/video processing and analysis algorithms and analysis of acquired data (detailed protocols and code are given in Appendix S1, to Section 2.3 and references therein). Images can describe individuals in terms of cell size, cell shape, coloration or movement (e.g. Pennekamp & Schtickzelle 2013; Fronhofer, Kropf & Altermatt 2014; Giometto *et al.* 2014). A focus of video analysis has been to quantitatively describe the movement behaviour of microbes (e.g. Fenchel 2001; Giometto *et al.* 2014), but it is also a promising tool to describe and quantify how individuals react to intra- and interspecific interactions (Fig. 1 [ii]) (Dell *et al.* 2014). An R package tailored to automatically extract such information from videos of protist microcosms was recently developed (Pennekamp, Schtickzelle & Petchey 2014a).

2.4 PARTICLE COUNTERS

Both the number of individuals as well as their body size are important traits in population biology, community and evolutionary ecology and thus of high interest to be measured (Table 1). Besides image and video analysis (Section 2.2), particle counters, such as the commonly used CASY Model TT Cell Counter and Analyzer (Roche®; detailed step-by-step protocols are given in Appendix S1, Section 2.4), can be used to measure size distributions and density of protist species, both in isolation (Giometto *et al.* 2013) and in communities (Mächler & Altermatt 2012). The CASY allows measurement of mean body size (with linear size ranging from 0·7 to 160 μm), its associated variability, community size spectra and total biomass. A limitation of particle counters (though not unique to them) is the measurement of low-density samples. Additionally, the CASY allows discerning the body size distributions of different species within the same sample only if the distributions are non-overlapping. Advantages of the particle counters over digital imaging include direct measurement of cell volume (CASY) and the rapid processing of samples with high densities.

2.5 MEASURING BACTERIA DENSITY: PLATING, OPTICAL DENSITY AND FLOW CYTOMETRY

Quantitative information on bacterial density is often highly desirable for understanding the dynamics of the protist species consuming them (Fig. 1 [ii] and [7]). Heterotrophic plate counts (HPC) or optical density

(OD) measurements can provide measures of bacteria density (e.g. Fox & Smith 1997; Beveridge, Petchey & Humphries 2010a). However, both these methods have constraints. For example, HPC assays are time- and work-intense and restrict the researcher to bacteria that are cultivable (measured as colony-forming units, CFUs), while giving no information on cell size. OD measurements are fast and indicative of biomass, but are limited to high cell densities, may suffer from artefacts (e.g. abiotic turbidity) and are bulk sample measurements, incapable of distinguishing cell size or viability on single-cell level.

An alternative is flow cytometry (FCM) (e.g. recently used by Limberger & Wickham 2011; DeLong & Vasseur 2012). FCM allows rapid quantification and characterization of suspended particles at the single bacteria-cell level. The method is fast (<1 min per sample) and thus enables high throughput measurements (a detailed protocol for a highly standardized approach is given in Appendix S1, Section 2.5). The method is highly reproducible with a typical error of below 5% on replicate measurements and usually measures several thousands of individuals per sample. Furthermore, FCM collects multivariable data for each particle, including light-scatter signals and fluorescence, which can distinguish bacteria from abiotic background, and be combined with fluorescent labels for interrogating the bacterial sample with respect to activity and viability (Hammes & Egli 2010).

2.6 RAMAN MICROSPECTROSCOPY

While image and video analysis yields information on optical features of individual cells, Raman microspectroscopy (RMS) yields information about their chemical composition and allows identifying different cell types, physiological states and variable phenotypes. RMS is a non-invasive and label-free method for biochemical cell analysis. RMS combines Raman spectroscopy (RS) with optical microscopy (Puppels *et al*. 1990). Wagner (2009) and Huang *et al*. (2010) provide an excellent and detailed description of RMS and its extensions and its use in microbiology. RMS can be combined with other methods, such as stable isotope probing (SIP) and fluorescence *in situ* hybridization (FISH), to reveal feeding relations and functional characters of cells (e.g. Huang *et al*. 2007; Li *et al*. 2013). RMS is a rather novel method in general and especially to ecological research. So far, it has not been broadly used in microcosm experiments although its potential is immense: enabling to measure the chemical composition on a single-cell basis, RMS could be used to precisely quantify trophic interactions or to measure the impacts of abiotic and biotic influences on ecological dynamics (e.g. food shortage, competition, predation pressure).

2.7 DNA SEQUENCING/BARCODING

While ecologist have been focusing on the phenotype of organisms for a long time, it is nowadays possible to work at the genotype level, and by that to study ecological and evolutionary dynamics, or set the research in a phylogenetic context (e.g. Violle *et al*. 2011). Many DNA sequencing methods are available to analyse protist community composition (Hajibabaei *et al*. 2011; Zufall, Dimon & Doerder 2013), to characterize genetic diversity of species complexes (e.g. Catania *et al*. 2009), or to understand the evolution of genes and genomes (e.g. Brunk *et al*. 2003; Moradian *et al*. 2007). DNA barcoding is a special case of sequencing, which focuses on the study of a short and conserved portion of the genome owing the property to disentangle the phylogenetic relationships between taxa (Pawlowski *et al*. 2012). Depending on the protist taxa, barcodes have been developed either on the mitochondrial genome or in the nuclear genome, and the best choice of genes depends on the specific protist taxa (Pawlowski *et al*. 2012). In some protists, ribosomal genes have been duplicated from the mitochondrial genome to the nuclear genome, potentially creating some noise in the data. It may thus be necessary to separate the nuclear from the mitochondrial materials, for example, by migration on agarose gel. In ciliates, the two nuclei (macronucleus and micronucleus) can be isolated by gradient separations, like Percoll gradients.

2.8 GENOMICS, PROTEOMICS AND EPIGENOMICS

All 'omics' methods aim at characterizing and quantifying the whole biological molecule content in a sample (DNA, RNA, proteins) and also allow addressing the subindividual level (similar to RMS, Section 2.4). Due to their small size, living conditions and underestimated diversity, protists are ideal study organisms for metagenomics and metaproteomics project. While not specifically developed for microcosm experiments, most 'omics' methods can be used directly, and the only crucial and organism-dependent step is molecule extraction. Although standard protocols of DNA (see Section 2.7) or RNA isolation can be used in protists (e.g. silica column methods, Xiong *et al*. 2012), slightly adapted protocols result in more accurate results. Cultured-cell protein extraction kits (e.g. Protein extraction from Tissues and Cultured Cells using Bioruptor®, Diagenode, Denville, NJ, USA) can be very useful in protists, some of them providing directly usable samples for mass spectrometry methods (Pierce Mass Spec Sample Prep Kit for Cultured Cells, Thermo Scientific®, Waltham, MA, USA).

Epigenetic phenomena have long been described (Strahl *et al*. 1999; Gutierrez *et al*. 2000; Swart *et al*. 2014) and studies mostly concentrate on the role of small RNAs on the macronuclear development in the ciliate two-nucleus group (Duharcourt, Lepere & Meyer 2009). Common techniques consist of a gel-based excision of small RNAs from total RNA extractions that are further used to construct libraries (e.g. Singh *et al*. 2014). Although not yet used in experimental protist microcosms, such libraries could serve as basis to assess the role of epigenetic changes in protist adaptation to environmental changes. In analogy, DNA methylation in the context of environmental change can be studied using sodium bisulfite conversion or immunoprecipitation (Bracht, Perlman & Landweber 2012).

2.9 RESPIROMETER

A key variable describing dynamics in ecosystems (Fig. 1 [v]) is the rate at which the organisms consume oxygen and produce carbon dioxide via respiration and the opposite via photosynthesis (see also Fig. 2). Coupled with 'light-dark-bottle' experiments (Pratt & Berkson 1959), measuring rate of oxygen use/production can inform about community respiration rate and net photosynthetic rate when autotrophs are present.

Diverse methods are used to derive respiration rate, but all are based on the principle 'What goes in must come out' to calculate changes in O_2 or CO_2 concentrations. They can be open or closed circuit (recommended for protists), often measuring oxygen concentrations using an oxygen cell (these have limited life and require frequent calibration). Dissolved O_2 concentration can be measured with electrochemical sensors (Pratt & Berkson 1959). However, more recently, non-invasive *in situ* measures of O_2 concentrations using oxygen optodes (e.g. PreSens™) have become more popular. MicroResp™ is a microplate-based respiration system to measure CO_2 concentration within 4–6 h, based on colorimetric detection (Campbell & Chapman 2003; Campbell, Chapman & Davidson 2003).

2.10 NUTRIENT DYNAMICS AND LITTER BAGS

Both the uptake of nutrients as well as the decomposition of organic matter is of primary research interest, especially in community ecology (Fig. 1 [iv], [6], [v]). Studies of free-living ciliates showed that elemental composition can influence the population dynamics of ciliate predators (e.g. rotifers) and thus the regulation of ciliate populations (Boëchat & Adrian 2006), and the selectivity of ciliates according to the elemental stoichiometry and hence food quality of their bacterial prey (Gruber, Tuorto & Taghon 2009).

Species like *T. thermophila*, whose nutrient requirements are very well understood, can be kept on chemically defined medium, where the exact composition of macronutrients (and thus the elemental composition) is precisely known and amenable to manipulation (Asai & Forney 2000). For other species, determination of elemental composition is possible by techniques such as RMS (see Section 2.4), combustion and infra-red spectrometry, and X-ray microanalysis (Vrede *et al.* 2002).

Decomposition is a critical ecosystem process due to its influence on nutrient cycling and availability, and protist have an important role in this process (Ribblett, Palmer & Coats 2005). Protists grazing on bacteria can promote decomposition, despite decreased bacterial biomass. Microcosm studies of decomposition rate include the effects of biodiversity (McGrady-Steed, Harris & Morin 1997) and effects of temperature change (Petchey *et al.* 1999) on decomposition. Decomposition rate can be estimated by measuring the weight loss of organic matter (e.g. of a wheat seed) over a specific amount of time (e.g. Davies *et al.* 2009), similar to the use of leaf litter bags for measuring decomposition in terrestrial ecosystems.

2.11 TIME SERIES

Times series obtained from experiments are a prerequisite to address numerous questions in ecology. They display the dynamic changes of characteristic variables such as density, biomass, population structure, genotype frequency or diversity (Fig. 1 [i] and [iv]). Protists are generally characterized by rather short generation times (usually a few hours) making them ideal model organisms to get comprehensive time series over many generations within only a couple of days/weeks. Typical measures of interest (see also Fig. 1, Table 1) are variability in population density and its derivatives, resilience, return rate or Lyapunov exponents (e.g. Lawler & Morin 1993), competition/coexistence, or synchrony (e.g. Vasseur & Fox 2009).

Recording entire time series instead of considering only one or two snapshots after starting an experiment gives a far more detailed insight of the ongoing processes. This is especially true for transient dynamics between two or more dynamical steady states (if there exist any at all for a given system) that can be highly complex due to inter- and intraspecific processes (Massie *et al.* 2010). Moreover, since comprehensive times series contain more information for analysis, derived predictions are likely to be more accurate and precise compared to before/after snapshot experiments. Combined with model fitting procedures such as trajectory matching, time series enable inferring not only qualitative but also quantitative information such as parameter values (e.g. intrinsic growth rate r, carrying capacity K, or half-saturation constant K_N).

2.12 INTERACTION STRENGTHS

Intra- and interspecific interactions (Fig. 1 [ii]) are key to understanding population dynamics and community structure (Fig. 1 [iii] and [iv]). Measurements of interaction strengths are usually done by measuring population growth in single-species versus pairwise two-species settings (for a comparison of methods and data requirement, see Novak & Wootton 2010; Carrara *et al.* 2014). The strength of competition can be measured as difference in equilibrium population density between single-species and two-species cultures or by competitive exclusion. Furthermore, competition coefficients can be estimated by fitting a Lotka-Volterra competition model to the growth curves.

Predation rates can be measured by direct observation of a single predator feeding on a known number of prey individuals in a small drop of medium over a short period of time. Otherwise functional response experiments can be used. Counting individual protists for functional response experiments is time-consuming; moreover, short generation time of most protists can be a confounding factor unless the duration of the experiments is very short. An alternative is to estimate predation strength by measuring population dynamics in a predator–prey system and inferring predation rates by fitting a suitable model, such as a Lotka-Volterra predator–prey model, to a time series of the two populations.

3 Manipulation methods

A considerable advantage of microcosm experiments is the high flexibility in doing various manipulations (Lawler 1998; Holyoak & Lawler 2005), covering manipulations of both abiotic as well as biotic conditions. Manipulations can cover almost all aspects of ecology and evolution (Fig. 1, and see also the extensive list of references in Appendix S1, Section 1.1) and are often highly specific to the question of interest (Table 1). Thus, in the following, it is not our goal to give all possible manipulations or to give a strict standardization, as this is neither wanted nor practicable. Rather, we give an overview of the common manipulations, pitfalls and opportunities and a selection of examples.

3.1 GENERAL EXPERIMENTAL DESIGN

One of the most significant strengths of protist microcosm is the varied and relatively straightforward manipulations that are possible (Lawler 1998). Another strength is the ease with which unmanipulated variables can be controlled, such as species composition, environmental conditions and system openness (which also allow the design of protist experiments in close analogy to mathematical models) (e.g. Altermatt *et al.* 2011a; Carrara *et al.* 2012; Giometto *et al.* 2014). Replication, randomization, blocking and independence are key, as with any good experiment (e.g. Quinn & Keough 2002). The ease of high replication can result in statistical significances that need to be carefully interpreted with respect to biologically relevant effect sizes. That is, effect size and not only statistical significance should be studied.

An important advantage of protist microcosms is that the experimental units are closed populations/communities, in which for example, the number and identity of species at start are known. Thereby, estimates of species richness or the potential occurrence of specific species interactions is *a priori* well known, an advantage compared to the often 'open' communities in natural systems (Gotelli & Colwell 2001).

3.2 MANIPULATION OF DENSITY

Many ecological processes show density dependence. Thus, manipulating density is of interest to study the direct effect of density on processes such as dispersal (e.g. Fellous *et al.* 2012a; Pennekamp *et al.* 2014b) as well as indirect effects, such as the sensitivity of dynamics to small

changes in initial density conditions (e.g. Worsfold, Warren & Petchey 2009). Generally, density is manipulated at the start of an experiment, but can also be manipulated during an experiment. Most experiments are either started with a fixed density of each species (e.g. all species/populations start with same density, Altermatt, Schreiber & Holyoak 2011b) or a fixed proportion of each species' density relative to its carrying capacity K (Carrara *et al.* 2012). The first case has the advantage that all starting densities are equal and thus should be equally affected by drift processes, but has the disadvantage that the starting densities may vary in orders of magnitude relative to a species' K. While the latter case may be generally more preferable, specific experimental considerations should take precedence.

As long as manipulations concern the range of densities up to carrying capacity, growing cultures to K and diluting them with culture medium to the desired density is sufficient. To get densities higher than K, cultures need to be concentrated. This can be done in two ways: first, by centrifuging cultures such that a pellet is created at the bottom of the tube, which contains the cells, whereas the cell-free medium (the supernatant) is removed. Secondly, one can remove medium and concentrate cells by reverse filtration (i.e. discard filtrate and preserve supernatant).

3.3 DISTURBANCE AND PERTURBATION MANIPULATIONS

Environmental disturbances (Fig. 1 [8]) correspond to forces that substantially modify the structure, resources and function of ecosystems during a discrete event on both large and small scales. Disturbances can either be a temporary change in the environment that affects the community (i.e. a pulse perturbation), but where eventually the environmental conditions return to the initial state, or be a permanent change in the environment (i.e. a press perturbation), or somewhere on the continuum between pulse and press. The consequences of natural disturbances on natural communities are often hard to study, as catastrophic disturbances are either impractical or unethical to be applied at large scales, whereas they can be easily applied to microcosm experiments.

A commonly applied disturbance in microcosm experiments is density-independent mortality, where either a part of the community is replaced by autoclaved medium (e.g. Warren 1996; Haddad *et al.* 2008; Altermatt *et al.* 2011a), or where a part of the community is killed (by heating or sonication), but the medium retained in the culture, such that chemical and nutritional conditions remain as constant as possible (e.g. Jiang & Patel 2008; Violle, Pu & Jiang 2010; Mächler & Altermatt 2012). This type of pulsed disturbance is easy to apply but does not allow species-specific resistance to disturbance, but rather reflects different recoveries from disturbances, strongly determined by a species' growth rate. Disturbances as persisting changes in the environmental conditions and possible species-specific resistance to the disturbance itself include change in temperature (e.g. to mimic global warming, Petchey *et al.* 1999; Laakso, Loytynoja & Kaitala 2003; Scholes, Warren & Beckerman 2005) and changes of the medium with respect to pH or chemical composition (e.g. Jin, Zhang & Yang 1991).

3.4 MANIPULATION OF NUTRIENT CONCENTRATION AND VISCOSITY OF THE MEDIUM

The availability of resources as well as time/energy spent collecting them is an important ecological variable. Nutrient concentration in protist microcosms is commonly manipulated (e.g. Luckinbill 1974; Li & Stevens 2010) by dilution of the medium (see Section 1.2) and/or adding sources of slow nutrient release such as seeds (e.g. autoclaved wheat or millet seeds) (e.g. Altermatt & Holyoak 2012). While for proteose peptone medium, the concentration of the proteose peptone (and additions of limiting nutrients such as iron via yeast extract) directly determines the food available to protists, manipulations of available bacteria are indirect via the concentration of nutrients available to the bacteria.

To manipulate the speed of movement/dispersal, the viscosity of the medium can be increased. The viscosity can be manipulated by adding methyl-cellulose (e.g. Luckinbill 1973) or Ficoll (GE Healthcare company, affects viscosity independent of temperature) (Beveridge, Petchey & Humphries 2010b). A higher viscosity directly decreases the speed of protists, which can be advantageous for direct microscopy (see Section 2.1) but also to manipulate community dynamics, for example by influencing the movement behaviour of predators and prey. This can for example stabilize ecological dynamics via its influence on the functional response (e.g. Luckinbill 1973).

3.5 MANIPULATION OF SPATIAL STRUCTURE OF THE LANDSCAPE

The importance of spatial structure for population dynamics has been appreciated since the very beginnings of ecological research and became an independent area of study with the birth of biogeography. Subsequently, 'space' has been added to community ecology ('metacommunity ecology', reviewed by Leibold *et al.* 2004) and more recently to ecosystem ecology ('meta-ecosystem ecology', Loreau, Mouquet & Holt 2003). Protist microcosms are particularly well suited to test concepts in spatial ecology, as they allow the building of complex landscapes and the manipulation of relevant parameters (e.g. patch sizes, connectivity, spatio-temporal dynamics or correlations of patch characteristics; for an overview of examples, see figures in Appendix S1, Section 3.5) with a very high degree of replication compared to semi-natural or natural systems (e.g. Legrand *et al.* 2012). Particularly, the entire dispersal process (emigration, transition, immigration) can be manipulated independently.

There are two basic types of dispersal used, namely passive dispersal (patches are physically not connected and part of the population/community is pipetted from one patch to another patch, see for example Warren 1996; Altermatt, Schreiber & Holyoak 2011b; Carrara *et al.* 2012) and active dispersal (patches physically connected through tubing, and protist swim actively between patches, see for example Holyoak & Lawler 1996a; Cadotte 2006, 2007; Fellous *et al.* 2012a). Passive dispersal allows a much higher control of dispersal timing, direction and rate, but possibly disrupts trade-offs, for example between colonization and competition (Cadotte 2007), and neglects that dispersers are often not a random fraction of the population. The choice of dispersal method may also depend on the linking to theoretical models, which may either assume discrete or continuous phases of growth and dispersal, subsequently simplifying the comparison, parameterization and/or fitting of models with experimental data.

The choice of possible landscape structures is large and includes single patch systems of varying sizes, simple two-patch landscapes, linear, star-like or dendritic landscapes (see also Holyoak & Lawler 1996a; Cadotte 2006; Schtickzelle *et al.* 2009; Altermatt, Schreiber & Holyoak 2011b; Carrara *et al.* 2012; Fellous *et al.* 2012a; Pennekamp *et al.* 2014b). While most work has been done on landscapes that are discrete (e.g. have discrete patches surrounded by non-habitat matrix or connected by small corridors), continuous landscapes of complex structure have been developed (Giometto *et al.* 2014; Seymour & Altermatt 2014).

3.6 MANIPULATION OF TEMPERATURE

Temperature (Fig. 1 [7]) is said to be the second most important (after body size) determinant of biological rates such as respiration, photosynthesis, mortality, resource uptake and predation (Brown *et al.* 2004) and one of the key drivers of ecosystem change. Understanding the ecological consequences of temperature variation is therefore of high priority. Experimental manipulation of microcosm temperature is relatively straightforward, by placing them in incubators, water baths, or other controlled temperature environments (CTEs). A central technical challenge is to provide sufficient numbers of independent CTEs so that pseudoreplication is avoided or can be accounted for statistically, for example using a mixed effect model. Other important considerations are as follows: what range of temperatures to use; whether to include temporally changing temperatures; how quickly temperatures should change (and that this can be realized in the liquid in the microcosms); and random or blocked positioning of microcosms within CTEs and minimizing the time during which microcosms are removed from the CTEs (e.g. for sampling). Protist microcosm studies addressing the biological role of temperature looked at its effects on individual metabolic rate (e.g. Fenchel & Finlay 1983) or movement speed (e.g. Beveridge, Petchey & Humphries 2010b), on population and community dynamics (e.g. Fussmann *et al.* 2014), an on affecting ecosystem processes, such as net primary production (Petchey *et al.* 1999; Fig. 1).

3.7 MANIPULATION OF THE BIOTIC ENVIRONMENT

The composition and dynamics of the biotic environment are not only studied as response variables (e.g. number of species, abundances), but are also often manipulated to study the consequences of the biotic environment on ecological dynamics (e.g. productivity, stability of the system). Probably the most common manipulation refers to diversity and identity of species used (such as comparing dynamics in single-species communities vs. multiple-species communities, e.g. McGrady-Steed, Harris & Morin 1997). Further aspects that can be manipulated are the trophic structure of communities (e.g. Lawler & Morin 1993), assembly history (e.g. Fukami & Morin 2003) or invasion dynamics (e.g. Mächler & Altermatt 2012). It is not our goal to describe all possible biotic manipulations, as they are directly derived from the ecological question of interest and standardization may not be directly possible or wanted. In the supplementary protocol, we are giving an overview of examples with respect to different biotic manipulations.

3.8 EXPERIMENTAL EVOLUTION AND SELECTION EXPERIMENTS

It is nowadays generally accepted that evolutionary dynamics are often co-occurring and interacting with ecological dynamics (Fig. 1). Experimental evolution and selection experiments in microcosms are a unique opportunity to study these processes in real-time with sufficient replication. Protists are well suited due to their short generation times and high population densities (see Section 1.1). Furthermore, they can be preserved over long time periods (Section 1.6 and 1.7), and genetic techniques (Section 2.7) including genomics (Section 2.5) allow relating phenotypic evolution to its genetic basis. Kawecki *et al.* (2012) give a good overview on the prerequisite and conductance of experimental evolution and selection experiments. Examples for the use of protists in experimental evolution and selection experiments comprise early selection experiments on *r*- and *K*-strategies in *Paramecium* (Luckinbill 1979), the evolution of body size and growth rates in response to predation using *Colpoda* in *Sarracenia* pitcher plants (TerHorst 2010), the evolution of virulence using *Paramecium* and its bacterial parasite *Holospora* (Magalon *et al.* 2010) and dispersal evolution during range expansions with *Tetrahymena* (Fronhofer & Altermatt, Submitted). Note that exactly because of their suitability for evolutionary experiments, protists can unintentionally undergo evolutionary changes during experiments that have been designed to analyse purely ecological questions, which may impact the observed patterns and interpretations (see Hiltunen *et al.* 2014).

Discussion

Ecology and evolutionary biology aim at understanding patterns and processes resulting from interactions among individuals, organisms and their environment. Thereby, the greatest challenge is to identify, understand and causally link processes between the different levels of organization by which an ecosystem can be described (individuals to ecosystem, Fig. 1). A comprehensive understanding becomes increasingly important as species abundances, species diversity and the stability of natural populations, communities and ecosystems are threatened due to, for example, anthropogenic effects. In particular, knowledge and understanding of responses to environmental changes can help us predicting such responses in future environments. However, gaining such insights in natural and semi-natural systems can be challenging, as multiple ecological and evolutionary processes are acting and interacting at different rates and at different spatiotemporal scales (Fig. 1).

Protist microcosm experiments have proven to be a suitable model system for a wide range of questions in ecology and evolutionary biology (Fig. 2, Lawler 1998; Petchey *et al.* 2002; Holyoak & Lawler 2005) (for an extensive list of studies and their historic context, see also references in Appendix S1 1.1). Thereby, experiments are providing a link between theory and more complex natural systems, as questions motivated by natural ecosystems can be addressed in simplified but highly controlled and replicated experiments, which are often designed and performed in close analogy to mathematical models (Fig. 3, see also Jessup *et al.* 2004; Benton *et al.* 2007). This has led to the development of a variety of experimental techniques, but with little overlap between disciplines (for example, the common use of protists in cell biology and molecular biology only minimally been integrated into ecology, see Asai & Forney 2000). In ecology, laboratory methods and techniques, for example introduced by Gause (1934), have been modified and improved in a manifold but often unstandardized way (for an overview of diversity of studies, see references in Appendix S1 Section 1.1) and protocols are rarely made available in sufficient detail to be fully reproducible.

The lack of a consistent use of generally available protocols hinders the comparison of results between different studies, limits meta-analyses as well as replication and repetition of experiments. Furthermore, the lack of standardization and availability of methods and protocols also constrains the use and dissemination of novel methods to their full potential. In the recent past, technology and methodology advanced rapidly and opened up possibilities to conduct and analyse experiments that have not been possible before. This is

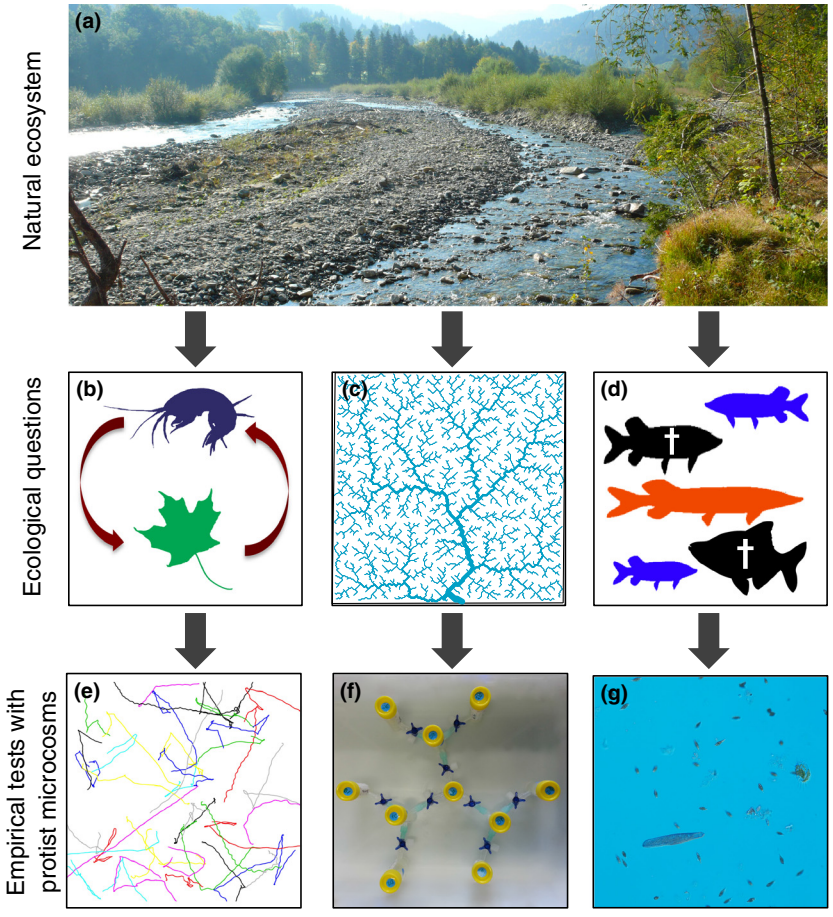

Fig. 3. Protist microcosm experiments are used to address questions in ecology and evolutionary biology derived from natural systems. For example, in a complex natural ecosystem such a river ecosystem (a), questions of interest are how interactions of species with other species or the environment affect behaviour or ecosystem processes (b), how spatial connectivity affects diversity (c) or how to predict the occurrence and sequence of extinctions (d). The questions are usually not system specific and often based on fundamental theoretical concepts. Protist microcosm systems allow to individually address these questions in simplified but highly replicated experiments, often in close analogy to mathematical models. For example, one can study the effects of resource availability on movement behaviour (e, movement paths of *Tetrahymena*), manipulate the connectivity of local communities by connecting patches with corridors made of tubing (f) or screen whole communities for the presence of species and eventual extinctions (g).

especially true for methods that allow integrating traits, behaviour and physiology of single cells/individuals into general ecological questions at the population, community or ecosystem level (see Sections 2.3, 2.5, 2.6 and 2.8). Researchers working with microcosms should be aware of these improvements, enabling them to address questions within their field of research at an unprecedented precision and replication.

We here give the first comprehensive overview of methods used for protist microcosm experiments in the fields of ecology and evolutionary biology. We provide a comprehensive list of methods and protocols in an online repository that is easily accessible and updateable. Providing such an online repository allows continuous editing as well as fast and simple exchange of information. This should facilitate comparability, repeatability and meta-analyses of future protist microcosm experiments. Standardization of methods can also facilitate large-scale, distributed experiments that would not be possible to conduct in a single laboratory. Such experiments are important, since they inform about the reproducibility of experiments and hence, the generality of their results. Furthermore, standardization will propagate the application of useful methods and hopefully ignite interdisciplinary research addressing questions that may be difficult to be answered by one discipline alone. For instance, a comprehensive understanding of the genetics (Section 2.7) of specific model organisms, such as *Tetrahymena thermophila*, could link the molecular bases of adaptive processes in eco-evolutionary feedback loops. Raman microspectroscopy (RMS, Section 2.6) provides information on the physiology of a single cell and could inform about physiological responses to stressors on the level of an individual. Video analysis (Section 2.3) allows detecting behavioural changes in response to changes of an individual's (a)biotic environment. Thus, employing such a set of complimentary methods and techniques from various disciplines in concert can improve our knowledge about the complexity of the cascading and interacting causalities schematically illustrated in Fig. 1.

There are, however, also particular challenges associated with microcosm-based experimental work that require continuous development in standardized methods and techniques. First, although a large number of traits from different protists species are known, one often cannot assume that the full complexity of an organism's niche (being the fundamental driver of eco-evolutionary dynamics) is approximated by typical measures (e.g. mean size). Advancing measurement methods (see Section 2) will lead to a more detailed understanding of the species' traits and how they link to environmental variables, for example. Secondly, even though the experiments are designed to focus on one or a few processes, multiple ecological (e.g. competition and predation), evolutionary (e.g. local adaptation) and stochastic (e.g. drift) processes may be acting simultaneously, making it difficult to get a mechanistic understanding of the system. Here, combining statistical, process-based modelling and experiments (parameterization, relating parameters to patterns) will help revealing the critical

links between patterns observed in the experiments and specific ecological and evolutionary processes. Thirdly, the advantages associated with microcosms while studying multiple spatiotemporal scales also pose challenges. Processes that act on different spatiotemporal scales may, for example, be difficult to be teased apart, especially in long-term experiments on large spatial scales. An example is rapid evolution that acts on ecological time-scales, which can be a few weeks in microcosm experiments, depending on an organism's generation time.

Nevertheless, protist microcosms are ideal systems to develop more mechanistic understanding of processes in ecology and evolution. Recent work highlights the utility of microcosms in understanding the causality of ecological and evolutionary processes (e.g. Drake & Kramer 2012). A next step is to have access to the molecular mechanisms underlying these processes. Our synthesis of the well-established (e.g. Sections 2.1, 2.2, 2.10, 2.12) and recent techniques (e.g. Sections 2.3 to 2.8) available for protist microcosm experiments shows that this system is ideal to achieve such a causal understanding. Tools exist to characterize the chemical composition and the whole biological molecular content of medium and individuals (e.g. Section 2.6) with characterized phenotypes, and experimental conditions can be set with a high degree of control and repeatability (see Sections 3.1 to 3.7). Promising directions can include the exploration of stress molecules implied in the response to environmental perturbations, the determination of the biological molecules implied in interindividual or interspecies communication, or else to the determination of the molecular bases of adaptation, with the possibility of using functional genetic tools in ciliate model species (Turkewitz, Orias & Kapler 2002). We acknowledge that the study of protists in natural systems still remains challenging, and work on how to bridge protist microcosm to natural systems is a worthy direction of future research (see pioneering work by Addicott 1974). Furthermore, only few (but influential) studies used protists to study macroecological patterns, for example comparing the abundance of cosmopolitan vs. local species (Fenchel & Finlay 2004). Still, there is much potential for research beyond metacommunities.

For microcosms to further claim their role as valuable research tools in ecology and evolution (see Table 1, Beyers & Odum 1993; Jessup *et al*. 2004; Srivastava *et al*. 2004; Cadotte, Drake & Fukami 2005; Benton *et al*. 2007), researchers have to embrace the full range of experimental techniques available and should rely not only on what they already know, but rather what set of tools is most suitable to tackle their question. We believe that our synthesis of established as well as novel techniques is important and needed. Together with the detailed protocols provided in the supplement and maintained in an online repository, it may help to significantly improve standardization and quality of research employing microcosm experiments.

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Data accessibility

This paper does not use data.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Maintenance, measurement methods and manipulation methods

Table of contents of supplementary information

Supplementary information for Altermatt et al. Methods in Ecology and Evolution.
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"Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution"

Altermatt F, Fronhofer EA, Garnier A, Giometto A, Hammes F, Klecka J, Legrand D, Mächler E, Massie TM, Pennekamp F, Plebani M, Pontarp M, Schtickzelle N, Thuillier V & Petchey OL

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1.1 Species used

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1.1 Species used

Introduction

Species generally used for protist microcosm experiments cover several major domains of life and a large part of eukaryotic phylogenetic diversity (Adl *et al*. 2005; Adl *et al*. 2012). Generally, and also in the following, the term "protist" covers free-living, unicellular eukaryotes that are not purely autotrophic (Fig. S1). This mostly includes species within the Cryptophyta, Foraminifera, Alveolata, Chloroplastida and Tubulinea (incl. Amoebozoa (Adl *et al*. 2005; Adl *et al*. 2012). Very typical and commonly used representatives are species of the genera *Paramecium*, *Tetrahymena*, and *Colpidium* (all Alveolates, used in >80 studies), as well as species of the genera *Bodo*, *Colpoda*, *Euplotes* and *Spirostomum* (all used in at least 30–50 studies). These species cover different trophic levels (purely bacterivorous heterotrophs, mixotrophs and predatory heterotrophs feeding also or exclusively on other protists). Table S1 gives a comprehensive list of species that have been used in microcosm experiment studies as discussed here. Many of the methods described in the following are also not restricted to protists, but can (and have been) also applied to single-celled autotrophic species (i.e., algae) or metazoans of similar size and ecological functional (e.g., rotifers).

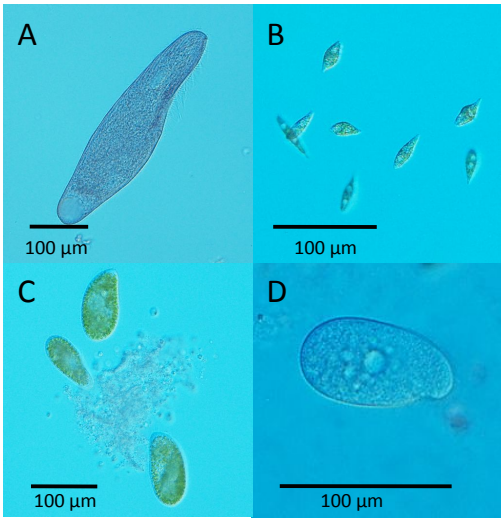

Fig. S1. Examples of different protist species used in microcosm experiments. A) *Blepharisma* sp., B) *Euglena gracilis*, C) *Paramecium bursaria*, D) *Colpidium* sp. All pictures by F. Altermatt/R. Illi.

1.1 Species used

Some of the species used can be cultivated in axenic conditions. However, most of the species thrive better when bacteria (see section 1.3) or microflagellates are present.

The selection of species is often a combination of practical reasons, such as distinctness, cultivability or availability, and the respective question of interest (e.g., functional types or size). All species can in principle be collected directly from natural populations in ponds, phytotelmata or other aquatic habitats (see detailed protocol below). This approach allows the use of co-evolved, potentially genetically diverse populations of natural co-occurring species. However, the difficulties faced during the isolation, cultivation and identification of naturally collected species often preclude this approach. Many studies have thus been based on species either already available in laboratory stocks or commonly available from culture collections. The most commonly used sources to order protist species are:

- UTEX culture collection of algae, University of Texas, Austin: http://web.biosci.utexas.edu/utex/media.aspx
- Culture Collection of Algae and Protozoa (CCAP), Scottish Marine Institute OBAN, Argyll: http://www.ccap.ac.uk/
- American Type Culture Collection, Georgetown University in Washington, DC: http://www.lgcstandards-atcc.org/en/Products/Cells_and_Microorganisms/Protozoa.aspx
- *Tetrahymena* stock center, University of Cornell, Ithaca: https://tetrahymena.vet.cornell.edu/recipes.php
- Scandinavian Culture Collection of Algae and Protozoa, Marine Biological Section, University of Copenhagen, Copenhagen: http://www.sccap.dk/
- Carolina Biological Supply Company, Burlington NC: http://www.carolina.com/
- Sciento Company, Manchester: http://www.sciento.co.uk

A difficulty/shortcoming of field collected species/strains is the often imprecise/vague identification of species. Most ecologists and evolutionary ecologists conducting protist microcosm experiments have relatively little taxonomic expertise regarding protists, and thus identifications and naming of species has to be taken with care. A set of identification manuals (Foissner & Berger 1996; Lee, Leedale & Bradbury 2000; Patterson 2003) as well as genetic barcoding techniques (Pawlowski *et al*. 2012), which are nowadays commonly available, should allow an identification at least to the genus level.

The advantage of the use of a common set of species across studies and laboratories is the availability of prior information (such as species traits, Table S2), and the possibility to link findings across studies. In this context, some species from a set of about 20 protist species originally isolated by Peter Morin from a pond at Rutgers University (McGrady-Steed, Harris & Morin 1997) have been very widely used across >50 studies, exemplifying the use of “model organisms” in ecology. The wider range of phylogeny, traits and trophic levels covered allows to select species for

1.1 Species used

specific experiments, e.g., to study predator-prey relationships (e.g., Holyoak 2000b; Vasseur & Fox 2009), compare trait-related relationships across orders of magnitude (Giometto *et al*. 2013), or to study how phylogenetic relationships are affecting competitive interactions (Violle *et al*. 2011). Importantly, it needs to be considered that A) many trait values are phenotypically plastic and can vary easily within one order of magnitude given the specific experimental conditions. B) protists often do not fall easily into well-defined categories that "higher" organisms do, and that are often used as inspiration for models and concepts to be tested with protists. For example, many protists may switch between different trophic roles, from heterotroph/mixotroph to autotroph (e.g., *Euglena gracilis*) or from autotroph to predatory (e.g., *Paramecium bursaria*). Thus, some of the classifications may be stricter than the actual behaviour/life history of the protists. C) Protists as used here cover the widest phylogenetically range possible within the Eucaryotes (Adl *et al*. 2012). Thus, comparisons that include phylogeny as an explanatory variable may be only meaningful within sub-groups (such as Alveolates, see for example Violle *et al*. 2011), as phylogenetic signals across major taxonomic groups may be mostly lost through multiple convergences.

The use of protists in ecology and evolutionary biology can be traced back to Gause (1934b; 1934a) and Dallinger (1878; 1887), who looked at ecological and evolutionary dynamics respectively. Both of them have been very much inspired by the work of Charles Darwin (1859), and are among the first experimental studies testing Darwin's ideas. In the 1950ies to 1970ies, a whole school of American Ecologists used protist experiments, and especially *Paramecium aurelia*, to address questions of species-coexistence, population dynamics and predator-prey interactions (e.g., Sonneborn 1950; Nelson 1958; Nelson & Kellermann 1965; Nelson 1967; Salt 1967; Gill 1972a; Gill 1972b; Gill & Nelson 1972; Vandermeer *et al*. 1972; Luckinbill 1973; Luckinbill 1974; Luckinbill & Fenton 1978; Luckinbill 1979; Veilleux 1979). This work was later on revived, especially by Peter Morin and colleagues (e.g., Lawler & Morin 1993; McGrady-Steed, Harris & Morin 1997; Petchey *et al*. 1999; McGrady-Steed & Morin 2000; Fox & Morin 2001; Fukami & Morin 2003; Jiang & Morin 2004; Morin & McGrady-Steed 2004; Jiang & Morin 2005; Steiner *et al*. 2006). It has been ever since used by a growing number of ecologists and evolutionary biologists (e.g., Lawler & Morin 1993; Warren 1996b; Warren 1996a; Fox & Smith 1997; Petchey *et al*. 1999; Fox, McGrady-Steed & Petchey 2000; Holyoak 2000b; Holyoak 2000a; Petchey 2000; Fukami 2001; Donahue, Holyoak & Feng 2003; Kneitel & Miller 2003; Laakso, Loytynoja & Kaitala 2003; Jiang & Kulczycki 2004; Kneitel & Chase 2004; Holyoak & Lawler 2005; Cadotte *et al*. 2006; Östman, Kneitel & Chase 2006; Cadotte 2007b; Fjerdingstad *et al*. 2007; Friman *et al*. 2008; Haddad *et al*. 2008; Jiang & Patel 2008; Davies *et al*. 2009; Schtickzelle *et al*. 2009; Worsfold, Warren & Petchey 2009; Chaine *et al*. 2010; Hammill, Petchey & Anholt 2010; Petchey, Brose & Rall 2010; TerHorst 2010; Violle, Pu & Jiang 2010; Altermatt *et al*. 2011; Altermatt, Schreiber & Holyoak 2011; Friman & Laakso 2011; Limberger & Wickham 2011; Violle *et al*.

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2011; Altermatt & Holyoak 2012; Carrara *et al*. 2012; Limberger & Wickham 2012; Mächler & Altermatt 2012; Clements *et al*. 2013a; Clements *et al*. 2013b; Giometto *et al*. 2013; Pennekamp & Schtickzelle 2013; Carrara *et al*. 2014; Clements *et al*. 2014; Fronhofer, Kropf & Altermatt 2014; Giometto *et al*. 2014; Pennekamp *et al*. 2014; Seymour & Altermatt 2014), and the types of questions addressed diversified extensively. Research areas now include the phylogenetic limiting similarity hypothesis (e.g., Violle, Pu & Jiang 2010), effects of disturbance and productivity on diversity (e.g., Haddad *et al*. 2008; Altermatt, Schreiber & Holyoak 2011), the significance of trade-offs (e.g., Cadotte 2007a; Violle, Pu & Jiang 2010), synchrony in population dynamics (e.g., Vasseur & Fox 2009), effects of environmental change on food web structure and species interactions (e.g., Petchey *et al*. 1999; Fox & Morin 2001), the study of predator-prey interactions and inducible defences (Kratina *et al*. 2009; Kratina, Hammill & Anholt 2010), the regulatory effects of biodiversity on ecosystem processes (e.g., McGrady-Steed, Harris & Morin 1997), invasion dynamics (e.g., Mächler & Altermatt 2012; Giometto *et al*. 2014), the significance of spatial dynamics on diversity and species interactions (e.g., Holyoak & Lawler 1996; Carrara *et al*. 2012), scaling laws in ecology (e.g., Fenchel 1974; Giometto *et al*. 2013), epidemiological dynamics (e.g., Fellous *et al*. 2012) and evolutionary and eco-evolutionary dynamics (e.g., Dallinger 1887; Schtickzelle *et al*. 2009; Hiltunen *et al*. 2014).

Table S1. List of species used in protist microcosm experiments (alphabetically sorted from higher to lower taxonomic levels). The name of each species as well as its higher and lower taxonomic classification (after Adl et al. 2012) is given. For each species, we give one or few representative references of studies that have been using it. SAR is a clade including the groups Stramenopiles, Alveolata, and Rhizaria.

Species name	**Higher taxonomic group**	**Lower taxonomic group**	**Reference examples**
Adercotryma glomerata	SAR	Foraminifera	Gross 2000
Allogromia sp.	SAR	Foraminifera	Gross 2000
Ammonia beccarii	SAR	Foraminifera	Gross 2000
Ammoscalaria pseudospiralis	SAR	Foraminifera	Gross 2000
Amoeba proteus	Amoebozoa	Tubulinea	Davies et al. 2009, Holt et al. 2002, Holyoak 2000, Lawler & Morin 1993, Livingston et al. 2013, Naeem & Li 1998
Amoeba radiosa	Amoebozoa	Tubulinea	Östman et al. 2006, Fox et al 2000, Krumins et al, 2006
Amphicoryna scalaris	SAR	Foraminifera	Gross 2000
Ankistrodesmus falcatus	Archaeplastida	Chloroplastida	Jin et al. 1991, McGrady-Steed et al. 1997 (genus), Davies et al. 2009 (genus), Fox et al. 2000 (genus)
Arcella vulgaris	Amoebozoa	Tubulinea	Li & Stevens 2010 Oikos, Li & Stevens 2010 CommEcol
Askenasia sp.	SAR	Alveolata	Lawler 1993, McGrady-Steed & Morin 1996
Aspidisca sp.	SAR	Alveolata	Fox et al 2000, Kneitel & Perrault 2006, McGrady-Steed & Morin 2000, Warren et al. 2003
Asterionella formosa	SAR	Stramenopiles	Fox 2004, Robinson & Edgemon 1998 (genus)
Atractomorpha echinata	Archaeplastida	Chloroplastida	Livingston et al. 2013
Bigenerina nodosaria	SAR	Foraminifera	Gross 2000
Blepharisma americanum	SAR	Alveolata	Fox & Morin 2001, Holyoak 2000, Krumins et

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			al. 2006, Livingston et al. 2013, Olito & Fukami 2009
Blepharisma japonicum	SAR	Alveolata	Clements et al. 2013 JAnim Ecol, Holt et al. 2004, Law et al. 2000, Spencer & Warren 1996 Oecologia, Weatherby et al 1998
Bodo designis	Excavata	Discoba	Fitter & Hillebrand 2009, Burkey 1997 (genus), Cochran-Strafira & von Ende 1998 (genus), Scarff & Bradley 2002 (genus)
Bodo saltans	Excavata	Discoba	Giometto et al 2013, Jürgens & Sala 2000, Kneitel & Perrault 2006, Östman et al. 2006
Boldia erythrosiphon	Archaeplastida	Rhodophyceae	Livingston et al. 2013
Bulimina marginata	SAR	Foraminifera	Gross 2000
Campylomonas reflexa	Cryptophyta	Cryptophyta	Livingston et al. 2013
Cassidulina leavigata	SAR	Foraminifera	Gross 2000
Chilomonas paramecium	Cryptophyta	Cryptophyta	Balciunas & Lawler 1995, Burkey 1997, Holt et al. 2004, Naeem & Li 1998, Warren & Gaston 1997, Scholes et al. 2005
Chilomonas spp.	Cryptophyta	Cryptophyta	Giometto et al. 2013 PNAS, McGrady-Steed et al. 1997, Davies et al. 2009, Fox et al. 2000, Robinson & Dickerson 1987
Chlamydomonas microsphaera	Archaeplastida	Chloroplastida	Jin et al. 1991
Chlamydomonas moewusii	Archaeplastida	Chloroplastida	Dickerson & Robinson 1985, Dickerson & Robinson 1986
Chlamydomonas noctigama	Archaeplastida	Chloroplastida	Rboinson & Edgemon 1988
Chlamydomonas reinhadrtii	Archaeplastida	Chloroplastida	Fox & Olson 2000, Fox 2004, Livingston et al. 2013, Naeem & Li 1998
Chlamydomonas terricola	Archaeplastida	Chloroplastida	Filip et al. 2012
Chlorella autotrophica	Archaeplastida	Chloroplastida	Hiltunen et al. 2013, Fox 2008 (genus), Hulot et al. 2001 (genus), Kurihara 1978 (genus), Li & Stevens 2012 (genus)
Chlorella pyrenoidosa	Archaeplastida	Chloroplastida	Jin et al. 1991
Chlorella vulgaris	Archaeplastida	Chloroplastida	Fox 2004, Mueller et al. 2012, Nakajima et al. 2009
Chlorogonium euchlorum	Archaeplastida	Chloroplastida	Giometto et al. 2013
Chlorokybus atmophyticus	Archaeplastida	Chloroplastida	Livingston et al. 2013
Chloromonas clathrata	Archaeplastida	Chloroplastida	Livingston et al. 2013
Chroomonas pochmanii	Cryptophyta	Cryptophyta	Livingston et al. 2013
Chrysopsis sp.	Archaeplastida	Glaucophyta	Krumins et al. 2006
Cibicidoides flordanus	SAR	Foraminifera	Gross 2000
Closterium acerosum	Archaeplastida	Chloroplastida	Livingston et al. 2013, Robinson & Edgemon 1988 (genus)
Closterium libellula	Archaeplastida	Chloroplastida	Li & Stevens 2010 Oikos, Li & Stevens 2010 CommEcol
Colacium vesiculosum	Excavata	Discoba	Livingston et al. 2013, Cadotte et al. 2006 (genus),
Coleps hirtus	SAR	Alveolata	Have 1993, Cadotte et al. 2006 (genus), Fukami 2004 (genus), Mata et al. 2013 (genus)
Collodictyon triciliatum	Collodictyonidae	Collodictyonidae	Petchey 2000
Colpidium campylum	SAR	Alveolata	Have 1990, Luckinbill & Fenton 1978, Östman et al. 2006
Colpidium cf. striatum	SAR	Alveolata	Balciunas & Lawler 1995, Holyoak & Lawler 1996 Ecology
Colpidium colpidium	SAR	Alveolata	Scholes et al. 2005
Colpidium colpoda	SAR	Alveolata	Have 1993
Colpidium kleini	SAR	Alveolata	Jiang & Patel 1993, Livingston et al. 2013, Violle et al. 2010
Colpidium striatum	SAR	Alveolata	Cadotte & Fukami 2005, Fox & Barreto 2006, Jiang & Morin 2005, Leary & Petchy 2009, Warren & Weatherby 2006
Colpoda cucullus	SAR	Alveolata	Bretthauer 1980, Fukami 2004, Jiang & Morin 2005, Krumins et al. 2006
Colpoda inflata	SAR	Alveolata	Cadotte & Fukami 2005, Krumins et al. 2006, Steiner 2005
Condylostoma sp.	SAR	Alveolata	Warren 1996 Oikos
Cosmarium sportella	Archaeplastida	Chloroplastida	Li & Stevens 2010 Oikos, Li & Stevens 2012, Robinson & Edgemon 1988 (genus)
Cryptomonas curvata	Cryptophyta	Cryptophyta	Giometto et al. 2013, Filip et al. 2012 (genus)
Cryptomonas erosa	Cryptophyta	Cryptophyta	Livingston et al. 2013

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Cryptomonas ovata	Cryptophyta	Cryptophyta	Bretthauer 1980, Elstad 1986, Fox 2004, Östman et al. 2006
Crytolophosis sp.	SAR	Alveolata	Fukami 2001
Cyclidium glaucoma	SAR	Alveolata	Giometto et al. 2013, Fox 2007, Davies et al. 2009 (genus), Kneitel & Perrault 2006 (genus)
Cyclotella sp.	SAR	Stramenopiles	Krumins et al. 2006
Dexiostoma campylum	SAR	Alveolata	Giometto et al. 2013, Riblett et al. 2003
Dictyosphaerium planctonicum	Archaeplastida	Chloroplastida	Dickerson & Robinson 1985, Dickerson & Edgemon 1988
Didinium nasutum	SAR	Alveolata	Veilleux 1979, Holyoak & Sachdev 1998, Luckinbill 1979, Warren 1996 Oikos
Dileptus anser	SAR	Alveolata	Davies et al. 2009, Petchey 2000
Dileptus monilatus	SAR	Alveolata	Jiang et al. 2011, Livingston et al. 2013
Dinobryon cylindricum	Archaeplastida	Chloroplastida	Dickerson & Robinson 1986, Robinson & Edgemon 1988
Entosiphon sulcatum	Excavata	Discoba	Fitter & Hillebrand 2009, Holt et al. 2004 (genus), Scholes et al. 2005 (genus), Warren & Weatherby 2006 (genus)
Eremosphaera viridis	Archaeplastida	Chloroplastida	Robinson & Edgemon 1988
Eudorina elegans	Archaeplastida	Chloroplastida	Dickerson & Robinson 1985
Euglena gracilis	Excavata	Discoba	Altermatt & Holyoak 2012, Davies et al. 2009, Dickerson & Robinson 1985, Kawambata et al. 1995
Euglena mutabilis	Excavata	Discoba	Giometto et al. 2013
Euplotes aediculatus	SAR	Alveolata	Altwegg et al. 2004, Carrara et al. 2012, Jiang & Morin 2005, Kratina et al. 2007
Euplotes affinis	SAR	Alveolata	Bretthauer 1980
Euplotes cf. eurystomus	SAR	Alveolata	Mata et al. 2013
Euplotes daidaleos	SAR	Alveolata	Filip et al. 2012
Euplotes eurystomus	SAR	Alveolata	Li & Stevens 2010 Oikos, Li & Stevens 2012, Naeem & Li 1998
Euplotes octocarinatus	SAR	Alveolata	Altwegg et al. 2004
Euplotes patella	SAR	Alveolata	Balciunas & Lawler 1995, Fox et al. 2013, Holyoak & Sachdev 1998, Spencer & Warren 1996 Oecologia
Euplotes plumipes	SAR	Alveolata	Altwegg et al. 2004
Euplotes surystomus	SAR	Alveolata	Li & Stevens 2010 CommEcol
Fragilaria capucina	SAR	Stramenopiles	Filip et al. 2012, Fitter & Hillebrand 2009
Frontonia angusta	SAR	Alveolata	Filip et al. 2012, McGrady-Steed et al. 1997 (genus), Fox et al. 2000 (genus), Have 1993 (genus)
Gavelinopsis praegeri	SAR	Foraminifera	Gross 2000
Gavelinopsis translucens	SAR	Foraminifera	Gross 2000
Glaucoma myriophylli	SAR	Alveolata	Bretthauer 1980
Glaucoma scintillans	SAR	Alveolata	Have 1990, Livingston er al. 2013, Violle et al. 2011
Glaucoma sp.	SAR	Alveolata	Jiang & Patel 2008
Globobulimina affinis	SAR	Foraminifera	Gross 2000
Globocassidulina subglobosa	SAR	Foraminifera	Gross 2000
Gonium pectorale	Archaeplastida	Chloroplastida	Dickerson & Robinson 1985, Dickerson & Robinson 1986
Haematococcus lacustris	Archaeplastida	Chloroplastida	Fox 2004, Livingston et al. 2013
Halteria grandinella	SAR	Alveolata	Have 1993, Jiang et al. 2009, Livingston et al. 2013, Violle et al. 2010,
Heliozoa sp.	SAR	Chromista	McGrady-Steed et al. 1997 (genus), Davies et al. 2009, Fox et al. 2000
Keronopsis sp.	SAR	Alveolata	Have 1993
Lacrymaria olor	SAR	Alveolata	Have 1993, Jiang et al. 2009, Cadotte & Fukami 2005 (genus)
Lenticulina cultrata	SAR	Foraminifera	Gross 2000
Leptopharynx sp.	SAR	Alveolata	Fukami 2001
Litonotus sp.	SAR	Alveolata	Östman et al. 2006
Loxocephalus simplex	SAR	Alveolata	Have 1990, Clements et al. 2013 JAnimEcol (genus), Jiang & Morin 2005 (genus), Steiner 2005 (genus)
Loxophyllum helus	SAR	Alveolata	Have 1993

1.1 Species used

Mallomonas caudata	SAR	Stramenopiles	Robinson & Edgemon 1988
Mayorella sp.	Amoebozoa	Tubulinea	Kneitel & Perrault 2006 (genus)
Micrasterias rotata	Archaeplastida	Chloroplastida	Li & Stevens 2010 CommEcol
Monas sp.	SAR	Chromista	Bretthauer 1980
Nassula sorex	SAR	Alveolata	Filip et al. 2012
Navicula pelliculosa	Archaeplastida	Stramenopiles	Filip et al. 2012, Limberger & Wickham 2011 PLoSOne, Limberger & Wickham 2010
Netrium sp.	Archaeplastida	Chloroplastida	Fox et al. 2000, McGrady-Steed et al. 1997, McGrady-Steed & Morin 2000
Nitzschia sp.	Archaeplastida	Chloroplastida	Fitter & Hildebrand 2009, Jin et al. 1991, Robinson & Edgemon 1988
Nonion commune	SAR	Foraminifera	Gross 2000
Ochromonas danica	Archaeplastida	Stramenopiles	Dickerson & Robinson 1985, Dickerson & Robinson 1986, Robinson & Edgemon 1988
Ochromonas sociabilis	Archaeplastida	Stramenopiles	Bretthauer 1980
Onychodromopsis flexilis	SAR	Alveolata	Limberger & Wickham 2011 Oecologia, Limberger & Wickham 2012
Oocystis apiculata	Archaeplastida	Chloroplastida	Robinson & Edgemon 1988
Ophiocytium maius	SAR	Stramenopiles	Robinson & Edgemon 1988
Oxyrrhis marina	SAR	Alveolata	Hiltunen et al. 2013
Oxytricha sp.	SAR	Alveolata	Fox et al. 2000, Krumins et al. 2006
Pandorina morum	Archaeplastida	Chloroplastida	Li & Stevens 2010 CommEcol, Robinson & Edgemon 1988
Paradileptus sp.	SAR	Alveolata	Have 1993
Paramecium aurelia	SAR	Alveolata	DeLong & Vasseur 2012, Fox 2008, Luckinbill 1973, Petchey 2000
Paramecium bursaria	SAR	Alveolata	Altermatt et al. 2011, Cadotte 2006 Ecol, Vandermeer 1969, Violle et al 2011 EcoLet
Paramecium caudatum	SAR	Alveolata	Fellous et al. 2012 PLoSOne, Duncan et al. 2011, Fels et al. 2008, Lunn et al. 2013
Paramecium multimicronucleatum	SAR	Alveolata	Dickerson & Robinson 1986, Naeem & Li 1998, Robinson & Dickerson 1987
Paramecium primaurelia	SAR	Alveolata	Luckinbill & Fenton 1978, Luckinbill 1979 AmNat
Paramecium tetraurelia	SAR	Alveolata	Cohen et al. 1998, Gonzales & Holt 2002, Jiang & Kulcycki 2004, Long & Karel 2002
Paramecium trichium	SAR	Alveolata	Östman et al. 2006
Pediastrum sp.	Archaeplastida	Chloroplastida	Livingston et al. 2013, Robinson & Edgemon 1988
Pelomyxa carolinensis	Amoebozoa	Archamoebae	Naeem & Li 1998
Peranema trichophorum	Excavata	Discoba	Spencer & Warren 1996 Oikos
Peridinium cinctum f. ovoplanum	SAR	Alveolata	Dickerson & Robinson 1985, Fox 2008 (genus), Robinson & Edgemon 1988 (genus)
Petalomonas sp.	Excavata	Discoba	Spencer & Warren 1996 Oikos
Phacus sp.	Excavata	Discoba	Fox et al. 2000
Planorbulina mediterranensis	SAR	Foraminifera	Gross 2000
Platydorina sp.	Archaeplastida	Chloroplastida	Dickerson & Robinson 1985
Pleodorina californica	Archaeplastida	Chloroplastida	Livingston et al. 2013
Polytomella sp.	Archaeplastida	Chloroplastida	Kneitel & Perrault 2006
Poterioochromonas malhamensis	SAR	Stramenopiles	Kadowaki et al. 2012, Saleem et al. 2012 (genus), Saleem et al. 2013 (genus)
Poterioochromonas stipitata	SAR	Stramenopiles	Östman et al. 2006
Pseudocyrtolophosis alpestris	SAR	Alveolata	terHorst 2010 AmNat, terHorst 2010 JEB
Pyrgo murrhina	SAR	Rhizaria	Gross 2000
Quuinequeloculina lamarckiana	SAR	Foraminifera	Gross 2000
Rhynchomonas nasuta	Excavata	Discoba	Fitter & Hillebrand 2009
Rosalina cf. bardyi	SAR	Rhizaria	Gross 2000
Rubrioxytricha ferruginea	SAR	Alveolata	Limberger & Wickham 2011 Oecologia
Saccammina sp.	SAR	Foraminifera	Gross 2000
Scenedesmus gladiosum	Archaeplastida	Chloroplastida	Livingston et al. 2013
Scenedesmus obliquus	Archaeplastida	Chloroplastida	Jin et al. 1991
Scenedesmus opoliensis	Archaeplastida	Chloroplastida	Li & Stevens 2010 ComEcol, Li & Stevens 2010 Oikos, Li & Stevens 2012

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Scenedesmus quadricauda	Archaeplastida	Chloroplastida	Dickerson & Robinson 1985, Dickerson & Robinson 1986, Robinson & Edgemon 1988
Selenastrum capricornutum	Archaeplastida	Chloroplastida	Jin et al. 1991
Sellaphora pupula	SAR	Stramenopiles	Livingston et al. 2013
Spathidium sp.	SAR	Alveolata	Fukami 2001, McGrady-Steed & Morin 1996
Sphaerocystis schroeteri	Archaeplastida	Chloroplastida	Robinson & Edgemon 1988
Spirogyra occidentalis	Archaeplastida	Chloroplastida	Livingston et al. 2013, Robinson & Edgemon 1988 (genus)
Spiroplectinella wrightii	SAR	Foraminifera	Gross 2000
Spirostomum ambiguum	SAR	Alveolata	Have 1990, Kratina et al. 2007, Krumins et al. 2006, Naeem & Li 1998, Spencer & Warreb 1996 Oikos
Spirostomum teres	SAR	Alveolata	Holt et al. 2004, Violle et al. 2011, Warren & Gaston 1997
Spumella sp.	Archaeplastida	Chloroplastida	Jürgens & Sala 2000, Riblett et al. 2008
Staurastrum gladiosum	Archaeplastida	Chloroplastida	Livingston et al. 2013, McGrady-Steed et al.1997 (genus), Davies et al. 2009 (genus), Fox 2008 (genus)
Staurastrum pingue	Archaeplastida	Chloroplastida	Robinson & Edgemon 1988
Steinia sp.	SAR	Alveolata	Lawler 1993
Stentor coeruleus	SAR	Alveolata	Bretthauer 1980, Cadotte & Fukami 2005, Jiang & Morin 2005, Spencer & Warren 1996 Oecologia
Stentor polymorphus	SAR	Alveolata	Have1993, Östman et al. 2006
Stephanodiscus sp.	SAR	Stramenopiles	Robinson & Edgemon 1988
Stichococcus sp.	Archaeplastida	Chloroplastida	Scraff & Bradley 2002
Stigeoclonium sp.	Archaeplastida	Chloroplastida	Jin et al. 1991
Stylonychia mytilus	SAR	Alveolata	Bretthauer 1980, Fox et al. 2000 (genus), McGrady-Steed et al. 1997 (genus), Filip et al. 2009 (genus)
Stylonychia pustulata	SAR	Alveolata	Limberger & Wickham 2012 Oecologia, Limberger & Wickham 2011 PLoSOne
Suctoria sp.	SAR	Alveolata	Fukami 2001
Synedra sp.	SAR	Stramenopiles	Robinson & Edgemon 1988
Synura sp.	SAR	Stramenopiles	Robinson & Edgemon 1988
Tachysoma pellionellum	SAR	Alveolata	Östman et al. 2006, Limberger & Wickham 2012 PLoSOne, McGrady-Steed & Morin 1996 (genus)
Tetrahymena pyriformis	SAR	Alveolata	Amezuca & Holyoak 2000, Glaser 1988, Olito & Fukami 2009, Vasseur & Fox 2009
Tetrahymena thermophila	SAR	Alveolata	Fjerdingstad et al. 2008, Fryxell et al. 2005, Chaine et al. 2009, Laakso et al. 2003, Nakajima et al. 2009
Tetrahymena vorax	SAR	Alveolata	Fox 2008, Holyoak & Sachdev 1998, Jiang & Patel 2008, Livingston et al. 2013
Textularia porrecta	SAR	Rhizaria	Gross 2000
Tillina magna	SAR	Alveolata	Scholes et al. 2005, Warren & Weatherby 2006, Holt et al. 2004 (genus), McGrady-Steed & Morin 1996 (genus)
Trachelomonas grandis	Excavata	Discoba	Dickerson & Robinson 1985, Dickerson & Robinson 1986
Trachelomonas hispida	Excavata	Discoba	Robinson & Edgemon 1988, Robinson & Dickerson 1987 (genus)
Trochammina shannoni	SAR	Rhizaria	Gross 2000
Urocentrum turbo	SAR	Alveolata	Have 1993
Uroleptus sp.	SAR	Alveolata	Kneitel & Perrault 2006
Uronema sp.	Archaeplastida	Chloroplastida	Cadotte 2006, Kuppardt et al. 2010, Lawler 1993, Livingston et al. 2013
Urostyla grandis	SAR	Alveolata	Limberger & Wickham 2012 PLoSOne, Fox et al. 2000 (genus), Lawler 1993 (genus)
Uvigerina mediterranea	SAR	Foraminifera	Gross 2000
Vischeria helvetica	SAR	Stramenopiles	Livingston et al. 2013
Volvox aureus	Archaeplastida	Chloroplastida	Li & Stevens 2012 Oikos, Robinson & Edgemon 1988
Volvox carteri	Archaeplastida	Chloroplastida	Li & Stevens 2010 CommEcol, Li & Stevens 2010 Oikos
Volvox rousseletti	Archaeplastida	Chloroplastida	Livingston et al. 2013
Vorticella campanula	SAR	Alveolata	Ollason 1977, Fox 2008 (genus), Fukami 2001

1.1 Species used

			(genus), Kneitel & Perrault 2006 (genus)
Vorticella convallaria	SAR	Alveolata	Ollason 1977
Vorticella microstoma	SAR	Alveolata	Östman et al. 2006
Vorticella similis	SAR	Alveolata	Spencer & Warren 1996 Oikos
Zygnema circumcarinatum	Archaeplastida	Chloroplastida	Livingston et al. 2013

Table S2. Overview on traits of some of the most commonly used species. The trait measurements for individual species may depend on the specific experimental conditions (e.g., temperature and nutrient levels affecting both growth rates as well as size). This table, however, is mostly aiming at showing overall patterns in traits and exemplifying the range of trait values (often over orders of magnitudes). The original source of the trait value is given for each trait. Size gives the diameter. If not indicated differently, trait values on size, growth rate and carrying capacity are from Carrara et al. 2012, and velocity is from Altermatt et al. 2012. When possible, mean and ±standard deviations of trait values are given.

Species name	**Size (*μ*m)**	**Growth rate r (1/d)**	**Carrying capacity K (Ind/ml)**	**Velocity (*μ*m/s)**	**Trophic status**
Blepharisma sp.	471.3 ± 57.1	0.67 ± 0.07	59.5 ± 4.7		predator
Chilomonas sp.	23.3 ± 3.7	0.98 ± 0.13	1572.4 ± 278.3	168.1	heterotroph
Colpidium sp.	81 ± 7.8	1.5 ± 0.08	1379.2 ± 76.6	470.2	heterotroph
Euglena gracilis	36.7 ± 6.4*	0.87*	84578*	69.1	mixotroph
Euplotes aediculatus	85.4 ± 8.6*	0.43*	359*	591.9	mixotroph
Paramecium aurelia	111.6 ± 15.1	0.86 ± 0.02	111.1 ± 2.6	1280.8	heterotroph
Paramecium bursaria	101.3 ± 12.9	0.23	1639	1090.2	mixotroph
Spirostomum sp.	843.8 ± 149.7	0.57 ± 0.15	13.6 ± 4.2	418.2	heterotroph
Tetrahymena cf. *pyriformis*	26.7 ± 4.8	2.24 ± 0.15	2996.8 ± 196.1	148.8	heterotroph

* data from Haddad et al. 2008

Materials

Equipment

For the isolation of protists the following equipment is needed:

- Stereomicroscope (see section 2.2) and general apparatus for cultivation (section 1.4).
- Sterile petri dishes.
- Sterile capillary glass-pipettes (glass Pasteur pipettes with latex bulbs).

Reagents

- Autoclaved and bacterized culture medium (see section 1.2).
- 80% Ethanol for sterilizing surfaces and equipment.

Procedure

The following procedure is for isolating species from natural communities or from species purchased from culture collections that are not pure:

1.1 Species used

1. Collect a water sample (100–200 mL) from the natural source of interest (pond, tree hole, pitcher plant etc.).
2. Bring the sample as quickly as possible to the laboratory, avoid warming of the sample (store and transport it in a cooler box at 10°C) and avoid strong exposition to sunlight.
3. Take a subsample of about 5 mL into a petri dish, dilute with 10 mL of the chosen culture medium, in order acclimate the species to the new osmotic conditions and to dilute densities of the protists.
4. Separately place five 0.5 mL drops of the culture medium in a petri dish.
5. Using the stereomicroscope, collect from the natural community sample (step 3) one individual of the focal species with a glass capillary pipette with as little water as possible.
6. Place this isolated individual into the first of the separate drops (step 4).
7. Take a new sterile pipette and isolate the focal individual from the drop and place it into the next one, again transporting it with as little medium as possible (<5% of the total drops volume).
8. Repeat at least five times, such that with each isolation step, the individual and potential co-occurring other individuals are diluted and "washed", eventually isolating the focal individual from all other cells.
9. From the final drop, transport the washed individual into a culture vessel containing up to 10 mL of bacterized medium.
10. Label the vessel with the name of the species isolated (or morphospecies), source of origin (site) and date.
11. Allow the isolated individuals to grow and reproduce (1 to 5 days)
12. Check for survival and potential contaminations. If the isolated individual survived and replicated, and no contaminations are present, the species is now present in a pure (monoclonal) culture and can be used for further experiments.
13. Add it to your long-term stock culture collection (section 1.6)

Timing: Collection of the sample >1 h, reparation all equipment: 0.5 h, isolating 1 h, growing the isolated individuals for 24 to 48 h, checking for success 0.5 h.

Troubleshooting (Tips and Tricks)

The two most common problems are: 1) the isolated species does not grow; 2) the isolation procedure was not successful and the isolated species is contaminated with other (mostly very small) protists species. It is advised to independently isolate at least 5 to 10 individuals, to ensure a higher success. Sometimes, isolated species grow better when they are initially placed in relatively little medium (1 mL, use microwell-plates), and only later on be transferred into more medium volume when the populations have reached a few dozen cells. Some species may not be cultivable within the chosen medium or the chosen medium concentration/laboratory conditions. Try different media (section 1.2) and different laboratory conditions, staying as close

to the natural environmental conditions as possible. When using bacterized medium, ensure that the bacteria concentrations are not so high that anoxic conditions occur. Using 10-fold diluted medium may solve this.

Often, the isolation process is not 100% perfect, and other species (bacteria and mostly protists smaller than <10 μm, such as "microflagellates"), are inadvertently isolated together with the focal species. To remove bacteria, the use of antibiotics is needed (see axenic cultures in section 1.2), while to remove microflagellates, steps 4 to 8 need to be repeated for another 5 to 10 times.

It is important to switch to new, sterilized pipettes for each serial dilution/washing step. However, the same pipette may be used multiple times to independently isolate several individuals/species in parallel. That is, use one pipette for each serial step, but the same pipette can be used multiple times for parallel isolations at the same step.

During the isolation process, individuals may die or get lost (e.g., get stuck to the glass of the pipette), thus to isolate one new species, it is generally necessary to go through the whole isolation process multiple times with independent individuals. The above-described procedure can also be used to create monoclonal populations of already established and well-running laboratory cultures, which may have accumulated genetic diversity by mutations over time.

Anticipated results

The goal is to have a well-growing culture of the isolated species, which can then be added to the stock culture collection (section 1.3) and for which species traits etc. can be measured. It is important to remember that a culture isolated from one single cell is initially a monoclonal population, and may only accumulate genetic diversity over time by mutations. An initially potentially higher genetic diversity can be achieved by isolating several individuals a time. However, it is then not known if this includes different cryptic species or different cells that are genetically identical as they originated from the same mother cell in the natural environment already.

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"Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution"

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1.2 Culture medium

Introduction

All experimental protist-microcosm studies keep protists in a freshwater-based medium containing nutrients and sometimes bacteria. The composition of the medium (e.g., nutrient content, pH, presence/absence of bacteria) has far-reaching consequences on dynamics, performance, and evolution of protist populations. Comparability across studies in terms of species traits, population and community dynamics and general cultivability thus strongly depends on the use of common media types. Generally, stock cultures are kept in an optimal medium, which prevents local extinctions and facilitates the maintenance of species. During experiments, media composition might be adjusted to mimic specific conditions, such as low nutrients, shared or partitioned set of resources among species, or viscosity to modify movement behaviour of protists (Luckinbill 1973; Haddad *et al*. 2008; Altermatt & Holyoak 2012), and are described in detail under section 3.4.

There is a large number of culture media for protists in the wider sense. Extensive summaries and manuals for making media are commonly available (e.g., Cassidy-Hanley 2012), especially at web-pages of culture collections, and it is not our goal to cover all of these media types, but rather identify the most commonly used. Useful websites summarizing a wider range of media recipes include:

- UTEX culture collection of algae, University of Texas, Austin: http://web.biosci.utexas.edu/utex/media.aspx
- *Tetrahymena* stock center, University of Cornell, Ithaca: https://tetrahymena.vet.cornell.edu/recipes.php
- Culture Collection of Algae and Protozoa (CCAP), Scottish Marine Institute OBAN, Argyll: http://www.ccap.ac.uk/media/pdfrecipes.htm

Generally, the water used for the medium is either deionized water, in which micro- and macronutrients are added to reach a reasonable osmolarity, or tap water or commercial well water. Deionized water has the advantage that the chemical composition of the final medium is well-known and highly reproducible. However, this approach is generally more laborious, and often less-defined media made of tap-water are used. Local tap-water should only be used when it is of constant quality and

not chlorinated. Before use, the tap water can be aged (to gas-out any chlorine). Nutrients and carbon-sources are added to the water.
All media are autoclaved at 121 °C prior to the use. Autoclaving for 20 minutes is recommended for a volume of 2L, larger volumes may take longer. Before use, the medium must cool down to the temperature used in the experiment (usually around 20 °C) and bacteria may be added as food source (see section 1.3).

We describe five different and commonly used media: Bristol medium, Chalkley's solution, Proteose peptone medium, Protozoan pellet medium, and wheat/hay (= wheat/lettuce) medium (Fig. 1). The former two are based on deionized water to which anorganic nutrients are added. These two media cannot be used per se for keeping protists, but need an additional carbon source. However, these two media are generally recommended to be used as a replacement of tap or well-water, in which the concentrations of inorganic nutrients is either not known or not standardized. The latter three medium types are common and simple approaches of media in which organic nutrients are added as a carbon source. Protists feed either directly on this carbon source, or indirectly through feeding on bacteria that grow in the medium. The use of bacteria, as well as the making of axenic or monoxenic media is described in section 1.3. The viscosity of the medium can be changed (e.g., for behavioural studies), by adding methyl-cellulose (e.g., Luckinbill 1973) (see section 3.4).

All media can be prepared by persons with basic laboratory skills (including technician and graduate students), and can be learnt within a few hours of instructions. Precaution needs to be taken during the handling of hot media (after autoclaving; only people that have been specifically instructed to the use of the autoclave at hand should use it) and during the handling of chemicals. Wearing lab coats and protective glasses is advised.

Fig. S1. Autoclaved bottle with protozoa pellet medium ready to use. Note the black stripes on the autoclave tape indicating that it was autoclaved, and also giving date and initials of when and by whom the medium was made. The sediments at the bottom are remains of dissolved protozoa pellets, and are generally discarded.

1.2 Culture medium

Materials

Equipment

For the making of all media the following equipment is needed:

- Autoclave to sterilize the medium as well as beakers etc. used to handle the final medium.
- Microbalance with a precision of at least 0.01 g to weigh the chemicals used for the different media.
- Graduated beakers to measure different volumes of liquid. We recommend a set of graduated beakers with the following maximum volume: 10 mL, 20 mL, 100 mL, 500 mL, 1000 mL and 2000 mL.
- Micropipettes to handle solutions in the range of 0.1 to 10 mL.
- Containers/flasks to make, autoclave and temporarily store the medium. We recommend using containers with a volumetric content about 50% larger than the actual medium volume to be made in order to avoid spilling during autoclaving. For making 1 L of medium, 1.5 L Erlenmeyer glass beakers have been proven highly suitable (or for 2 L medium, 3 L Erlenmeyer glass beakers).
- Aluminium foil to cover the lid of the medium container and maintain it sterile after autoclaving.
- Spatula to handle chemicals.
- Labelling tape and pen to label the medium bottle.
- All glassware and tools used in the making of the medium should be rinsed with deionised ultrapure (or equivalent) water to ensure that no soap or acide residue remains on the surface of the glassware after it has been washed.

Reagents

All media are made of either deionized or well water, and chemicals and nutrients that are added either as solutions or solid particles. For media in which different stock solutions are prepared, we give the components of the stock solutions and concentrations therein, for all other media we only list the reagents needed.

Bristol medium

- Deionized water (dH_2O)
- Stock solutions described in Table S1

Table S1. Stock solutions needed for Bristol medium.

Component	Amount	Concentration stock solution
NaNO3	10 mL/L	10 g/400mL dH2O
CaCl2*2H2O	10 mL/L	1 g/400mL dH2O
MgSO4*7H2O	10 mL/L	3 g/400mL dH2O
K2HPO4	10 mL/L	3 g/400mL dH2O
KH2PO4	10 mL/L	7 g/400mL dH2O
NaCl	10 mL/L	1 g/400mL dH2O

1.2 Culture medium

Chalkley's solution

- Deionized water (dH_2O)
- Stock solutions described in Table S2

Table S2. Stock solutions needed for Chalkley's medium,

Component	Amount	Concentration stock solution
NaCl	5 mL/L	2 g/100mL dH2O
KCl	5 mL/L	0.08 g/100mL dH2O
CaCl2	5 mL/L	0.12 g/100mL dH2O

Proteose peptone medium:

- Bristol medium
- proteose peptone, e.g. from BD Diagnostic Systems No.: 211684 or BD Diagnostic Systems No.: 212750. Available through retailers like Fisher Scientific.
- $FeCl_3$ Solution at a concentration of 270 mg $FeCl_3 \cdot 6H_20$ per 10 ml (10 μM $FeCl_3$)
- Facultativly: 0.2% yeast extract (e.g., Becton Dickinson or Oxoid L21).

Protozoan pellet medium:

- tap/well water or Chalkley's solution
- Protozoan Pellet (provided by Carolina™ Biological Supply Company, Burlington NC)

Wheat/hay-wheat/lettuce medium

- tap/well water or Chalkley's solution
- organic wheat seeds or dry organic hay/straw or dried/baked organic lettuce

Procedure

Bristol medium

To get 1 L of total medium, the following procedure is advised:

1. Fill about 900 mL of deionized water (dH_2O) into an autoclavable beaker with a minimum volume of 1.5 L.
2. Add each of the components of table S1 in the order specified while stirring continuously.
3. Bring total volume to 1 L by adding dH_2O.
4. Cover the beaker and autoclave the medium at 121 °C for 15–20 minutes.
5. Before use, the medium must cool down to the temperature used in the experiment (usually around 20 °C).
6. Label the medium bottle with the name of the medium type, the initials of the person who made it, and the date when it was made.

1.2 Culture medium

7. The medium can be stored at 4 °C for a few weeks, it should be discarded when contaminations with bacteria are observed (i.e., when medium gets cloudy).

Timing: Preparation of medium: 1–2 h, autoclaving 0.5 h, cooling down 12 h.

Chalkley's solution

To get 1 L of total medium, the following procedure is advised:

1. Fill about 900 mL of deionized water (dH_2O) into an autoclavable beaker with a minimum volume of 1.5 L.
2. Add 5 mL each of the stock solutions of table S2 in the order specified while stirring continuously.
3. Bring total volume to 1 L by adding dH_2O.
4. Cover the beaker and autoclave the medium at 121 °C for 15–20 minutes.
5. Before use, the medium must cool down to the temperature used in the experiment (usually around 20 °C).
6. Label the medium bottle with the name of the medium type, the initials of the person who made it, and the date when it was made.
7. The medium can be stored at 4 °C for a few weeks, it should be discarded when contaminations with bacteria are observed (i.e., when medium gets cloudy).

Timing: Preparation of medium: 1–2 h, autoclaving 0.5 h, cooling down 12 h.

Proteose peptone medium:

Proteose peptone medium is a modified Bristol's medium, and generally 1% or 2% proteose peptone medium is used. This medium is generally used for axenic cultures, and especially well-suited to grow *Tetrahymena* sp. under axenic conditions (Cassidy-Hanley 2012). 1%–2% Proteose peptone medium is rich enough to promote high cell densities. The medium must be autoclaved and not filtered for sterilization, as some particulate matter is required to induce formation of food vacuoles in *Tetrahymena* (Cassidy-Hanley 2012). Sterilized medium can be frozen in aliquots at –20 °C for storage. To get 1 L of total medium at pH ~6.8, the following procedure is advised (Asai & Forney 2000; Cassidy-Hanley 2012):

1. Fill 950 mL of ready-made Bristol medium into an autoclavable beaker with a minimum volume of 1.5 L.
2. For a 1% Proteose Peptone medium, add 10 mL proteose peptone. For a 2% Proteose Peptone medium, add 20 mL proteose peptone.
3. Add 100 μl $FeCl_3$-solution.
4. Facultative: add 0.2% yeast extract (e.g., Becton Dickinson).
5. Bring total volume to 1 L by adding Bristol medium.
6. Cover the beaker and autoclave the medium at 121 °C for 15–20 minutes.
7. Before use, the medium must cool down to the temperature used in the experiment (usually around 20 °C).

1.2 Culture medium

8. Label the medium bottle with the name of the medium type, the initials of the person who made it, and the date when it was made.
9. The medium can be stored at 4 °C for a few weeks, it should be discarded when contaminations with bacteria are observed (i.e., when medium gets cloudy).

Timing: Preparation of medium: 1–2 h, autoclaving 0.5 h, cooling down 12 h.

Protozoan pellet medium:

This medium is among the less-defined media, but very commonly used due to its simple preparation and suitability for relatively many species. This medium is generally only used for bacterized cultures. It can be used for a very wide range of protozoa cultures. For long-term or stock cultures, heterotrophic cultures can additionally receive two autoclaved wheat seed per 100 ml medium. The content of the Protozoan pellet medium (and Protozoan pellets themselves) is not very well defined. Protozoan pellets are supposedly made of dried, compressed organic material (alfalfa). The chemical composition with respect to nutrients of Protozoan Pellet medium is described in table S3. To get 1 L of total medium, the following procedure is advised:

1. Fill 1 L of deionized tap water or ready-made Chalkley's medium into an autoclavable beaker with a minimum volume of 1.5 L.
2. Add 0.44 g/L^{-1} ground up Protozoan pellets.
3. Cover the beaker and autoclave the medium at 121 °C for 15–20 minutes.
4. Before use, the medium must cool down to the temperature used in the experiment (usually around 20 °C).
5. Label the medium bottle with the name of the medium type, the initials of the person who made it, and the date when it was made.
6. The medium can be stored at 4 °C for a few weeks, it should be discarded when contaminations with bacteria are observed (i.e., when medium gets cloudy).

Timing: Preparation of medium: 1–2 h, autoclaving 0.5 h, cooling down 12 h.

Table S3. Physio-chemical description of Protozoan Pellet medium made with local, nutrient-poor well-water. Mean and standard deviation (sd) values of 4 replicates are given.

Component	**Value (mean±sd)**
DOC (mg C/L)	259.6±7.4
TOC (mg C/L)	407±6
DN (mg N/L)	24.9±0.2
TN (mg N/L)	33.7±0.4
Chloride (mg/L)	72.4±0.4
Nitrate (mg N/L)	10.8±0.1
Sulfate (mg/L)	101.2±0.1
Conductivity (μS/cm 20 °C)	1424±3.5
pH	34.4±0.1
Alcalinity (mmol/L)	10.8±0

1.2 Culture medium

Total hardnes (mmol/L)	6.9±0
Silicic Acid (mg/L)	137.4±1.6
o-P (μg P/L)	225±19.8
DP (μg P/L)	1216±48.1
TP (μg P/L)	2660±58.2
Na (mg/L)	42.4±0.3
K (mg/L)	54±0.1
Ca (mg/L)	189±0.6
Mg (mg/L)	45.8±0.2
Ammonium (μg/L)	1501±29.1
Nitrite (μg N/L)	7.8±0.1
Mn (μg/L)	8.7±0.4

Wheat/hay-wheat/lettuce/Cerophyll medium

This is the least standardized type of medium, consisting of an organic nutrient source (dried plant material) suspended in water. The amount, type and origin of the plant material may vary, and includes wheat seeds (e.g., Haddad *et al*. 2008; Altermatt, Schreiber & Holyoak 2011), straw/hay, dried/baked lettuce (e.g., Sonneborn 1950; Fellous *et al*. 2012a; Fellous *et al*. 2012b) or rye leaves (Cerophyll) (Cassidy-Hanley 2012). Only use plant material grown without pesticide (i.e., from organic farming). This medium is generally only used for bacterized cultures. To get 1 L of total medium, the following procedure is advised:

1. Fill 1 L of deionized tap water or ready-made Chalkley's medium into an autoclavable beaker with a minimum volume of 1.5 L.
2. Add carbon sources, there are 3 options to add carbon sources:
   A. Add 20 wheat seeds.
   B. Alternatively: add 20 wheat seeds and 1 g of dry straw.
   C. Alternatively: add 1 g of dried/baked lettuce (dried/baked at 110 °C for multiple hours, discard dark brown/black portions).
3. Cover the beaker and autoclave the medium at 121 °C for 15–20 minutes. This step can be skipped for hay or dried lettuce, and is even common practice to revive dormant stages of protists. Wheat seeds need to be autoclaved, as they otherwise germinate in the medium.
4. Before use, the medium must cool down to the temperature used in the experiment (usually around 20 °C).
5. Label the medium bottle with the name of the medium type, the initials of the person who made it, and the date when it was made.
6. Generally, the wheat seeds or hay/lettuce particles remain in the medium/vessel.
7. The medium can be stored at 4 °C for a few weeks, it should be discarded when contaminations with bacteria are observed (i.e., when medium gets cloudy).

Timing: Preparation of medium: 1–2 h, autoclaving 0.5 h, cooling down 12 h.

1.2 Culture medium

Troubleshooting (Tips and Tricks)

In some protist microcosm studies, vitamin powder (e.g., 0.06 g/L Herpetivite powdered vitamin supplement, Research Labs, Los Gatos, California, USA) has been added to the medium to improve performance and well-being of the cultures (Donahue, Holyoak & Feng 2003; Fukami 2004). Also, in several studies soil or soil-extracts have been added to the medium (McGrady-Steed & Morin 2000; Scholes, Warren & Beckerman 2005; Altermatt *et al*. 2011). However, even when autoclaving the medium thoroughly, contaminations by microbes from this soil (from dormant and often very persistent spores) is a problem, and soil-additions are hard to standardize.

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"Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution"

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1.3 Bacteria

Introduction

Bacteria to add

For non-axenic cultures, it is advised to add a set of known bacteria as food source. Generally, this happens in a two-step process. In a first step, bacteria cultures are individually grown to carrying capacity in medium to be used in the experiment. From these stock cultures, a small inoculum is then transferred to the actual medium used in the experiment, where bacteria are allowed to grow for a short time (e.g., 12 to 24 hours), before the medium is then used to cultivate protists. We recommend individually growing an extensive volume (e.g., 1 L) of each bacterium species to carrying capacity, and then make 1 mL aliquots of inocula. These can then be frozen in glycerol, and be used across experiments for a standardized set-up of bacteria populations. While different non-pathogenic bacteria species have been successfully added and used in protist microcosm experiments, the control of the bacterial community is often not very extensive. Thus, while the experimenter usually incoulates the microcosms with a few known bacteria species, there may be other species present in the protist stock cultures or subsequentely invade the experiment. A better control of the bacterial communities in protist microcosm experiments would thus be a desired improvement for future work.

Commonly used freshwater bacteria species include *Bacillus subtilis*, *B. brevis* (=*parabrevis*), *B. cereus, Enterobacter aerogenes, Proteus vulgaris*, *Serratia fonticola,* or *S. marcescens*. Generally, two to three species are used in a mixture. Please be aware that even the non-pathogenic strains of some of these species are only allowed to be used in "Biohazard level 2" labs in some countries. It is advised to use non-pathogenic and Biohazard level 1 strains/species only.

Adding bacteria in standardized aliquots

For standardized experiments, and to allow a consistency in bacterial resources, it is advised to add the same set of bacteria to the experiments/cultures over time. Thereby, bacteria species are initially grown in isolation to high densities, then split into aliquots and stored at –80 °C. Subsequently, the same set of bacteria can be used from these stocks to start experiments with protists.

Removing bacteria

1.3 Bacteria

The advantage of axenic cultures is the higher level of standardization and reproducibility. To maintain axenic cultures, or to transform non-axenic cultures into axenic ones, the medium needs to be treated with antibiotics, and subsequently sterile techniques need to be used continuously. To remove bacteria, a combination of 250 µg/ml penicillin G, streptomycin sulfate and 1.25 µg /ml amphotericin B (Fungizone-GIBCO) is added to cultures kept in any type of media. If this is not successful, the addition of 2 µl/ml Normocin™ (InvivoGen) has been reported to successfully eliminate bacteria (Asai & Forney 2000). Axenic cultures are often used for single species experiments (especially *Tetrahymena* sp.) (e.g., Asai & Forney 2000; Fjerdingstad *et al*. 2007; Pennekamp & Schtickzelle 2013), while almost all experiments containing multiple species of protists are done under non-axenic conditions (e.g., Petchey *et al*. 1999; Haddad *et al*. 2008; Altermatt, Schreiber & Holyoak 2011). Importantly, an often diverse but undocumented diversity of bacteria and "microflagellates" may persist in non-axenic conditions. It is not uncommon to notice that many species often thrive much better under non-axenic cultures, and that it is much more difficult to maintain these species under axenic conditions.

Materials

Equipment

For the handling of bacteria (addition or removal to protist cultures), the following equipment is needed:

- Sterile working bench.
- Bunsen burner (or other flame source)
- Spatula or wire loop to transfer bacteria.
- Micropipettes to handle solutions in the range of 0.1 to 10 mL.
- Sterile beakers and jars.
- Aluminium foil to cover the lid of the medium container and maintain it sterile after autoclaving.
- Labelling tape and pen to label cultures.
- Stock cultures of the respective bacteria species (includes *Bacillus subtilis*, *B. brevis* (=*parabrevis*), *B. cereus, Enterobacter aerogenes, Proteus vulgaris*, *Serratia fonticola,* or *S. marcescens*), ordered at bacteria stock centres.

Reagents

- Protist culture medium (see section 1.2).
- Penicillin G.
- Streptomycin sulfate.
- Amphotericin B (Fungizone-GIBCO).
- 2 µl/ml Normocin™ .
- Glycerol.

1.3 Bacteria

Procedure

Adding bacteria

Bacteria are added from high-density cultures to the respective culture medium, where they are usually allowed to grow before protists are added. In many past studies, three different bacteria species have been added, but the procedure is identical for single species. Thereby, the following procedure is advised:

1. Using a sterile workbench, add each bacteria species received from the stock centre individually to 500 mL sterile culture medium. To transfer bacteria, sterilize the tube cap and spatula used for the transfer using a Bunsen burner. Maintain sterile working conditions throughout all subsequent working steps.
2. Grow the bacteria monocultures to carrying capacity (about 2–4 days) at 20 °C.
3. Make as many 1 mL aliquots of the bacteria-culture as desired (for long-term comparisons, this is ideally hundreds of aliquots). Therefore, 1/n mL of each bacteria monoculture (with n being the total number of bacteria monocultures) are added individually to 3 mL micro test tubes (e.g., Eppendorf®).
4. Mix the bacteria culture with 50% glycerol (50% glycerol, 50% bacteria inoculum, i.e., 1 mL glycerol to 1 mL total bacteria inoculum).
5. Store at –80 °C.
6. For use in experiment, slowly defrost one mixed bacteria culture, and add to 100 mL of sterile culture medium.
7. Let the bacteria grow for 24 h.
8. Mix this bacteria culture with the respective total amount of culture medium needed for the experiment. We recommend adding 5% of this bacteria inoculum to the total medium volume.
9. Start experiment immediately.

Timing: 1–2 h for step 1. 2–4 days for step 2 (culture growing). 1–2 h for steps 3 to 5. 24 h for step 6 and 7 (growing phase).

Removing bacteria

To get axenic cultures, the following procedure is advised:

1. Add a combination of 250 µg/ml penicillin G, 250 µg/ml streptomycin sulfate and 1.25 µg /ml amphotericin B (Fungizone-GIBCO) to the focal protist culture (kept in any type of media).
2. Subsequently maintain sterile working procedures (all work done in a sterile bench and cultures only opened after sterilizing caps with a Bunsen burner), only use sterile equipment (pipette tips, jars, etc.)
3. Let the culture grow at general maintenance conditions (section 1.6) for four days.
4. Check in a subsample for the presence of bacteria with a confocal microscope at 500- to 1000-fold magnification.

1.3 Bacteria

5. Additionally, plate a subsample onto sterile agar-plates to check for the formation of bacteria colonies.
6. If there are still bacteria found in the culture, add 2 µl/ml Normocin™ (InvivoGen) to successfully eliminate bacteria (Asai & Forney 2000) and repeat steps 2 to 5.

Timing: 1–2 h for step 1. 2–4 days for steps 2 and 3 (culture growing). 1–2 h for steps 4. 24 h for step 5 (growing phase). 1 h for step 6.

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1.4 Apparatus

Introduction

A laboratory equipped with general microbiological apparatus is required for protist microcosm experiments (Fig. S1). Furthermore, general laboratory glassware is needed. Protists cultures can be maintained and handled with general laboratory equipment. Importantly, all equipment used must be inert with respect to chemical leaking into the medium (e.g., using silicon tubes or glass jars). Jars and pipettes used must be rinsed with deionized water before autoclaving/use, to get rid of detergents. For experiments, glass jars or polystyrol-multiwell plates have been proven successful. Care needs to be taken when vessels are self-made, as for example silicone glue used to seal containers mostly contains antifouling chemicals that leak into the medium and kill protists (even from silicone glue recommended for aquaria use).

Materials

Equipment

For general lab-procedures, the following equipment is needed:

- Labcoat.
- Disposable gloves.
- Labelling tape and water proof pens.
- Autoclave bags (to autoclave/dispose biohazard waste).
- 80% denaturated alcohol (to clean surfaces).
- 2% bleach (to dispose cultures).

For the general procedures involving medium preparation, experimental set-up, and analyses of basic protist microcosm experiments, the following equipment is needed:

- Microbalances (precision 0.1 mg).
- Autoclave (Fig. S2).
- Incubators (temperature range 5 to 40 °C, light controlled) (Fig. S3) or temperature controlled walk-in chamber (Fig. S4)
- pH meter.
- Stereomicroscopes with zoom and dark field illumination (i.e., dissection microscope, Fig. S5).
- Sterile bench.

1.4 Apparatus

For the handling and culturing of protists in microcosms, the following equipment is needed:

- 200 mL glass jars (e.g., Erlenmeyer jars) to grow protist cultures.
- 2 L autoclavable containers to prepare the medium.
- Measuring glass beakers.
- Micropipettes (1–10 μL, 10–100 μL, 100–1000 μL, 1–5 mL).
- Petridishes (Polystyrol).
- Small vials to take subcultures (e.g., scintillation vials, 10 or 50 mL PP tubes).

Fig. S1. An exemplary laboratory in which protist microcosm experiments can be conducted. Photo by Florian Altermatt.

Fig. S2. Autoclave used to sterilize protist medium and equipment used for protist experiments. Photo by Florian Altermatt.

1.4 Apparatus

Fig. S3. Example of an incubator with individual protist microcosms (showing the experiment by Mächler & Altermatt 2012). The incubator is temperature-controlled (20 °C) and has constant fluorescent lighting. The position of the replicates of each treatment is randomized across the incubator to avoid biases due to position in the incubator. Photo by Elvira Mächler.

Fig. S4. Temperature controlled walk-in chambers in which protist experiments can be conducted. At each shelf, homogeneous light-sources are installed (note the insulation above each light to avoid warming of the shelf above it). Photo by Florian Altermatt.

1.4 Apparatus

Fig. S5. Two working spaces equipped with zoom stereomicroscopes and cameras. Microscopes are equipped with dark field illumination. Note: for working on the microscopes, blinds of the windows would be lowered to avoid reflections and uncontrolled illumination. Next to the microscopes, a calendar is given to reserve slots for individual work-projects. Photo by Florian Altermatt.

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1.5 Laboratory practices

Introduction

Experiments with protists might or might not be carried out in sterile conditions, depending on what needs to be measured and/or kept under control; regardless, a clean and tidy laboratory can make the difference between success and failure. This can be obtained by defining clear rules about how to operate common equipment, where to find and replace materials, how to access and handle cultures of protists and bacteria safely; in this appendix we outline these "rules of etiquette", that should be notified to all the personnel with access to the laboratory and be displayed in form of checklists.

It is recommended to follow general laboratory protocols and safety rules (wearing lab-coats, cleaning benches with Ethanol before and after work, etc.). The following biosafety rules should be followed: glassware should be washed at 80 °C, and protist cultures should only be disposed after killing all protists (e.g., collecting all culture waste and autoclave it or add 2% bleach; only thereafter dispose into the waste water system).

Specific members of the personnel should be explicitly in charge for taking care of recurrent tasks, such as maintaining organism stock cultures (see section 1.6), preparing commonly used media (see section 1.2), and refurnishing the laboratory with chemicals and consumables of common use.

Materials

Equipment

For general use:

- Autoclave for sterilizing medium, pieces of equipment (glass containers, pipettes, consumables, etc.), and biohazardous waste.
- Sterile bench for dealing with axenic cultures.
- Pipettes.
- Disposable gloves.
- Some paper towels.
- Plastic bags for biohazardous waste.

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For microcosm set-up:

- Adequate volume of sterile protist medium (see section 1.2).
- Bacterial culture(s) (agar slant or plate).
- Loop for getting bacterial sample off agar.
- Flame for sterilizing.
- Adequate number of autoclaved microcosms vessels, and a few spare (e.g. jars, tubes, flasks).
- 150 ml measuring cylinder.
- Pipettes with teat, or Gilson-type pipette with tips.
- Protist cultures, checked for the presence of unwanted organisms (e.g. microflagellates), and at appropriate density.
- Fine permanent marker.
- Medium permanent marker.
- Stickers.
- Sterile wheat seeds.

Reagents

- 70% Ethanol.

Procedure

General laboratory care

1. Provide initial training to personnel.
2. Display checklists regarding general laboratory etiquette as well as instructions on how to use common instruments.
3. Keep up-to-date journals regarding when and by whom instruments are used.

Rules of etiquette for the daily routine

1. Do not allow food or beverages in the laboratory.
2. Keep the laboratory doors closed.
3. Wash hands when accessing the laboratory.
4. Wear disposable gloves and sterilize them with ethanol 70% when working in sterile/axenic conditions.
5. Clean the sterile bench with ethanol 70% before and after use; leave nothing in it aside from dedicated items.
6. Wash hands when leaving the laboratory.
7. At the end of the day:
   - tidy and clean the benches with ethanol;
   - remove, sterilize and dispose biohazardous waste;
   - ensure that adequate supplies remain, if not arrange for more.

Periodic tasks

1. Maintain a stock of commonly used media.
2. Maintain a stock of commonly used consumables.

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Microcosm setup

Setting up microcosms for bacterivore protists requires two main steps: 1) inoculating fresh, sterile medium (Protist Pellet Medium, hereafter PPM, see also section 1.2 and supplement thereof) with bacteria, and 2) adding protists to the bacterized PPM.

Step 1: adding bacteria to the sterile PPM

1. You will probably have the sterile PPM in 1 litre volumes in one or more large flasks. Working under a sterile bench, pour about 100 ml into a small autoclaved vessel.
2. Using sterile technique, take a loop of bacteria from the bacterial culture and dip and swirl it into the media in the small vessel.
3. Put the small vessel in a warm (25°C or so; not critical) place for a couple of hours, to let the bacteria grow.
4. Under the sterile bench, divide the now bacterized media in the small vessel into however many large flasks you have.
5. Put the large flasks in a warm place overnight (see TIMING).

Step 2: adding the protists to the bacterized PPM

CRITICAL STEP: all the steps specified below need to be performed in a sterile environment if it is important to avoid the presence of bacteria other than those inoculated during *step 1* (*adding bacteria to the sterile PPM*) from the microcosm vessels.

1. Clear and wipe down an appropriately large amount of desk space.
2. Put the flasks of bacterized medium at hand. If you're being very careful, and have multiple large flasks of bacterized media, mix these up, so to minimize any existing difference between flasks.
3. Pour the appropriate volume of PPM in each of the microcosm vessels (MV). This can be done in two ways:
    A. By means of a precision scale.
    i. Take one empty MV and put it on the scale.
    ii. Tare the scale so that it reports zero weight with the empty MV on it.
    iii. Pour the exact volume of PPM required, by means of a pipette.
    iv. Write down the weight shown by the scale (as distilled water has a density of 1 g/ml, the number of grams shown should be very close to the number of ml poured).
    v. For all other MVs, put them on the scale, tare the scale and pour PPM until the scale shows the same value noted at step iv. CRITICAL STEP: tare the scale for each and every MV used.
    B. Using a MV as a reference for all the others. This method is less precise but faster to execute than the one at point A.

   i. Take one MV and put in the appropriate volume of tap water (say 100 ml). Mark on the outside of the vessel the level of the liquid using a fine permanent marker. Pour away the liquid.
   ii. Use this reference MV to put similar lines on all other MVs (without removing their lids).
   iii. Pour in the bacterized PPM to the line on a MV, or better add this in two steps (first half of the large flask, then second half).

   CRITICAL STEP: at step 3, the large flask containing the bacterized PPM needs to be well swirled before each pour, otherwise the bits of PP will remain in the bottom, and be poured only into the last few MVs.

4. If needed, place the required number of wheat seeds in each MV.
5. Now randomly assign MVs to treatments and label them (with permanent marker on the MV, or on a sticker stuck to the MV).
6. Estimate the density in the source cultures of each species of protist in the experiment.
7. Put in the appropriate volume / number of each species of protist in the appropriate MVs.
8. Record the number / volume you put in, and the density of the source culture, for each species. From this you can calculate the initial population density in the MVs.
9. Put the MVs into the appropriate incubator.

Correct handling of the microcosms

1. Ensure that microcosms are out of the experimental environment for as short a time as possible.
2. You may find it useful to remove samples from multiple microcosms in the room with the incubator, and then count these wherever. This avoids lots of going back and forth, or removing multiple microcosms from incubators for prolonged periods.
3. Lids should be off for as little time as possible. Best practice is to never put a lid down. I.e., take it off, keep it in your hand, and put it back on. Don't put lids down on the bench.
4. Don't attempt to carry more than one microcosm/sample in either hand. Don't attempt to carry three or more at once.
5. If you have to move microcosms between rooms, either carry only one (you need your other hand to open doors), or move them on a trolley or a tray.
6. During an experiment, ensure that the volume of medium in each microcosm is correct. This may mean topping up, perhaps during any removal and replacement of media that may be occurring. The top-up can be done with fresh medium to deal with medium removal, or with sterile, deionized water to deal with evaporation.

1.5 Laboratory practices

Troubleshooting (Tips and Tricks)

Put one person in charge of dealing with emergencies such as power failures, instrument faults and equipment breakdown; keep the contact details of repair technicians at hand.

When setting up microcosms:

- *How to avoid errors adding the correct species to the correct MV?* Add one species at a time. Separate on the desk all the MVs that require this species, then double check this, even triple checking is worth it, since this is critical. Add the species to these MVs. Do this for each species separately.
- *Adding prey and predator protist species?* Add the prey as described, wait a day or two, then add the predator, to allow for time for prey to increase in density somewhat.
- *Adding species from a mixed stock culture?* You might need, for example, to add a predator without putting in the prey from the stock culture. You need to use a micropipette to count out individual predators. It really helps to have a stock culture where the predators are as numerous as possible, and the prey as rare as possible; this can be obtained by simply giving time to the predator to reduce the prey density before collecting it.

Anticipated results

A laboratory running smoothly; microcosms accurately set up.

Supplementary information for Altermatt et al. Methods in Ecology and Evolution.
DOI: 10.1111/2041-210X.12312

"Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution"

Altermatt F, Fronhofer EA, Garnier A, Giometto A, Hammes F, Klecka J, Legrand D, Mächler E, Massie TM, Pennekamp F, Plebani M, Pontarp M, Schtickzelle N, Thuillier V & Petchey OL

1.6 Long-term maintenance of stock cultures

Introduction

Stock cultures of microorganisms kept in a laboratory provide the organisms required for conducting experiments (Fig. S1). As such, the stock cultures are extremely important resources, and should be maintained in a manner that ensures their long-term persistence.

The most important method to ensure long-term persistence is regular sub-culturing, whereby a number of individuals are transferred from an existing stock culture into a new microcosm containing abundant resources for the species being sub-cultured. Frequency of sub-culturing needs to be tailored to the dynamics of each species; usually every two to four weeks is sufficient.

Long-term access to protist strains is not usually achieved by freezing (in contrast to bacteria for example, but see section 1.7), though some species are maintained long-term as cysts, and revived when required. This method is not described below.

Fig. S1. Stock cultures in 250 ml glass jars with aluminium caps. Each row is a single species, with the most recent subculture in the front, and later ones towards the back. Photo by Owen Petchey.

Materials

Equipment

- Sterile culture vessels.
- Labels and a pen.

1.6 Long-term maintenance of stock cultures

- A sterile pipette (or pipette tip and pipette).

Reagents

- Fresh culture medium, containing appropriate high abundance resources for the species being sub-cultured.

Procedure

1. Remove the most recent stock culture from the incubator.
2. Estimate the density of the most recent stock culture, from which individuals will be taken to start the new stock culture.
3. Calculate the volume of stock culture containing at least 100 individuals.
4. Prepare the new stock culture in an appropriate vessel.
5. Label the new stock culture appropriately, (e.g., species name, source of species, culture media, person responsible, date).
6. Swirl the source stock, withdraw the appropriate volume of culture, and squirt it into the new stock culture.
7. Replace all stock cultures in the incubator. Discard the oldest stock culture, if necessary. Make sure that at least the second most recent stock culture (i.e. the one used as a source for the fresh one) is kept as a backup.
8. Enter data about the transfer in the stock culture lab book / records.

Timing

Appropriate media (at least a two day process) and sterile culture vessels should be prepared in advance.
Steps 1-6 above require approximately half a day for 20 stock cultures.

Troubleshooting (Tips and Tricks)

The following practices can help avoid unexpected or avoidable loss of stock cultures.

- Redundancy in the form of replicate stock cultures, housed in independent incubators, ideally in different locations.
- Regular monitoring of stock culture population sizes to prevent extinctions due to population fluctuations and small population sizes.
- Keeping detailed records about each of the stock cultures, including their population sizes.
- Having a single person responsible for maintenance of a set of stock culture.
- Not using stock cultures to seed experiments. Rather, make a set of separate stock cultures specifically for an experiment.
- Keeping predators on diverse prey assemblages, for longer persistence.

Anticipated results

Guaranteed long-term persistence of stock cultures.
Comprehensive and detailed records, including population sizes at subculture, of each stock culture.

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1.7 Long-term preservation

Below, we first describe the use of Lugol's solution to preserve dead protists in samples (e.g., for counting/identification, section A) and second describe the procedure to store protists alive, using cryopreservation (section B).

A) Lugol's solution

Introduction

Lugol's solution can be used to store samples of protists for several weeks or months. Some cells can be damaged during the procedure, so it is important to pay attention to the concentration of the Lugol's solution you use and not to store the samples for too long. The literature on the effect of the concentration of Lugol's solution on the proportion of cells damaged during the procedure is inconsistent, varying across a few percentage. A specific feature of Lugol's solution is that the protists are stained (they turn to red-brown color; they can be easily seen and counted under a microscope in a bright field) and they are heavy, so they sink to the bottom of the vial. Thereby, one can concentrate the sample by removing part of the liquid above them (or use an inverted microscope to count/observe them). However, Lugol's solution can slightly affect the cell size and shape due to shrinking, which can invalidate comparisons between preserved and unpreserved cells regarding these features.

Materials

Equipment

- Brown glass vials with screw tops to store Lugol's solution and samples.
- Pipettes.

Reagents

- Lugol's solution (also known as Lugol's iodine) at 5 % iodine potency. This solution can be made of 5 % (weight/volume) iodine and 10 % (weight/volume) potassium iodid (KI) mixed in deionized water, resulting in a total iodine content of 126.5 mg/mL

Procedure

The aim is to have a 0.5% concentration of Lugol's solution in the stored sample, higher concentrations lead to the loss of larger percentages of cells. Therefore, to store

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1 mL of sample, about 5 microliters of Lugol's solution have to be added to have a final concentration 0.5%.

1. Take an empty vial and add the right amount of Lugol's solution.
2. Add the sample with protists that you want to preserve. Adding the Lugol's solution to an empty vial and then adding the sample ensures that it mixes properly.
3. Close the vial and gently turn it upside down and back to mix the sample (do not shake it too much).
4. Remember that you cannot store samples in Lugol's solution indefinitely. Storage up for several weeks to a few moths is usually fine.

Important: Lugol's solution is light sensitive. Store samples in the dark, or (better) in brown glass bottles in the dark.

B) Cryopreservation

Introduction

There are several reasons why long-term storage of protist cultures using cryopreservation (or cryoconservation, i.e., storage at ultra-cold temperatures, below –130 °C), usually in liquid nitrogen (LN2), is desired (McAterr & Davis 2002; Day & Stacey 2007; Cassidy-Hanley 2012).

Firstly, cryopreserved stocks act as a renewal backup (cell banking) from which standard liquid cultures of strains with a specific interest can be recreated when needed. This is the primary *raison d'être* of protist culture collections (see section 3.1.1). Recreating cultures from a frozen stock is needed after bacteria/fungal contamination or accidental loss/extinction of the culture in the liquid medium. However, regularly reinitializing protist cultures is also necessary to prevent undesired genetic changes due to evolutionary changes during prolonged vegetative growth. For example, it is advised to restart *Tetrahymena* cultures every 6 months (Cassidy-Hanley 2012). This is necessary to prevent major genetic changes in the germinal micronucleus, transcriptionally inactive and hence under strong genetic drift. Specifically, this is needed to preserve specific mutations when the wild type has a selective advantage, causing a high risk of the mutation of interest to be lost due to random assortment of macronuclear chromosomes during asexual reproduction (Cassidy-Hanley 2012). It is however important to note that the low survival during thawing makes there is no 100% guarantee of genetic stability even with cryopreservation.

Secondly, cryopreservation of protist cultures can be a key point in some studies, for example in experimental evolution (Kawecki *et al*. 2012). Indeed, it allows taking a snapshot of a culture/strain under specific conditions and at a certain time. Such cryopreserved cultures can then be subsequently revived by thawing, to be

1.7 Long-term preservation

compared on phenotypic or genetic aspects, such as evolved versus non-evolved strains (Kawecki *et al*. 2012).

Standard protocols for the cryopreservation of protists are published (McAterr & Davis 2002; Day & Stacey 2007; Cassidy-Hanley 2012), or are readily available at webpages of culture collections (e.g., http://web.biosci.utexas.edu/utex/protocols.aspx). Freezing implies a phase of culturing the protists under specific conditions to prepare the cells and ensure the highest cell viability, the use of specific cryoprotectants, and a progressive and controlled cooling down before long-term storage in liquid nitrogen. Cryopreservation in principle works for all protists species, but we focus here mostly on *Tetrahymena* as a well-developed example. We use it to detail the material, reagents and protocols necessary to implement long-term cryopreservation in LN2 in a laboratory. We go beyond the mere description of freezing/thawing protocols by delivering information about key points for successful establishment of LN2 cryopreservation in the research laboratory, such as consequences of material choice, or the importance of a reliable inventory system.

For a given protist species, changes in the protocol will likely reside in specific points only, such as culture conditions prior to adding the cryoprotectant, or centrifugation force and duration. We advise searching the literature and the internet using species (or genus) names associated to keywords such as “cryopreservation”, “cryoconservation”, “cryogenic”, “freezing”, or “liquid nitrogen” to gather more specific information. It is important to recognize that reviving protists after cryopreservation does not always work, and may be less straightforward than with bacteria. We thus recommend testing survival rates for each specific protist species/strain and cryopreservation method before using it as a routine.

Extra general information on cryopreservation technique, safety, and material (especially recent advances in cryogenic material) can also be obtained from companies selling cryogenic equipment, such as Thermo Scientific (http://www.thermoscientific.com), Thaylor-Wharton (http://www.taylorwharton.com) or Air liquide (http://www.airliquide.com).

The preferred storage for long-term cryopreservation is in liquid nitrogen (–196 °C), because viability of frozen cells can tremendously decrease in case temperature increases above –130 °C, even for a short period of time. At –196 °C, metabolic reactions are slowed down so extensively that living cells can be maintained for very long time (potentially indefinitely). Handling liquid nitrogen needs careful training of staff and the necessary precautions.

CAUTION: *Safety note associated to use and handling of liquid nitrogen (LN2)*
It is important that staff is trained in the use of LN2 and associated equipment. Indeed, there are several safety risks associated to the use and handling of LN2 that can be important and should not be minimized, despite they can be largely controlled by enforcing clear procedures and a limited extra equipment:

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- LN2 is extremely cold (–196 °C) and immediately burns skin or eyes in case of contact. Never touch or immerge body parts into LN2, and wear adequate protection equipment (coats, full-face visor and use insulated gloves) at all times whilst handling vessels containing LN2 or manipulating cold items.
- A very important safety consideration is the potential risk of asphyxiation when escaped nitrogen vaporises and displaces atmospheric oxygen. Oxygen depletion can very rapidly cause loss of consciousness, without any sensation or prior warning because nitrogen is odourless, colourless, and tasteless. Vessels containing LN2 should be kept in well-ventilated areas in order to minimize this risk. In particular, if a pressurized LN2 vessel must be moved between levels, for example for refilling at an external LN2 source, never go in the lift with the vessel to avoid being trapped in a confined space in case of lift malfunction. Large volume LN2 vessels should be accompanied with an oxygen detector triggering an alarm in case oxygen level drops below 19%, or a mechanical ventilation installed in the room holding the LN2 vessel.
- A third risk is associated to the tremendous amount of force that can be generated if LN2 is rapidly vaporised inside any closed space such as a cryotube. The liquid-to-gas expansion ratio of nitrogen is 1:694 at 20 °C, and this will rapidly lead to explosion of sealed vials. This safety risk must be particularly controlled when cryotubes are stored in the liquid phase of LN2, because LN2 can enter the cryotube. Whereas this risk of explosion is relatively limited in the case of plastic cryotubes with screwtop closure, because accumulating pressure will lead to leaks in the seal that will relieve the pressure, dangers associated to LN2 spraying out of the tube (injury or dissemination of the cryotube content) must be taken into account. To thaw cryotubes kept in the liquid phase, a good practice is to move them in the vapour phase for 24 h, to allow any trapped LN2 to slowly evaporate; an easy way to apply this procedure in a liquid phase cryoconservator (see below) is to keep the top box of a rack above the maximal level of the liquid phase.

Materials

Equipment

We list here the standard equipment needed for successful cryopreservation of protists in LN2:

- Basic material to work with protist cultures under sterile conditions, e.g., flow hood, autoclave (see section 1.4).
- Basic material to prepare culture media (see section 1.2) and handle cultures, such as beakers, pipettes, etc.
- A centrifuge to concentrate cultures, fitted with an appropriate rotor accepting large tubes, such as 50 mL conical tubes.
- A vacuum pump to aspirate the supernatant after centrifugation.
- A water bath to heat up medium and cryosamples for fast thawing.
- A set of tweezers to safely manipulate cryotubes when they float in LN2.

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- A system allowing a controlled –1°C/min cooling rate. The best is a cooling unit that can be programmed for such a precise cooling rate. If such a device is not available, a semi-controlled alternative system, that proved very efficient, combines a –80°C freezer with special cryoboxes for cooling down the samples (e.g., isopropyl alcohol-filled Thermo Scientific Nalgene® Cryo 1 °C "Mr. Frosty", or alcohol-free Biocision® Coolcell).
- A LN2 cryoconservator, which is essentially a deeply insulated jar where LN2 is stored, creating a liquid phase down and a vapour phase up; often the limit between the two phases can be adjusted by the user to favour one or the other phase. An extensive range of sls in available, with smaller ones having capacities of 80 to 90 cryotubes placed on aluminium canes, to huge vessels with a capacity > 20,000 cryotubes placed in cryoboxes. Cryotubes can be either stored in the vapour or the liquid phase of LN2, each with advantages and disadvantages. This choice has important consequences for the selection of an appropriate cryoconservator and must not be neglected. For safety reasons, it is often recommended, especially by companies selling cryogenic equipment, to use vapour phase storage. Indeed, this limits the risks associated to LN2 entering the tubes when submerged, which may lead to cryotube explosion during thawing (see safety note above) and/or cross-contamination between samples if contaminants float in the LN2; this latter risk is extremely important when working with biologically hazardous organisms. However, storage in the vapour phase is accompanied by a trade-off limiting either cryoconservator capacity (big liquid phase & small vapour phase) or its autonomy (small liquid phase & big vapour phase), because autonomy straightly depends on the quantity of LN2 in the liquid phase. Furthermore, temperature is less stable and forms a vertical gradient in the vapour phase (from –180 °C to –140 °C), which might be critical for some protist species. Recently, a specific type (dry phase) of cryoconservator has been developed, where LN2 circulates into a closed circuit, with thermal transfer elements ensuring cryotubes are maintained at appropriate low temperature; this technology ensures cryotubes are not in direct contact with LN2, either liquid or vapour. Despite attractive in its principle, this design may have two major disadvantages for some laboratories: dry phase cryoconservators are largely more costly than liquid/vapour phase ones, and their autonomy in the absence of external LN2 refilling is usually very short (a few days only). Whatever its type, *a fortiori* for dry and vapour phase or when external supply of LN2 can be erratic, a LN2 cryoconservator should be constantly monitored and alarmed for temperature and LN2 level, because any failure in maintaining the minimum level of LN2 in the cryoconservator will lead to irremediable loss of the frozen samples. Note that electronic ultra-low (–135 °C) freezers exist, but their mechanical complexity requires an external LN2 backup in case of failure, and their temperature is high compared to LN2; so they are currently rarely used for protist cryopreservation. Regular advances in technology might

lead to changes in the perspectives expressed here in a near future, so we advise laboratory planning to acquire a cryopreservation system to enquire about the most recent available equipment and their features before choosing for a specific solution.

- An external source of LN2 for regular refilling of the cryoconservator. Depending on the local availability, the LN2 refilling could be performed manually, by pouring LN2 into the cryoconservator (but see safety note above), or manually/automatically from a pressurized source of LN2 attached to the cryoconservator. Many modern cyoconservators can indeed be fitted with automatic LN2 level monitoring systems that trigger refilling from the external source when needed (often user adjustable). Except in the rare cases where a pressurized LN2 circuit is available, this external LN2 source is a pressurized tank, which must itself be refilled either from a larger tank or directly from a truck. Local constrains about the regular delivery of LN2 must be taken into account with prime importance when choosing the cryopreservation system to ensure sufficient autonomy even in adverse conditions. A LN2 cryoconservator can often survive absence of electricity power for a prolonged time (even up to a month), but in case of shortage of LN2, there is no way to maintain the integrity of cryosamples.
- Cryoboxes and sterile plastic cryotubes. Cryotubes in the 1.2 to 2 mL volume range (e.g., Thermo Scientific Nalgene® #5000-0020 or Nunc® #340711) have been proven adequate for protist culture freezing; tubes with external thread limit the risk of contamination from handling compared to internally-threaded cryotubes. A large variety of cryotubes and cryoboxes exist; specific features of some brands and models are worth mentioning. A small cryotube size allows using cryoboxes holding 100 (10*10) or even 169 (13*13) tubes boosting the overall capacity of a cryoconservator compared to the classical 81 (9*9) cryoboxes with limited extra cost. Also, cryotubes and cryoboxes with integrated barcode can be useful for easier referencing (see inventory control system below). Be sure to use cryotubes and cryoboxes suitable for LN2 storage, as some can only be used in freezers at temperatures above –100 °C.

A reliable inventory control system, designed to organize the contents for ease of location and retrieval, is vital for efficient cryopreservation in the laboratory (as well as being important in other techniques). The key point is that small cryosamples cannot be kept out of LN2 for more than 30 s to 1 min, making hunting for a specific sample inside the cryoconservator very difficult without an external inventory system. Finding a missing sample can rapidly turn into a nightmare, with non-negligible risks for the samples and the user.

A reliable inventory control system is based on three complementary subsystems: (1) an individual tube labelling system, (2) a database recording the position of each sample together with its associated important data, and (3) a system

limiting errors, particularly preventing the possibility to deposit/move/withdraw a sample without updating its record in the database.

Such an inventory control system can in principle be developed on paper or on simple electronic supports provided extreme care is taken to label, position and record the fate (moving, thawing, etc.) of each cryosample. We, however, strongly recommend to use/develop a system specifically designed for it, combining the use of barcodes for individual error-proof cryotube labelling, and a database system allowing both to record all important information associated to cryosamples (date, content, exact position in the cryoconservator, etc.) and to ensure the integrity of the inventory.

Commercial systems exist to implement such a referencing solution from one hand to another, from barcoded tubes to specialized laboratory software for inventory database (e.g. Labcollector®, www.labcollector.com). However, it is also possible to create a customized and cheaper solution based on a general database management (e.g., Microsoft Access®, or FileMaker Pro®) or spreadsheet software, connected to a printer to create custom "wrap around" LN2 resistant labels (e.g., Brady® # 800537), and a barcode scanner. Prefer 2D barcodes (e.g., matrix) over 1D barcodes, as they are smaller and fitted with error-correction preventing reading errors.

A key point for data integrity, whatever the system, is to develop a carefully thought set of practices and rules to limit human errors as much as possible by having the system enforcing/preventing specific actions. For example, letting the database system automatically allocate an empty space (vs. user chosen) for each new cryotube and print it on a label to be affixed on the tube allows for easier and less error-prone placement of the cryotube and recording of its associated data. Similarly, enforcing every cryotube, when thawed, is recorded as such in the database ensures the current content of the cryoconservator is correctly reflected, allowing for easy sample search and inventory *in silico*. Recording freezing success (yes or no) for each cryotube, once it is known whether a culture has successfully developed after thawing, also allows to accumulate some knowledge that may be helpful to troubleshoot reasons for freezing failure.

Reagents

- Standard growing culture medium, with possible addition of suitable antibiotics to prevent contamination, whose impact can be bigger on fragile cultures freshly thawed.
- Starvation medium: 10 mM Tris (pH 7.5, adjusted by adding HCl), sterilized in the autoclave.
- DMSO (Dimethyl Sulfoxide), ACS reagent grade (e.g. Fisher #D1281 or Sigma-Aldrich #472301). DMSO must be sterilized by filtration using a 0.2 micron syringe filter which has been pre-washed with alcohol and rinsed with DMSO. CAUTION: DMSO is readily absorbed through the skin and can penetrate some rubber gloves, leading to potential introduction of harmful agents into the body.

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Procedure

Freezing usually implies a phase of culture under specific conditions to prepare the cells and ensure the highest cell viability, the use of specific cryoprotectants, and a progressive and controlled cooling down. Thawing also requires specific precautions to limit the thermic shock and ensure cells go rapidly back to normal reproduction. All solutions and material in contact with the cell cultures must be sterile.

Freezing

This protocol has been optimized for *Tetrahymena* by Nicolas Schtickzelle, Linda Dhondt (both Université catholique de Louvain, Biodiversity Research Centre, Belgium) and Michèle Huet (Station d'Ecologie Expérimentale du CNRS, Moulis, France) on the basis of the protocol described by Cassidy-Hanley (2012) but is likely a good basis for many protists. It spans a period of 13 days; optimized weekday for each step is indicated to avoid working during weekends.

The quantities given allow the preparation of 8 cryotubes per culture sample. As revival success cannot be 100% guaranteed for each thawed tube, we strongly advise against decreasing the number of cryotubes per culture sample. If more cryotubes are desired, adapt the quantities but be sure to respect the filling amount per recipient for optimal cell survival; for example to make 16 cryotubes, perform two 50 mL cultures, each in a separate 500 mL Erlenmeyer, instead of one single 100 mL culture. To avoid variation between lots, these cultures can be mixed together to get one single homogeneous culture, and then divided back (at step 3, and again at step 5). Timing information is indicative, given for one culture frozen as a set of 8 cryotubes, and does not include time needed to prepare the material and reagents.

<u>1st day (Wednesday – 0.5 h): Preculture</u>

1. Put 400 μL of stock culture with 5 mL of culture medium in a 50 mL tube.

<u>3rd day (Friday – 0.5 h): Culture</u>

2. Transfer each pre-culture in a 500 mL Erlenmeyer flask filled with 50 mL of culture medium; culture them at 30 °C to log phase (c. 500,000 cells/mL according to strain) with 150 rpm shaking. Temperature and good culture aeration are important to ensure optimal recovery.

<u>6th day (Monday – 1 h): Starvation</u>

3. Measure cell density in the culture and adjust, if necessary, to c. 500,000 cells/mL. Transfer into a 50 mL tube that can be centrifuged.
4. Centrifuge (1100 g for 3 min at room temperature) and remove the supernatant by aspiration.
5. Dissolve the pellet in 10 mL of Tris, transfer into a 500 mL Erlenmeyer flask and complete with Tris to reach a final 50 mL volume.
6. Culture them for 3 days at 30 °C with 150 rpm shaking.

<u>9th day (Thursday – 1 h): Freezing</u>

7. Label the appropriate number of cryotubes, and enter their details in the inventory system. The label on each cryotube should include the exact position where it will go in the cryoconservator.

1.7 Long-term preservation

8. Transfer the content of each Erlenmeyer into a 50 mL tube.
9. Centrifuge (1100 g for 3 min at room temperature) and remove the supernatant by aspiration, leaving 500 μL of Tris to dissolve the pellet.
10. Add carefully 2 mL of DMSO (final DMSO concentration 8%), stir gently (cells become fragile by DMSO, so avoid shocks).
11. Put immediately 300 μL in each cryotube and incubate at room temperature for 30min to allow DMSO to penetrate the cells (so-called equilibration period).
12. Cool down at –1 °C/min, overnight. Whatever the device used for this controlled cooling down, group cryotubes together according to the position they will occupy in the cryoconservator, to ease their transfer (see below).

<u>10th day (Friday – 0.5 h): Transfer in LN2</u>

13. Fill 2 expanded polystyrene boxes with a few centimetres LN2: one will receive the cryotubes out of the -80°C freezer (or cooling unit), the other will receive the cryobox extracted from the cryoconservator. This allows keeping all cryotubes (new or existing) deeply frozen during manipulation. Be sure to regularly check the LN2 level in the two boxes and refill if necessary to maintain a level allowing cryotubes to be fully submerged in LN2.
14. Rapidly move the cryotubes from the freezer to LN2, using tweezers or if possible by overturning the box in which they are and let cryotubes drop into LN2. Do not let any cryotube/cryobox outside LN2 for more than 30 seconds. Once they are in the expanded polystyrene box, soaked/floating in LN2, they are safe and you can take the necessary time to carefully select the appropriate cryotube for placement in the cryobox. No hurry means no mistake.
15. Put each cryotube in the cryobox, at the exact position indicated in the label.
16. When all tubes are placed into the cryobox, put the cryobox back into the cryoconservator, and proceed by loading remaining cryotubes into the next cryobox, until all are placed.

<u>13th day (Monday – 0.5 h): Viability check</u>

17. Take out one tube per series and thaw it (see procedure below) to check the success of the freezing procedure, i.e. a viable culture is obtained.

Thawing (0.5 h)

1. Use the inventory system to locate tubes to be thawed, and plan in which order they will be removed from the cryoconservator so as to minimize the time frozen cryosamples are out of the LN2.
2. If cryotubes are conserved in the liquid phase, move them into the vapour phase during 24 h to minimize risks of explosion (see safety note above). Use procedure with two expanded polystyrene boxes (described at step 15 of freezing protocol) if cryotubes from several cryoboxes need to be gathered and placed into a single cryobox to be stored in vapour phase, ensuring no cryotube/cryobox is left out of LN2 for more than 30 seconds.
3. Prepare all the material (pipettes, tweezers…) to ensure no delay will subsequently happen during the thawing procedure.

1.7 Long-term preservation

4. Prepare a set of 50 mL tubes (one for each cryotube to be thawed), each containing 50 mL of standard culture medium at room temperature, and label them. Antibiotics should be added to minimize potential contamination.
5. Preheat the water bath at 42 °C and place in it one or several tubes (e.g., 50 mL conical) containing an appropriate quantity of standard culture medium (1.5 mL * number of cryotubes to be thawed); be sure the top of the tube does not touch the water to avoid contamination. Once at 42 °C, take the tube out of the water bath and wipe it with an alcohol-soaked tissue prior to opening under the hood to minimize the risk of contamination.
6. Fill 2 expanded polystyrene boxes with a few centimeters LN2: one will receive the cryotubes to be thawed, the other will receive the cryobox extracted from the cryoconservator. Do not let any cryotube/cryobox outside LN2 for more than 30 seconds. Be sure to regularly check the LN2 level and refill if necessary to maintain a level allowing cryotubes to be fully submerged in LN2.
7. Take out the first cryobox from the cryoconservator, put it in one of the LN2-filled expanded polystyrene box, and extract the selected cryotube(s). Repeat, one cryobox at a time, until all cryotubes to be thawed are extracted and grouped in the other LN2-filled expanded polystyrene box.
8. Place the first cryotube into the 42 °C water bath, and shake gently for c. 30 s.
9. Take the cryotube out of the water bath and wipe it with an alcohol-soaked tissue prior to opening under the hood to minimize the risk of contamination.
10. Add 1.5 mL of culture medium from the 42 °C prewarmed tube and shake gently to ensure the pellet is fully dissolved.
11. Transfer the content of the tube into the appropriate labelled 50 mL tube containing 5 mL of culture medium, and culture at 30 °C.
12. Repeat steps 6 to 10 for each cryotube to be thawed.
13. After 24 to 48h, check the presence of live cells.
14. Update the inventory system, indicating the tube(s) that were thawed and whether thawing was successful or not.

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2.1 Sampling and counting

Introduction

Observing properties of microbial microcosms, such as organism size and population density, often cannot be carried out *in situ*, and usually cannot be performed for the entire microcosm or every individual therein. Hence, observations are virtually always made on a sample of the microcosm, and usually involves removing this sample from the microcosm (though see below for exceptions).

Important questions include what volume sample to remove, whether samples will be returned to the microcosm, when to sample, and whether to homogenise microcosms before sampling. There is no single correct answer for any of these questions, though most often microcosms are homogenized by swirling prior to sampling. How to answer these questions for one's specific situation is discussed in the troubleshooting section.

How frequently to sample depends on the goals of the experiment, and on the rates of interest. For example, at least daily sampling during exponential growth phase is desirable to get a good estimate of growth rate while less frequent sampling may be enough to get estimates on the carrying capacity.

Note that these protocols deal with sampling alone (i.e., physically withdrawing a sample). Other sections (e.g., section 2.2 Microscopy) deal with taking measurements from the samples.

Materials

Equipment

- A sterile pipette (or pipette tip and pipette).
- A vessel into which the sample will be placed. This might be for storage until a measurement is made, or directly into a measurement vessel (e.g., a Sedgewick Rafter counting chamber or a petri dish).

Reagents

- Culture media with which to replace sampled volume.

2.1 Sampling and counting

Procedure

1. If sampling is sensitive to movement of the microcosms, do not move the microcosms. If sampling is not sensitive to movement of the microcosms, or requires it, remove microcosms from their experimental environment.
2. Swirl the microcosm if the contents needs to be homogenized.
3. Prepare the pipette ready for extracting liquid (i.e. put a clean tip on) and keep this in one hand; it is important to do not put the pipette on the bench at this stage to avoid contaminations.
4. Use the other hand to move the microcosms lid aside.
5. Remove the required volume of culture, and immediately reseat the lid.
6. Do not remove the lid and place it on the bench; only move the lid aside, and keep it in hand.
7. Squirt the sampled volume into the appropriate vessel.
8. If necessary, replace the same volume of removed media with fresh media, following general good practices (cross reference to these).
9. Replace the microcosms as soon as possible in the experimental environment.
10. Make whatever measurements are required.

Figure S1. A sample being taken from a microcosm. Photo by Florian Altermatt and Owen Petchey.

Timing

Preparation of fresh media for replacement takes at least two days. Steps 1-11, with good organization and practice, take as little as one minute.

2.1 Sampling and counting

Troubleshooting (Tips and Tricks)

What volume to sample? This will depend on the population densities of the species to be sampled. Lower population densities required larger samples, higher population sizes required smaller samples. If microcosms have been homogenized before sampling, and this has distributed individuals randomly, one can assume the observed number of individuals in a sample is Poisson distributed with mean of the population density in the microcosm. Low population sizes and small samples can easily result in zero individuals counted, which should be avoided if possible, as zeros can make some analyses problematic.

Should samples be returned to the microcosm? Smaller samples generally need not be (e.g., 1 ml or less), and their equivalent volume can be replaced with fresh culture media. Larger samples can be replaced, but care should be taken not to introduce contaminants (e.g. by using disposable vials to place the sample while making measurements, or by disinfecting the non-disposable ones with ethanol followed by rinsing them with deionized water).

How often to sample? This will depend on how fast are the dynamics, and recording of any transient dynamics is required. Some experiments may require only recording of the end state, while others may require highly resolved time series. Whether to homogenise before sampling, for example by swirling, depends on whether disturbance in spatial heterogeneity are acceptable. If they are not acceptable, perhaps because the experiment concerns the consequences of small-scale spatial heterogeneity, population densities should be estimated at a fixed position in the microcosm, or at several fixed positions. There is relatively little evidence that swirling as often as every other day has large effects on population dynamics.

Anticipated results

A sample ready to take measurements from.

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2.2 Estimating abundances by eye (Microscopy)

Introduction

Protist ecology has successfully used optical microscopes for estimating protist densities and for observing cell features since its very beginning (Gause 1934; Vandermeer 1969; Luckinbill 1973). Different methods and different microscopes can be used for counting protists depending on their cell size, their population density and whether they are in monoculture or in combination with other protist species. Most protist species, having a body length in the range of 10 to 300 µm and densities of 10 to >10,000 individuals/ml, can be counted using dissecting microscopes (=stereomicroscope, see Fig. S1); these microscopes are provided with a single objective which allow to zoom up to a magnification of ~160x. Compound microscopes, capable of higher magnification (usually up to 1000x), allow the detection and the counting of very small organisms (e.g., microflagellates and small amoebae) and the observation of cells in detail (e.g., for evidence of parasitism).

Inverted microscopes are compound microscopes in which the light source is set above the stage and the objectives are below the stage. Their magnification is more limited than in normal compound microscopes (usually up to 40x objectives); on the other hand they allow the observation of deeper containers (such as Sedgewick Rafter cell counters: see below under "equipment").

Most modern microscopes have an internal light source for lighting the observed sample, and they allow different kinds of lighting. Dark-field illumination, which emphasizes cells over a dark background, is the classic method used for counting protists with dissecting microscopes; it can be obtained by either using a stage with dark field capability, or more recently a separate LED ring. Dark-field microscopy allows telling species apart even when they are present in the sample at the same time.

In principle, all microscopes can be accessorized with cameras and/or video-cameras (see section 2.3), allowing automated counting or species delimitations. However, even in this case it is commonly advised to regularly check the cultures by eye, as this can give information on the conditions of the cultures otherwise not available.

2.2 Estimating abundances by eye (Microscopy)

Fig. S1. Two working spaces equipped with high-end stereomicroscopes and cameras. Microscopes are equipped with dark field illumination. Note: for working on the microscopes, blinds of the windows would be lowered to avoid reflections and uncontrolled illumination. Next to the microscopes, a calendar is given to reserve slots for individual work-projects. Photo by Florian Altermatt.

Materials

Equipment

- Dissecting microscope with dark field illumination.
- Multiple lens (compound) optical microscope.
- Inverted optical microscope.
- Vials: many types are available. Commonly used are:
  i. *disposable Petri dishes*, used for counting protists with dissecting microscopes; keep at hand at least three times as many 5 cm diameter plastic Petri dishes as microcosms to count, in a plastic tub or box.
  ii. *microscope slides*, which allow the observation of individual cells in detail.
  iii. *Sedgewick Rafter cell counters;* they consist of a vial holding 1 ml of volume, 1 mm deep, with a reference grid with units of 1 mm x 1 mm. They are useful for characterizing the micro-plankton from field samples.
  iv. *Haemocytometers* and other counting chambers (see photograph *c*); they are provided with a reference grid with units of 1 μm x 1 μm, allowing the count of very small protists and of bacteria.
- A plastic tub to put used Petri dishes in.
- Some paper towels.
- Clicker counter.
- Container for disposing of pipette tips or Pasteur pipettes.
- About 1m of bench space.
- Tissue for cleaning microscope optics.
- Pen for writing on datasheet.

2.2 Estimating abundances by eye (Microscopy)

- Datasheet for recording information. Such a sheet should report the information as follows:

Date: ……. Sampler name: ……
Experiment: …….……..……..

Experimental Unit unique ID	Time	Species	Volume.1	Volume.2	Volume.3	Cell count	Notes

- 200 µl pipette and 1000 µl pipette (Gilson-type) with their sterile tips or balance accurate to 0.01g, sterile Pasteur pipettes with teats.

Reagents

- Ethanol 70%.
- Immersion oil.
- Protist Pellet Medium (PPM) for dilution (the diluent), in a jar (must not contain any protists).

Procedure

Counting protists with a dissecting microscope can be done using two different methods: using a Gilson-type pipette, or using a balance.

Counting protists using a Gilson-type pipette

1. Enter onto the datasheet that you are using this method. Do not switch between methods.
2. Ensure that microcosms are out of the experimental environment for as short a time as possible.
3. Get a plastic Petri dish ready to receive a sample.
4. Loosen the lid of the microcosm, so it can be removed with one hand.
5. Get a pipette with sterile tip ready in one hand.
6. Swirl the microcosm to well mix the contents.
7. Remove lid with one hand (do not put down the lid), withdraw a sample with the pipette that you have in your other hand, replace the lid. The volume of this sample is “Volume.1” on the datasheet.
8. Put the lid back onto the microcosm, and move the microcosms to a safe place. We are finished with it, and don’t want to risk knocking it over, or putting something back into it.

9. Place the withdrawn medium in small drops on a Petri dish; dispose the drops in lines and rows (see picture *a*).
10. Make sure that the counter is set to zero.
11. Look at the drops under the dissecting microscope (see photograph *b,c*). Adjust illumination and magnification as required. If the drops contain few enough individuals, count now using a clicker counter to make a running total across all the drops (you need to record only the total number of individuals in all drops, not the number in each drop). Move from one drop to the other to avoid missing any.
12. Dilute the sample if the cells are too many to be counted reliably. Use the 1000 µl pipette to put a suitable amount of diluent into the Petri dish and mix well with the sample, by squirting in and out of the pipette. Record the new volume (the sum of the original volume and the volume of diluent added) in column "Volume.2" of the datasheet. Now withdraw a fraction of the diluted volume and repeat steps 9 to 11. The new sampled volume is now "Volume.3" on the datasheet.
13. Write on the datasheet the number of cells observed (in column "Cell count"), the volumes of liquid sampled, and the time (in column "Time"). If there was no dilution, enter dashes in columns "Volume.2" and "Volume.3" (do not leave these blank).
14. Set the counter back to zero.
15. Dispose of the Petri dish or put it in a plastic tub for washing it when the counting session is over.

Counting protists using a balance

1. Enter onto the datasheet that you are using this method. Do not switch between methods.
2. Ensure that microcosms are out of the experimental environment for as short a time as possible.
3. Get a plastic Petri dish ready to receive a sample: put it onto the balance, tare the balance, put the dish back on the desk.
4. Loosen the lid of the microcosm, so it can be removed with one hand.
5. Get a sterile Pasteur pipette ready in one hand.
6. Swirl the microcosm to well mix the contents.
7. Remove lid with one hand (do not put down the lid), withdraw a sample with the pipette that you have in your other hand, replace the lid.
8. Put the lid back onto the microcosm, and move the microcosms to a safe place. We are finished with it, and do not want to risk knocking it over, or putting something back into it.
9. Distribute the sample in small drops onto a clean Petri dish.
10. Place the dish on the balance and record the weight in the "Volume.1" column.
11. Look at the drops under the dissecting microscope. Adjust illumination and magnification as required. If the drops contain few enough individuals, count

now using a clicker counter to make a running total across all the drops (you need record only the total number of individuals in all drops, not the number in each drop). Move from one drop to the other to avoid missing any.

12. If the drops contain too many individuals to count, you need to dilute. Put a suitable amount of diluent into the Petri dish, and mix well with the sample, by squirting in and out of the pipette. Put the dish onto the balance again, and record the new weight (due to the volume of diluent plus the original amount of volume) in column "Volume.2" on the datasheet.
13. Get a fresh plastic Petri dish ready to receive a sample of the diluted sample: put it onto the balance, **tare the balance**, put the dish back on the desk.
14. Mix the diluent well with the sample, by squirting in and out of the pipette. Now do steps 9, 10, 11 on this diluted liquid, except the volume in step 9 is now "Volume.3" on the datasheet.
15. Withdraw a new sample from this diluted liquid and put it in small drops onto a new Petri dish. The new sampling volume is now "Volume.3" on the datasheet.
16. Make sure on the datasheet you have written: the number of cells observed (in column "Cell count"), the volumes of liquid sampled, and the time (in column "Time"). If there was no dilution, enter dashes in columns "Volume.2" and "Volume.3" (do not leave these blank).
17. Put used Petri dishes into the plastic tub for this.

Counting protists with a Counting chamber

1. Enter onto the datasheet that you are using this method. Do not switch between methods.
2. Ensure that microcosms are out of the experimental environment for as short a time as possible.
3. Get a plastic Petri dish ready to receive a sample.
4. Loosen the lid of the microcosm, so it can be removed with one hand.
5. Get a pipette with sterile tip ready in one hand.
6. Swirl the microcosm to well mix the contents.
7. Remove lid with one hand (do not put down the lid), withdraw a sample with the pipette that you have in your other hand, replace the lid. The volume of this sample is "Volume.1" on the datasheet.
8. Put the lid back onto the microcosm, and move the microcosms to a safe place. We are finished with it, and don't want to risk knocking it over, or putting something back into it.
9. Place the medium in the counting chamber.
10. Place the counting chamber under the microscope.
11. Make sure that the counter is set to zero.
12. Count the cells in the sample using the counter; move along the grid in a regular, standardized way.
13. Record the number of cells in column "Cell count" of the datasheet.

2.2 Estimating abundances by eye (Microscopy)

14. Dilute the sample if the cells are too many to be counted reliably. Therefore, add a new sample from the microcosm of volume = Volume.1 into a Petri dish or scintillation vial. Use the 1000 µl pipette to add a suitable amount of diluent (about 5 to 20 times the volume of the sample is usually appropriate) and mix well by squirting in and out of the pipette. Record the new volume (the sum of the volume of the sample and the volume of diluent added) in column "Volume.2" of the datasheet. Now withdraw a subsample of Volume.2 and repeat steps 9 to 13. The newly sub-sampled volume is now "Volume.3" on the datasheet.
15. Write on the datasheet the number of cells observed for each species in the column "Cell count", the sample volume in column "Volume.1", and the dilution volumes in case a dilution was necessary.
16. Dispose of the counting chamber (if disposable) or wash it thoroughly before processing a new sample.

Clean up after the counting session

1. Wash the plastic Petri dishes, wiping the surface with a sponge, and giving them a quick rinse, then stacking them to dry.
2. Wipe down surfaces.
3. Tidy workspace so that others could use it.
4. Clean the eyepiece with ethanol 70% after use.
5. Cover the microscope after use.
6. Ensure adequate supplies are available for next sampling event.

Timing

You will be quite slow at first, but will speed up a lot. For a monoculture, this process should take a couple of minutes for one microcosm. Cleaning up after the sampling session could take about 15 minutes.

Troubleshooting (Tips and Tricks)

- Remember that the total magnification of a microscope is the product of the magnification offered by the objective in use and of the magnification due to the eyepiece (fixed; either 2x, 10x or, less commonly, 50x).
- Switch the microscope on, *then* dime the illumination up; dime the illumination down *before* switching the microscope off.
- *What volume to sample?* Often removing 0.5ml is a good option. However, when population sizes are low, this may result in high sampling error (e.g., zero counts when individuals are present). When population sizes are high, considerable diluent will be required (a couple of ml). With experience, you will be able to adjust the volume sampled, and the volume of diluent, to get good counts.
- *What is a good count?* You should aim at sampling a volume that allows counting a least ten individuals minimum across the whole sample, and not

more than about 15 per drop maximum. We control the maximum number of individuals by dilution. We can't always have so much control over the minimum number of individuals, since this is in large part determined by the population density in the microcosm.

- *What if we need to have accurate counts of rare species?* We then have to sample a larger volume. We could withdraw 5 ml, place it into a sterile Petri dish, count individuals in it, and put it back into the media. If we are fine with replacing 5 ml of fresh media at each sample, we do not have to worry about sterility here.
- *What if my communities contain multiple species?* Use the same method, but be ready to count some species in the undiluted sample, and others in the diluted sample. Two different dilutions may be required. It may even be useful to couple sampling of small volumes for abundant species, with larger volumes for rare species. All this adds lots of time to the processes. Sampling a community with 10 or more species can take over 15 minutes.
- You may find it useful to remove samples from multiple microcosms in the room with the incubator, and then count them elsewhere. This avoids lots of going back and forth, or removing multiple microcosms from incubators for prolonged periods.
- If no dilution was necessary, the mean cell density per ml equals to:
- If a dilution was necessary, the mean cell density per ml is estimated as follows:
  *(Cell count / Volume.3) * (Volume.1 / Volume.2)*

Anticipated results

Reliable estimates of cell densities.

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2.3 Image and video analysis

Introduction

Digital image and video analysis has successfully been used to reduce the amount of time spent by researchers at the microscope performing manual counts and enables to quantify a variety of traits simultaneously (Pennekamp & Schtickzelle 2013; Dell *et al*. 2014). The fast collection and processing of a large amount of data especially enables the use of microcosm experiments in a trait-based community ecology approach (McGill *et al*. 2006). Fig. S1 gives a general overview of the process of sampling, processing and analysing protist experiments using image and video analyses.

Examples of successful application of image and video analysis in protist microcosm experiments include measures of population dynamics in constant and fluctuating environments (Laakso, Loytynoja & Kaitala 2003; Fjerdingstad *et al*. 2007), dispersal (Pennekamp *et al*. 2014), movement (Fronhofer & Altermatt 2014; Fronhofer, Kropf & Altermatt 2014; Giometto *et al*. 2014), morphology (Fjerdingstad *et al*. 2007; Pennekamp *et al*. 2014), and behaviour (Schtickzelle *et al*. 2009; Chaine *et al*. 2010), spanning levels of organization from the individual to the community level.

Digital image analysis is especially suited when more than abundance data is to be collected (Dell *et al*. 2014), for instance morphological or behavioural data on a large number of individuals across many treatments and replicates. It is also highly convenient to scan large numbers of protist genotypes for ecologically important life history variation including traits like dispersal (Fjerdingstad *et al*. 2007; Pennekamp *et al*. 2014), which is of high relevance for the field of phenomics and was used for instance with nematodes (Yemini *et al*. 2013).

Because the experimenter will only take image and video samples from the experimental cultures, the subjective component of manual counts, which depends on the experience and skills of the experimenter, is reduced. Thus, a number of people with different degrees of experience can collect data for a single experiment without observer bias, and images/videos from different experiments can be easily compared as long as the experimental settings remained fixed.

However, these advantages come at the cost of setting up and fine-tuning such an automated system, which requires some financial investment in the appropriate hardware and time of a skilled technician or lab member to mould hardware and

2.2 Image and video analysis

software into a validated workflow. Accordingly, one-shot experiments to answer a specific question, may be still be solved faster by manual observations and measurements. In addition, the resolution of cameras collecting abundance and morphological/behavioural data simultaneously is generally too low for measurements at the sub-individual level, such as specific organelles or features such as the buccal cavity of certain protist species. However, taking images/videos at different magnifications would circumvent this problem without major modifications.

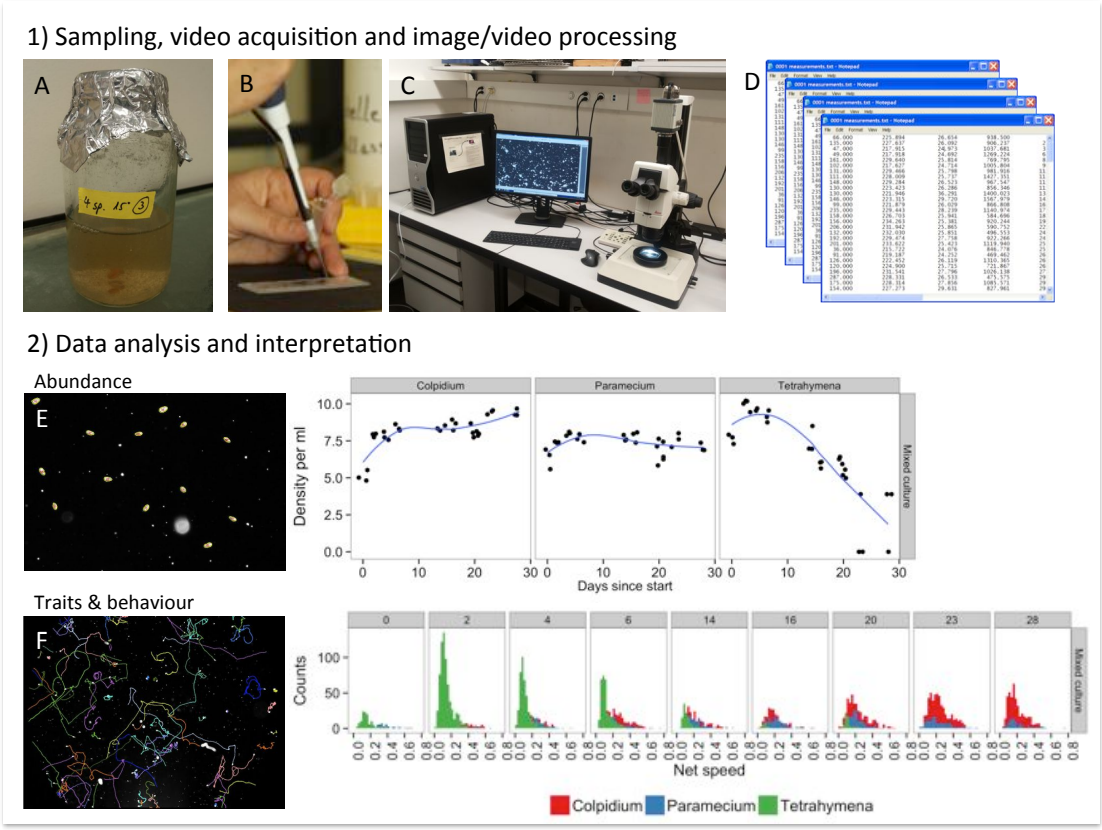

Fig. S1. Overview of the different steps in an automated image/video analysis work flow: 1) microcosms are sampled (A) and a fixed volume transferred to a counting chamber (B). The chamber is placed on the microscope stage and videos are taken via a camera coupled to the microscope, which can be controlled remotely from a computer (C). Image/video analysis software such as ImageJ is then used to process, segment and extract the information on images/videos and transformed into quantitative data in a machine-readable format (D). 2) The data stored in a database is then ready for further processing, analysis and interpretation. Two examples are shown illustrating the identification of target individuals: the abundance of protists can be estimated from a photo and up scaled to the density in the microcosm (E). (By sampling on multiple occasions through time, the population dynamics of different species are captured.) The second example is the re-construction of movement trajectories from videos (F). Using video, behavioural traits such as movement speed are captured and trait distributions in communities can be analysed.

2.3 Image and video analysis

Alternative methodologies that provide abundance and trait data simultaneously include flow cytometry (see section 2.5), particle counters (see section 2.4) and integrated systems combining flow cytometry with automated image analysis such as FlowCAM® (Fluid Imaging Technologies) (Sieracki, Sieracki & Yentsch 1998). The former can quantify morphological traits such as cell size and cell shape and be combined with fluorescence staining to yield information on DNA contents (Van Nevel *et al*. 2013), but cannot identify more complex shapes and no behavioural interactions can be measured due to its invasive nature and its measurement technique (see section 2.8 for details).

Whereas the above applications are well developed for characterizing traits and abundance of single species systems, complex communities with many species with overlapping morphologies are still a considerable challenge, but recent work on digital video analysis shows that it is feasible given sufficient differentiation in morphology and/or behaviour (especially movement behaviour, which is often a distinct feature of protist species). Work with microcosms is insofar easier that usually a limited and known number of species inhabits a community and reference data for the different morphologies and behaviours is readily available from monocultures. After individuals of each species can be reliably distinguished from videos of a complex community, the next step is the automated quantification of interspecific interactions such as predation or interference competition (Delgado *et al*. 2014). These applications however require powerful tracking algorithms that can deal with so-called occlusions, the overlapping of two cells without loosing track of the individual identities (morphological properties and species identity) (Dell *et al*. 2014). Such tracking algorithms become increasingly available (e.g., Ctrax (Branson *et al*. 2009) or idTracker (Pérez-Escudero *et al*. 2014)) but no demonstrations are so far available for protists. Another issue with video tracking is that except for some sophisticated 3D systems, most tracking is still performed in two dimensions. This is a simplification, which is likely to disappear in the coming years as both hardware and software are becoming available to do such tasks efficiently (Dell *et al*. 2014).

For an optimal use, the illumination during image/video acquisition should be fixed and optimized to yield the best contrast between the protists and the experimental arena. Measurements are taken in counting chambers or directly *in situ* in culture vessels. The resulting images/videos are then processed to separate protists (foreground) from the experimental arena (background) in a step called segmentation. Different algorithms are available whose performance depends on the properties of the protists (e.g., movement) and the nature of the surrounding medium (e.g., debris particles in the medium). After segmentation, the number and morphological properties and spatial position of all identified individuals are extracted from each image. For videos, this information is available for each frame allowing to consequently track the movement of the individuals through time using dedicated tracking software (Dell *et al*. 2014). So far, automated image/video analysis systems are mainly used in single species microcosms. Efforts to develop systems for automated sampling in marine environments and activated sludge of water treatment

facilities have shown some success (e.g., Amaral *et al*. 2004) and should be facilitated by the low species numbers in microcosms and the availability of high quality data to calibrate the classification algorithms used (e.g. artificial neural networks or random forest classification).

A variety of measurements can be taken from images and videos (see Fig. S1 for a general overview of the process of sampling, processing and analysis). Several recent publications describe in detail how to setup an image/video acquisition and processing pipeline in ecology and evolution (e.g., Kühl & Burghardt 2013; Mallard, Le Bourlot & Tully 2013; Mesquita, Amaral & Ferreira 2013; Pennekamp & Schtickzelle 2013; Dell *et al*. 2014), providing scripts for the automated image analysis (Pennekamp & Schtickzelle 2013) or plug-ins for software like ImageJ (Mallard, Le Bourlot & Tully 2013). Therefore, we here focus on giving an overview of the equipment required and point to specific difficulties and limitations of image/video analysis in microcosm systems. Furthermore, integrated systems using a combination of flow cytometry and image analysis such as FlowCAM® (Fluid Imaging Technologies) (Sieracki, Sieracki & Yentsch 1998) rely on the same principles as the respective techniques and will therefore not be covered explicitly here.

Material

Equipment

Microscope or stereomicroscope with a C-mount video adapter to connect a camera

The magnification and illumination (brightfield versus darkfield) of the experimental chamber depend on the optical equipment used and is discussed in section 2.2. Protists are generally transparent, therefore darkfield microscopy improves the contrast between them and the background of the chamber. However, protist can appear coloured due to the ingestion of pigmented food particles such as algae. Image analysis usually disregards colour, if it does not provide additional information value and images and videos in grey scale are analysed for computational efficiency. The use of fluorescence techniques usually requires specialized hardware as well. Individualized adapters for mounting cameras to microscopes are for example provided by Micro Tech Lab company, Graz, Austria (www.LMscope.com).

Digital (video) camera

The abilities of the acquisition hardware depend on the research question and range from high-resolution and frame rate cameras for detailed individual level descriptions of movement behaviour and morphology (e.g., Hamamatsu Orca Flash 4.0 sCMOS Camera), to powerful customer cameras (e.g., Canon Mark III 5D) that can be coupled to microscopes via adaptors which capture relevant variation on the individual level, but do not allow for sub-individual measurements. For some setups, high-end web cams may be sufficient and provide a better price-to-usability ratio. If colour is used for protist detection/species classification, cameras need to have colour capability.

2.3 Image and video analysis

Larger sensors usually allow capturing a larger area of the viewing field increasing the total volume sampled.

Light
Inhomogeneous illumination, shadows due to unilateral light sources and changes in light during a sequence are to be avoided, although techniques exist that can deal to a certain degree with such problems and certain segmentation approaches (difference imaging) can deal with changing light conditions. However, it is always better to avoid these nuisances in the first place by sufficiently testing the setup.

Chamber
Images/videos are acquired from samples either pipetted in re-usable counting chambers (e.g., Sedgewick-Rafter) or disposable chambers used for instance in urinary analyses, which cannot be cleaned but allow for faster image acquisition. These counting chambers usually guarantee a sufficient depth-of-field such that all individuals are in the focal plane. They do however restrict the volume sampled, which may be problematic if species are at low abundance. Sampling several samples or taking repeated samples from different areas of the counting slide would mitigate this limitation. Condensation on the walls of the disposable chambers might impede the recording of videos. To avoid such inconvenience, the use of cell culture flasks with ventilated top is recommended.

Sampling *in situ* using transparent culture vessels such as Petri dishes or cell culture flasks may be used for non-invasive sampling of the cultures, if the depth-of-field can be extended for example due to the use of diaphragms build into the microscope or customized solutions.

Software
Many software solutions for image and video analysis exist, however, ImageJ (Image Processing and Analysis in Java; developed at the National Institutes of Health) is among the most popular. ImageJ is a widely used open source solution, which is fast, user-friendly and well-supported by a user community. Many cutting edge segmentation methods are quickly adopted and plug-ins for specific tasks such as tracking are readily available. For video tracking, Dell et al. (2014) provide an overview of software ranging from commercial to open source solutions. In addition, software to automate the merging of results and following analysis is generally advised.

Computer hardware and disk space for storage
The analysis of digital images and videos can be a computationally demanding task, depending on the resolution and number of images to process, and the complexity of the image analysis task. Especially the sophisticated tracking algorithms can require considerable time to connect a large number of individuals through lengthy video sequences. Therefore, powerful computer hardware, especially the availability of large amounts of RAM (> 8 GB) and fast CPU are a requirement. In addition, large

amounts of disk space are required to store videos, which often need to be in an uncompressed format to be processed by image analysis software (e.g., ImageJ) and therefore can quickly accumulate to terabytes of storage space; for long-term storage, we recommend to compress files with lossless formats to reduce space requirements but still allow re-analysis at the original quality if needed.

Reagents

Usually video analysis does not require any reagents, however adding methylcellulose may help to slow down dynamics such they can be capture by video equipment which lacks very high frame rates (see also section 3.4).

Individual marking of protists is difficult, and sophisticated image analysis approaches to distinguish individuals by subtle differences in their appearance ("fingerprinting", Pérez-Escudero *et al.* 2014), are also difficult due to low morphological differentiation and limitation to low numbers of individuals in controlled settings. However, fluorescence marking and quantum dots (Daims & Wagner 2007; Lard *et al.* 2010) are two ways of visually separating two morphologically and behaviourally identical populations or to improve the tracking abilities under difficult settings.

Procedure

The major procedure of this protocol is setting up the image/video acquisition system (including the illumination and sampling chamber) and the automation of the image/video processing rather than the videoing itself to which the same requirements apply as to the sampling and general microscopy (see section 2.2). Other information about procedures is available in the following references.

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"Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution"

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2.4 Particle counter

Introduction

We describe how to measure protist body size with the particle counter CASY Model TT Cell Counter and Analyzer, Roche® (in the following, simply CASY). We will use 'cell size' as a synonymous of body size, even though the method can also be used to measure body size of multicellular organisms (e.g., rotifer species). The CASY measures the volume of individuals via the Electrical Current Exclusion principle (please refer to the CASY user manual for in-depth description). The CASY allows measuring the body size distribution of ensembles of individuals and was found to resolve protists with typical linear size between 2 µm and 60 µm (according to specifications, the measurement range is 0.7 µm to 160 µm), both in isolation (Giometto *et al*. 2013) and assembled in multi-species communities (Mächler & Altermatt 2012). The typical measurement time is less than 60 s and requires the sampling of a volume between 100 µL and 4 mL, depending on cell density and size. If two or more species are present in the same sample, their body size distributions can only be discerned if they are non-overlapping. The CASY can process high cells densities and allows direct measurement of the cell volume with high resolution (512,000 measuring channels).

The instrument requires sampling of a typical volume between 100 µL and 4 mL; thus, if a non-invasive / destructive measure is required, alternative methods (e.g., digital imaging) are to be preferred. The CASY also allows measuring cell densities in user-defined size windows. The presence of debris in the sample, however, might result in imprecise counts; thus, alternative techniques (see sections 2.2 and 2.5) are recommended for measuring abundances.

The main limitation in the use of the CASY to measure protist size distributions relies on the low signal to noise (debris) ratio when the protist density is too low or the sample presents many impurities. The most common alternative method to measure protist body size is optical microscopy: digital images of individuals are recorded with a stereomicroscope equipped with a digital camera, cell lengths and widths are measured and volumes are calculated using known formulas for regular solids (e.g., a prolate spheroid). However, the imaging method is limited in the number of cells that can be processed in short times and relies on the calculation of 3-dimensional volumes from 2-dimensional images.

2.4 Particle counter

The use of the CASY is suggested when measurement of a large amount of individuals is needed or when the study species presents irregular cell shape (or a cell shape that is not a solid of revolution). An additional advantage of the CASY system is the possibility of measuring body size distributions of organisms that are too small to be observed with a stereomicroscope (e.g., *Bodo saltans*). Possible applications of the CASY include the study of cell size regulation and the plasticity of body size in the presence of predator/prey species or in different environmental conditions.

Materials

Equipment

The following equipment is needed for the measurement of protist body size distributions:

- CASY Model TT Cell Counter and Analyzer.
- CASY measuring capillary tubes. Available diameter sizes: 45 µm, 60 µm, 150 µm, 200 µm (the 200 µm capillary is currently out of production). The choice of capillary is related to the size spectrum of the sample. Capillaries can get clogged if the sample contains particles larger than the diameter of the capillary.
- CASYcups. Measurement cups with lid.
- Micropipettes to handle the sample and the CASYton (see Reagents).

Reagents

- CASYton, an electrolyte used for cell suspension. The CASYton is used to dilute the sample below the maximum concentration processed by the CASY. We suggest filtering the CASYton with a 0.2 µm filter before use, to reduce debris counts. If the 45 µm capillary is used, CASYton should be de-gassed with an ultrasonic bath and a vacuum pump.
- CASYclean: solution for the weekly cleaning of CASY (see user manual).

Procedure

The following procedure is advised for the measurement of body size distributions:

1. Switch on the CASY.
2. Check that the *Waste* Container is empty and fill the *Storage* container with clean CASYton.
3. Fit the desired capillary and the external electrode on the main unit.
4. Place a CASYcup with 10 mL of CASYton on the sample platform, with both the capillary and the external electrode in the solution.
5. Setup. Select the appropriate measuring setup under *File - Setup Management* and click *Activate* or manually change the measurement and display parameters in the *Measure* and *Display* menus. Refer to the user manual to save user defined measuring setups.
6. Background measurement. Perform a measurement to check the background counts. If the total counts are too high (> 100 counts/mL for the 150 µm, 200

2.4 Particle counter

μm measuring capillaries and > 200 counts/mL for the 60 μm capillary, refer to user manual for the 45 μm capillary), perform a 3x *Clean* cycle and replace the CASYcup with one containing clean CASYton. Repeat the measurement until the background is below the threshold. Please refer to the troubleshooting section or the user manual for typical errors displayed by the CASY in this step.

7. Sample preparation. Pipette 10 mL of CASYton in a clean CASYcup. Mix the sample and pipette the desired volume of the cell suspension in the CASYcup. Close the CASYcup with the provided lid and mix gently. The aliquot of sample depends on its concentration and the measuring capillary. Typical values are 1 mL of sample diluted in 10 mL of CASYton with the 150 μm and 200 μm measuring capillaries. Always prepare the sample just before performing the measurement.
8. Measurement. Place the CASYcup containing the cell suspension on the sample platform. Perform a measurement and visualise the body size distribution on screen (see Fig. 1). If the concentration of the sample is high enough, two peaks will appear on the CASY display: the leftmost peak is due to debris in the solution and (possibly) smaller organisms (which might be resolved using a smaller measuring capillary), while the rightmost peak (or peaks, if more than one species is present) is relative to the study species. Although the instrument measures cell volume, body size is displayed on screen in terms of the *Equivalent Diameter*, that is, the diameter of a cell assuming it is spherical. Please note that the Equivalent Diameter is generally smaller than what is commonly reported as a typical linear size, such as cell length; thus, reporting the cell volume instead of the Equivalent Diameter is suggested. In the *Display - Analysis* menu, cursors can be set to compute cell density, mean body size and other information. Note, however, that these calculations do not subtract the background due to the debris.
9. Exporting data and analysis. The measurement can be exported in TXT format and imported in the desired software for statistical analysis (e.g., *R* or *Mathworks Matlab*). Subtraction of the debris peak is required when the body size distribution of the study species overlaps with the debris peak. The debris peak is typically found to be exponentially decaying in the region adjacent to the viable cells peak. An exponential fit of the debris size distribution in such region allows the extraction of the species' size distribution.
10. Fill a CASYcup with clean CASYton, place it in the sample platform and perform a *Clean* cycle.
11. To perform another measurement, repeat from step 7.

Please note that the above procedure is a typical one. Details of the measurement (e.g., number of cycles per measurement, volume per cycle, number of measurements per sample) depend on the concentration and body size distribution of the sample. Abundant organisms (e.g., density > 10^3 mL^{-1} and equivalent diameter > 10 μm with

2.4 Particle counter

measuring capillary 150 µm and 200 µm) like *Euglena gracilis* (see Fig. S1a) are ideal for use with the CASY and one measurement per sample usually suffices to measure a smooth body size distribution. Less abundant species (e.g., density < 10^3 mL^{-1} and equivalent diameter > 10 µm with measuring capillary 150 µm and 200 µm) like *Euplotes aediculatus* might require more than one measurement per sample to obtain a smooth size distribution (see Fig. S1b). When measuring samples containing multiple species, one might need to use different sized measuring capillaries to correctly resolve the whole community size distribution.

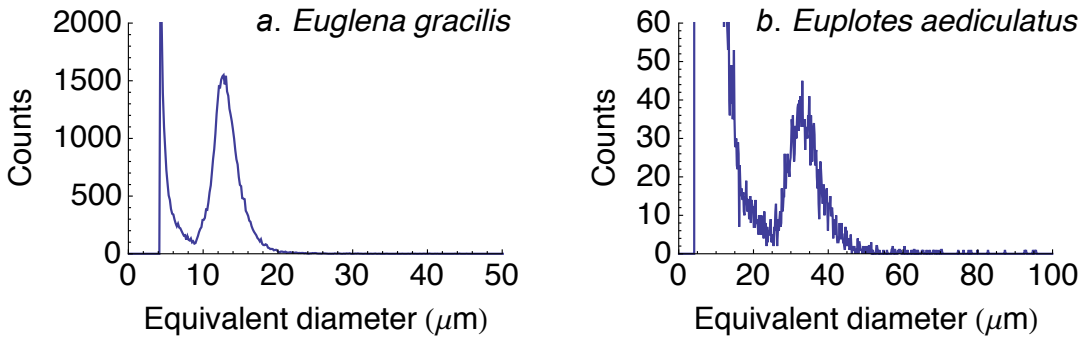

Fig. S1. Body size distributions measured with CASY: in each panel, the leftmost peak is the Debris peak and the rightmost peak is the peak relative to the study species. Straight lines join data points. *a*: Body size distribution (not normalized) of *Euglena gracilis*. Shown are the total counts in 3 measurements from the same sample with measured density 1.4 x 10^4 mL^{-1} (measuring capillary: 200 µm, cycles: 12, measurement volume: 400 mL, dilution: 5). *b*: Body size distribution (not normalized) of *Euplotes aediculatus*. Shown are the total counts in 9 measurements from the same sample with measured density 300 mL^{-1} (measuring capillary: 200 µm, cycles: 12, measurement volume: 400 mL, dilution: 5).

Timing

The typical measurement time is 15 s to 60 s per sample, depending on the measuring capillary and the number of cycles. However, the identification of the proper dilution required and the necessity to perform several measurements per sample might increase considerably the processing time. The cleaning procedure (3x Clean cycle) lasts approximately 20 s. The change of measuring capillary takes approximately 1 min. The weekly cleaning takes at least 4 h.

Troubleshooting (Tips and Tricks)

Step	Problem	Possible reason	Solution
Background measurement	Error message: *Concentration too high*	Impurities in the system	Place a CASYcup with clean CASYton on the sample platform and perform cleaning cycles until the counts are

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			low
Measurement	Error message: *Concentration too high*	The concentration of the sample is too high	Further dilute the sample or choose a larger capillary
Background measurement or *Measurement*	Error message: *The measurement time is too short*	The wrong capillary is selected in the setup	Choose the correct measuring capillary in the setup
Background measurement or *Measurement*	Error message: *Large air bubble detected*	Air bubble in the calibrated vertical tube	Remove the sample, place a CASYcup filled with clean CASYton and perform a cleaning cycle. If the error persists, perform a weekly cleaning cycle. Avoid the formation of bubbles or foam while mixing the CASYcup containing the cell suspension

Important

Do not place the CASY in the proximity of strong electromagnetic or electrostatic fields, as this can strongly interfere with the measurements and result in erroneous counts (without giving a warning message). Typical devices that can generate such fields in laboratories are fluorescent lamps and water baths.

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2.5 Measuring bacteria density: Flow cytometry

Introduction

Bacteria are the basic food resource for many protists, and commonly added in protist microcosm experiments (see section 1.3). However, in most studies bacteria have either been not or only crudely measured (e.g., using plating or optical density measurements), assuming that their dynamics are on a much faster scale than protist dynamics, and thus not limiting. This, however, may be oversimplifying (Fig. S1), especially as bacteria nowadays can be measured using flow cytometry (FCM) with at least at the same if not higher temporal resolution than protists. We thus postulate that ecologists using protist microcosm experiments should consider also measuring bacteria.

FCM has been used extensively in aquatic microbiology during the last decades, and the ongoing development of affordable and easy-to-use instrumentation has generalized the application thereof. FCM allows rapid quantification and characterization of suspended particles on single bacteria-cell level. The method is fast (<1 min per sample), and thus enables high throughput measurements. The method is highly reproducible with a typical error of below 5% on replicate measurements. Moreover, FCM analysis of a sample usually measures several thousands of individual events, thus providing a strong statistical relevance for the obtained data. A FCM measurement collects multi-variable data for each particle, including light-scatter signals and fluorescence. The former is indicative of the size of the particles, while fluorescence is used in the simplest form for distinguishing bacteria from abiotic background. This is achieved through labelling the cells with a fluorescent dye such as SYBR Green or DAPI. Such staining can also provide information on the nucleic acid content of the bacteria, often related to the cell size. Finally, an array of fluorescent dyes exist that can be used to interrogate the bacterial sample with respect to activity and viability (Hammes & Egli 2010). The value of FCM comes from the use of highly defined staining and analysis protocols (Prest *et al*. 2013), resulting in a high reproducibility.

In the context of protist experiments, flow cytometric (FCM) analysis can be used to (1) accurately quantify the density of bacteria in a microcosm or similar experimental environment, and if required, (2) estimate the average cell size of bacteria. For this purpose, a set of experimental procedures is described, based on the work of Prest et al. (2013) and SLMB (2012). In recent protist experiments, FCM has

been used to measure bacteria density (e.g., Limberger & Wickham 2011), however, we here give for the first time a detailed standardized protocol.

The described methods can be used on commercially available FCM instrumentation (discussed below). Standard laboratory safety precautions (e.g., protective clothing, gloves, etc.) are advised.

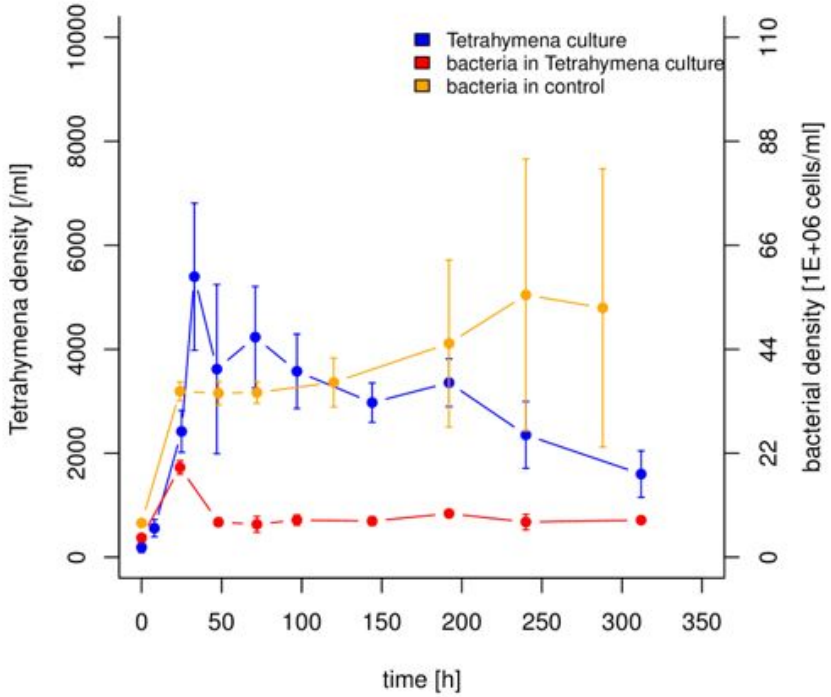

Figure S1. Density of *Tetrahymena* and a mixed bacteria culture (for bacteria used see Giometto *et al*. 2014) over 310 hours. Bacteria dynamics in the control are highly significantly different compared to bacteria dynamics in a *Tetrahymena* culture.

Materials

Equipment

- A flow cytometer equipped with a 488 nm blue laser (>15 mW) and detectors for green fluorescence (520 ± 20 nm), red fluorescence (< 610 nm) and high angle sideward scatter (90 °; SSC). The instrument is cleaned and calibrated according to the manufacturer's procedures.
- Sterile Eppendorf tubes (2 mL).
- Heating block (37°C).
- Pipettes and sterile tips.

Reagents

SYBR® Green I working solution

- Dilute SYBR® Green I (SG; Invitrogen) stock solution 100x in sterile filtered (0.1 µm; Millipore) dimethyl sulfoxid (DMSO) and store refrigerated (5 °C) until use (Prest *et al*. 2013). Alternatively, the SG dilution can be prepared with sterile TRIS buffer (10 mM, pH 8) (Hammes & Egli 2010).

2.5 Measuring bacteria density: Flow cytometry

Particle-free water/buffer for dilution

- Prepare particle-free dilution media by filtering (0.1 µm; Millipore) commercially available bottled mineral water (e.g., Evian®). Alternatively, sterile buffer (e.g., TRIS buffer, 10 mM, pH 8) can be used filtered similarly.

Procedure

Sample preparation

1. Collect the sample (1 mL) and homogenise by vortexing (10 sec).
2. Dilute the sample 100x (10 µL sample in 990 µL) in particle-free mineral water or buffer). The dilution step may be omitted if a low cell density (< 10^7 cells/mL) is expected; a larger dilution may be used if needed.
3. Transfer 200 μL of the diluted sample into a labelled Eppendorf tube.
4. Warm for 3 min at 37 ± 2 °C in a heating block.
5. Add 2 μL of SYBR® Green I working solution. The volumes of the sample and stain may be altered, but a stain dilution of 100x should be maintained.
6. Vortex briefly and incubate in the dark for 10 min at 37 ± 2 °C.
7. Transfer just before measurement 50 μL of the stained sample into 450 μL of particle free water to achieve a 10-fold dilution. The volumes may be adapted for different instrument requirements. This final dilution may be omitted if the cell density is already low enough.
8. Vortex briefly and measure.

Flow cytomtric measurement

1. Load the sample in the FCM and measure.
2. Use a pre-prepared template for measuring bacteria.
3. The "trigger" or "threshold" should be set on green fluorescence, and the instrument set-up should be in such a manner as to allow visualisation of all cells stained with SYBR Green I. Specific instrument settings will differ between instruments, but an example is demonstrated in the "Expected Results" section below.
4. Visualise the sample on a two-dimensional density plot of green fluorescence (520 nm) and red fluorescence (>610 nm) and optionally a second two-dimensional plot of green fluorescence and sideward scattered (SSC) light.
5. Distinguish between bacterial cells and background with electronic gating.
6. Distinguish between small low nucleic acid (LNA) content bacteria and large high nucleic acid content (HNA) bacteria with electronic gating.
7. Extensive details on the FCM methodology are supplied in PREST and SLMB.

Timing

15 minutes for sample preparation, 2 minutes for measuring. Can be automated for high throughput measurements (see Van Nevel *et al*. 2013).

2.5 Measuring bacteria density: Flow cytometry

Troubleshooting (Tips and Tricks)

Cell concentrations:

Most commercial FCM instruments measure accurately in the range of 10'000 – 1'000'000 cells/mL. The method description included several dilution steps that should suffice to reach this range of cells. However, the dilution steps can be adapted if the required concentrations range is not reached.

Extensive details on the FCM methodology are supplied in Prest et al. (2013) and SLMB (2012).

Anticipated results

Figure 2 shows FCM density plots of a bacterial culture during a protist growth experiment. The bacteria were stained with SYBR Green I as described above and analyled at an appropriate dilution. Figure 2A shows the green and red fluorescence intensities (arbitrary units) of ca. 1500 bacterial cells in a clear cluster, separated from background and instrument noise with electronic gating. Figure 2B shows the forward and sideward scatter intensities of the same cells, which are indicative of cell size, again forming a relatively homogenous cluster.

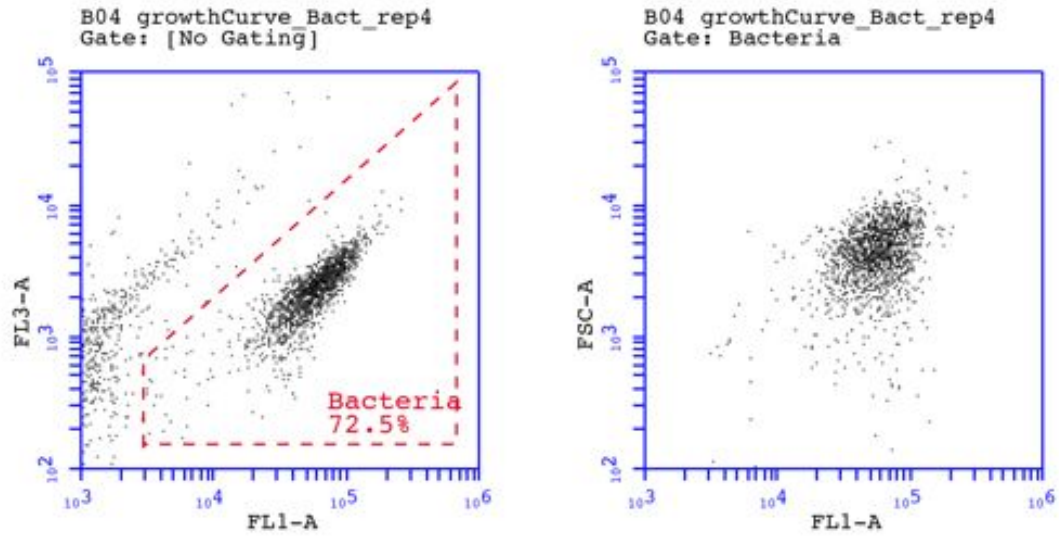

Figure 2. Flow cytometric density plots of an undefined bacterial community stained with SYBR Green I. FL1 = green fluorescence (530 nm); FL3 = red fluorescence (>610 nm); FSC = forward scatter; SSC = sideward scatter.

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2.6 RAMAN microspectroscopy

Introduction

Raman microspectroscopy (RMS) yields information about the chemical composition of individual cells. Raman spectra result from the inelastic scattering of photons from a sample (Raman effect). That is, the scattered photons posses a wavelength/energy that is different from that of the incident light (monochromatic laser). The change in wavelength/energy during the scattering process is caused by the interaction of the photon with vibrational modes of the various chemical bonds of the molecules within a sample (e.g., C=O or C–H) (Wagner 2009). Raman scattering provides detailed information about the chemical composition of a sample (molecular structure, cellular composition or, physiological state of the sample), which is summarized in the Raman spectrum (Huang *et al*. 2010).

Two extensions of RMS are of special interest for experiments with microorganisms. First, a combination with stable isotope probing (SIP). Li et al. 2013 (2013) demonstrated that RMS is able to detect isotopic shifts to higher wavelengths (or lower wavenumbers, wavelength^{-1}), so called "red-shifting", in the Raman spectra when replacing ^{12}C with ^{13}C carbon. The calculated red shift ratio (RSR) is highly correlated with the ^{13}C-content of the cells. Thus, combining SIP with RMS bears great potential for ecological experiments, such as tracking the flow of elements through food webs on a single-cell basis (Abraham 2014). Moreover, using SIP with RMS is non-invasive, which stays in contrast to destructive methods such as 16S-rRNA sequencing. Second, a combination with fluorescence in-situ hybridization (FISH). Because FISH adds detailed information about the spatial structure of a cell, the combination with RMS (Raman-FISH) gives an interesting tool for single cell structure function analyses in protist populations/communities (Huang *et al*. 2007).

The herein given protocol includes all necessary steps after the sampling procedure and preparations needed before analysis with RMS. This comprises the cleaning of protists and bacteria as well as the transfer to quartz slides used later for RMS, that is we cover all preparation steps specific to protists. We do not provide a protocol for the RMS analysis itself since highly specific expertise is known, such that RMS should be performed in collaboration with individuals that have the expertise and the devices to analyse samples of microorganisms.

2.6 RAMAN microspectroscopy

Materials

Equipment

- MgF_2 or CaF_2 microscope slides (© Crystran Limited).
- (Plastic) Petri dishes (60 x 15 mm).
- Micropipettes (10, 100, 1.000 μL).
- Stereomicroscope (magnification 10–50 times, depending on organism size).
- Eppendorf tubes.

Reagents

- Bacterial buffer (or similar liquid) to clean protists. This liquid should not contain any of the elements that may be part of the later analysis, such as carbon when using stable isotope probing (SIP).

Procedure

Isolate and clean ciliates from culture liquid.

This has to be done to remove influences that might potentially disturb/influence the spectra obtained from RMS. This is especially true when labelling individuals by stable isotope probing and or fixation chemicals. However, we do not recommend the use of fixation chemicals since they might influence the RMS output when being absorbed/adsorbed by a cell.

1. Put 3 mL of bacterial buffer in a plastic Petri dish (5 cm in diameter).
2. Select the protists under the stereomicroscope with a micropipette out of the sample volume and put the individuals in the Petri dish containing bacterial buffer. Take care that as little as possible is transferred from the rest of the culture to guarantee a high dilution and cleaning! E.g., if 100 μL of culture liquid are transferred together with twenty ciliates the dilution is 100 / 3.000 ≈ 3.3 %.
3. Select the protists out of this Petri dish as described in the previous step and put them in another Petri dish containing bacterial buffer.
4. Repeat step 3 several times to make sure that the protists are well cleaned. The number of repetitions depends on the volume of culture liquid transferred which each ciliate. The larger the volume the more repetitions it takes to get properly cleaned protists.

Isolate and clean bacteria from culture liquid

Be aware that other organisms might get destroyed during centrifugation!

1. Take 1 mL of experimental volume and put this in an Eppendorf tube.
2. Centrifuged this volume at 3000 rpm for 10 min.
3. Remove as much of the liquid phase as possible (using a micropipette) and re-suspend the residue at the bottom (bacterial pellet) with 200 μL.
4. Repeat steps 2 and 3 two times (or more often if desired).

2.6 RAMAN microspectroscopy

Prepare slides for RMS

1. Put cleaned protists individuals or bacteria in small droplets on MgF_2/CaF_2 slides. These slides are highly light translucent which is a prerequisite for successful application of monochrome light (laser) used in RMS.
2. Let them dry until all liquid is vaporized.
3. The organisms are now ready for RMS analysis.

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2.7 DNA Sequencing and Barcoding

Introduction

DNA sequencing of protist species is done to analyse population dynamics (Hajibabaei *et al*. 2011; Zufall, Dimon & Doerder 2013) or genetic diversity of species complex (e.g., Catania *et al*. 2009), for comparative studies (Gray *et al*. 1998) or to understand the evolution of gene and genomes (Brunk *et al*. 2003; Chen, Zhong & Monteiro 2006; Moradian *et al*. 2007). DNA barcoding is a special case of sequencing, focusing on a short and conserved portion of the genome in order to disentangle the phylogenetic relationships between taxa (Pawlowski *et al*. 2012). The use of DNA barcoding or sequencing enables to estimate nucleotide diversity and fixation indices (Fst), consequently to access the genetic structure and gene flow within and among populations. The genetic variability can also be compared to life history traits or phenotypic plasticity resulting from local adaptation (Krenek, Petzoldt & Berendonk 2012) in order to understand the pattern of evolution. DNA barcoding has been of great interest in phylogenetics to discover morphospecies or cryptic species and to identify the species' composition in a particular environment. Barcodes have been used to study the composition and interaction between species coming from the same environment, like soil (Blaxter 2004) or water column (Stern *et al*. 2010; Hajibabaei *et al*. 2011) and identify cryptic or morpho-species frequent in protists (e.g., Barth *et al*. 2006).

The choice of the gene or barcode of interest should be carefully made depending on the taxonomic level and species one works on. Knowing that the mitochondrial genome evolves faster, the accumulation of sequence variability between organisms would be higher, enabling to discriminate the intraspecific relationships or recent phylogenetic splits. Many barcodes have been previously developed (Nassonova *et al*. 2010; Pawlowski *et al*. 2012) either on the mitochondrial genome (e.g., Cox-1, Cob, SSU of rDNA) or in the nuclear genome (e.g., ITS1-2, SSU and LSU of rDNA).

Approaches

The procedure for DNA sequencing and barcoding consists of three steps: DNA extraction, gene/barcode choice and amplification by PCR, and sequencing methods.

2.7 DNA sequencing and barcoding

First, DNA should be extracted from the cell. Different procedures have been developed to isolate the whole DNA: Chelex solution (Walsh, Metzger & Higuchi 1991), various DNA isolation kits (e.g., Epicentre) or modified phenol/chloroform extraction (Couvillion & Collins 2012). The extraction of whole DNA is sufficient for known barcodes or single copy gene sequencing. However, many protist species are polyploid (>45 in *Tetrahymena thermophila*), and some events of duplication of particular genes well known. Furthermore, ribosomal genes have been duplicated from the mitochondrial genome to the nuclear genome. In that particular case, all copies will be amplified without distinction. Since these two genomes do not evolve at the same speed, a mixture of amplified sequences will increase the noise on the chromatogram. This will complicate the readability of the resulting sequence and can lead to false interpretations. When one wants to create new barcodes and ensure their specificity, the mitochondrial genome should be separated from the nuclear genome as a necessary precaution. The separation between nuclear and mitochondrial materials could be achieved by migration on agarose gel (0.4% at 50V for 6h) with total DNA isolated by modified chloroform extraction (V. Thuillier et al. unpub. results). Depending on the organism being studied, the upper and brighter band in the agarose gel corresponds to the nuclear DNA and the mitochondrial DNA appears around 40kb. The band of interest could be excised and purified by a kit (e.g., wizard SV kit). In ciliates, two nuclei are found: the macronucleus participates in the somatic division and the micronucleus, which is responsible for the germinal line. Both genomes are particularly similar given that the micronucleus genes are copied several times to form the macronucleus (Prescott 1994). Therefore, in order to analyse nuclear genes, the two nuclei should be separated by gradient separations, such as Percoll gradients (Allen 1999; Asai & Forney 2000).

Second, the choice of the gene of interest or barcode should be carefully made depending on the taxonomic level and species one works on. Knowing that the mitochondrial genome evolves faster, the accumulation of sequence variability between organisms would be higher, enabling to discriminate the intraspecific relationships or recent phylogenetic node. Many barcodes have be developed (Nassonova *et al*. 2010; Pawlowski *et al*. 2012) either on the mitochondrial genome (e.g., Cox-1 cytochrome oxydase 1, in *Tetrahymena*, in Amoebae Cob cytochrome b, SSU of rDNA ribosomal small sub-unit, Slapeta, Moreira & Lopez-Garcia 2005; Chantangsi *et al*. 2007; Nassonova *et al*. 2010; Kher *et al*. 2011) or in fast evolving nuclear portions (e.g. ITS1-2 internal transcribed spacer 1-2 in *Carchesium polypinum*, diatoms, and *Tetrahymena thermophila,* SSU rDNA 5.8S in *Paramecium aurelia* or LSU rDNA ribosomal large sub-unit, Chen, Zhong & Monteiro 2006; Catania *et al*. 2009; Gentekaki & Lynn 2009; Moniz & Kaczmarska 2010). The PCR conditions and primers used are described in the corresponding publications. New barcodes could also be designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) that helps to design primers in association with NCBI database. A classical procedure for the PCR (Chen, Zhong & Monteiro 2006) could be tested and modified if necessary knowing that the Tm (melting point temperature) has a strong influence

on the PCR functioning. An optimal PCR protocol can be achieved by testing across a temperature-magnesium gradient.

Finally, the PCR products could be sequenced by Sanger Sequencing method or Next Generation Sequencing (NGS) (llumina, Solexa, Solid, see Valentini, Pompanon & Taberlet 2009). The use of Sanger method is favoured when the number of sequences and barcodes are limited. NGS costs have much decreased recently. NGS are usually used in metagenomics or in comparative studies. For Sanger methods, resulting sequences should be cleaned, most often achieved visually on the chromatogram in MEGA (open source software, http://www.megasoftware.net/), Sequencher (open source software, http://www.genecodes.com/download/external-tools-download) or Geneious (private software, http://www.geneious.com/download). For the analysis of the sequences, many software exist and are well explained (Hall 2013) depending on the purpose. The treatment and analysis of the sequences generated requires expertise in bioinformatics and the detailed procedure is out of scope of this paper. NGS are usually used in metagenomics (Hajibabaei *et al*. 2011), surveys of microorganism diversity (Medinger *et al*. 2010) or in comparative studies. Sequencing data are available and compiled into various databases like GenBank (NCBI, http://www.ncbi.nlm.nih.gov/) and BOLD (Barcode of Life Data Systems, http://www.barcodinglife.org/) for the barcoding sequences.

Materials

Equipment

A standard molecular biology laboratory (including a fume hood) and respective equipment is needed.

Reagents

DNA extractions (Phenole/Chloroform extraction), Lysis buffer (pH 9.5):

- 10mM Tris, pH 7.5
- 0.5M EDTA
- 1% SDS, completed with ultrapure water

DNA extractions (modified Chloroform extraction, modified by V. Thuillier et al.), Lysis buffer (pH=8):

- Tris 20 mM pH 7.5
- EDTA 1 mM
- NaCl 100 mM
- SDS 10%
- ddH_2O

Choice of Barcode and PCR amplification:

Choice of Cox-1 barcode with (Chantangsi *et al*. 2007) forward primer 5'-ATGTGAGTTGATTTTATAGA-3' and reverse primer 5'-CTCTTCTATGTCTTAAACCAGGCA-3'.

2.7 DNA sequencing and barcoding

Procedure

DNA extractions (Phenole/Chloroform extraction):

1. Collect $2.5*10^5$ cells in 50 μLTris (10 mM, pH 7.5).
2. Re-suspend and add 200 μL of pre-heated Lysis buffer (60 °C).
3. Add two volumes water and incubate at 60 °C at least 1 h.
4. Cool to room temperature, add 50 μg/mL proteinase K and incubate at 37 °C overnight.
5. Purify with one volume phenol/chloroform/isoamid.
6. Precipitate with one-tenth volume sodium acetate (pH 5.2) and one volume of isopropanol.
7. Wash pellet in 70 % ethanol.
8. Re-suspend in 75μL Tris-EDTA.
9. Add 0.8 μg/μL RNase A and incubate for 30min at 37 °C.
10. Purify with one volume phenol/chloroform/isoamid.
11. Precipitate with one-tenth volume sodium acetate (pH 5.2) and one volume of isopropanol.
12. Wash pellet in 70% ethanol.
13. Re-suspend in the desired volume of Tris-EDTA.

DNA extractions (modified Chloroform extraction, modified by V. Thuillier et al.):

1. Dilute the amount of cells in order to have a final volume of 200 μl ultrapure H_2O.
2. Add 500 μL of lysis Buffer (pH=8) and vortex them for few seconds until all is homogenized. Then, add 10 μL proteinase k (mg/μl).
3. Inverse the tube 2-3 times.
4. Incubate at 37 °C for 20 min, then vortex for few seconds.
5. Inactivate the enzyme by incubation for 20 min at 65 °C.
6. Add 10mg/mL RNAse A, mix gently and incubate for 30 min at 37 °C. Vortex for few seconds.
7. Separation with 750 μl of chloroform-isoamid (24:1). Then, homogenise and centrifuge at 12 000 rcf for 10min at room temperature. Collect the upper phase (aqueous phase).
8. Separation with 750 μL chloroform-isoamid (24:1) and repeat the same process.
9. Precipitation with 1 mL ethanol 100% (-20 °C) at room temperature. Mix carefully and incubate for 15 mins.
10. Centrifugation at 10000 rpm for 30mins and return the tube to eliminate the ethanol.

2.7 DNA sequencing and barcoding

11. Washing with 1 mL ethanol 70% and centrifuge for 5 min at 8000 rpm. Remove the ethanol with a pipette. Dry only if it rests some ethanol for few minutes.
12. Dissolution in 20 μL of water.

Choice of Barcode and PCR amplification (Chantangsi et al. 2007 for COX-1):

1. Initial denaturation step of 94 °C for 4 min.
2. Followed by 5 cycles consisting of (each cycle): 30 s at 94 °C; 1 min at 45 °C; 105 s at 72 °C.
3. Followed by 35 cycles consisting of (each cycle): 30 s at 94 °C; 1 min at 55 °C; s at 72 °C.
4. Final extension step at 72°C for 10 min.

Classical procedure for the PCR (Chen, Zhong & Monteiro 2006):

5. Initial denaturation step of 94°C for 10 min.
6. Followed by 30 cycles consisting of (each cycle): 1 min at 94 °C, 1 min at Tm; 1 min at 72 °C.
7. Final extension step at 72 °C for 10 min.

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2.8 Genomics, proteomics, and epigenomics

Introduction

The aim of 'omics' approaches (genomics, transcriptomics, proteomics, epigenomics) is to characterize whole molecular content in a sample (DNA, RNA, proteins, epigenetic factors). A sample may refer to a part of an individual (organ, tissue, organelle, etc.), an entire individual, a population, a community or an environmental sample. 'Omics' approaches are largely used by ecologists and evolutionary biologists because they may inform on the diversity of environmental samples, on the molecular bases of organism adaptations, on the modality of genome evolution, on organism-environment interactions, and on the processes of ecosystem functioning (Vandenkoornhuyse *et al.* 2010; Gilbert & Dupont 2011).

Although the general framework is the same for all taxa and all 'omics' (see below), the variety of molecules and applications of these approaches implies that it exists a huge number of available methods and protocols, even in the restricted protist group. Thus, it is an important decision to choose the most appropriate methods from all available ones, in order to answer the question of interest.

Rather than providing an exhaustive and surely incomplete list of detailed protocols, in this supplement, we have chosen first to briefly present the general framework of 'omics' methods. Then, we present relevant examples of specific methodologies within each 'omics' that we think of particular interest to study protist microcosms. For these selected examples, we detail the main steps required to obtain the data and refer to published manuscripts in which readers will be able to find the detailed protocols. Finally, we also provide at the end of each example a list of general and sometimes protist-specific review papers.

'Omics' general framework applied to experimental protist microcosms

High-throughput methods have rapidly spread in the field of ecology and evolution because they allow capturing massive molecular data on a specific sample. The general workflow of these methods is presented in Figure S1. Depending on the question raised, two strategies can be adopted to collect information. The first consists in performing random shotguns to capture all environmental molecules in a sample. In microcosms, this strategy can be used to assess the physiological responses of communities to changing or stressful environmental conditions, to determine the

changes in magnitude or rates of material and energetic fluxes within and between recreated ecosystems (Gotelli, Ellison & Ballif 2012), or else to reconstruct the global molecular content of target individuals (whole-genome, -transcriptome, -proteome or -epigenome). The second strategy consists in performing single molecule surveys within a sample. This can be particularly useful to follow the species composition within a community, to determine the role of target molecules in organisms' adaptation to perturbations (e.g., specific alleles, interfering RNA, heat shock proteins), or to identify common molecules between communities (Gilbert & Dupont 2011).

After the pre-experimental step and the resulting microcosm manipulation, samples to analyse are taken to the molecular biology laboratory in order to perform the extraction step. Depending on the question, either media or cells will be kept to perform extractions. This can be achieved by centrifugation or by using filters. To separate cells of different sizes, it can be useful to perform successive filtering using different meshes. More sophisticated methods can also be used to precisely determine the number of cells that will be extracted (see sections 2.2-2.4). Also, some applications may require the isolation of unique cells, which can be achieve by micromanipulations or fluorescence-activated cell sorting (FACS). An additional step can consist in the isolation of a particular cell component like the cell membrane, micro or macro-nucleus in ciliates, phagosomes or pellicles.

There exist numerous techniques and protocols to perform extractions. The extraction-step will depend upon the biological sample, the target molecules and the analytical tools used to obtain the data. Although manufactured kits may be more expensive than traditional methods (e.g., chelex, trizol, phenol/chloroform), they may be advantageous because some are adapted to cell cultures, the quality of extracts is often high, and some kits couple the isolation of molecules to the post-extraction treatments needed to the analytical step.

The choice of post-extraction treatments will then depend upon the molecule type and the desired coverage of the data. For example, deep and high-resolution proteomes will be obtained by the cross-use of 2-dimensional gel electrophoresis and high performance liquid chromatography/mass spectrometry (Wright *et al.* 2012), which means that the protein extract loaded on a gel will be treated with trypsin after excision. Another example of a post-extraction treatment is the purification of mRNA from total RNA extraction with oligo(dT) magnetic bead, that will be further fragmented, amplified and ligated with adaptators specific to the Next Generation Sequencer used to obtain transcriptomes.

Once massive data are generated, they are analysed using bioinformatics tools. In non-model organisms, the challenge is the *de novo* assemblage and characterization of the data, while model organisms have published biological molecules accessible on web databases (e.g., NCBI and Swiss Prot). Some databases are taxon-specific, species-specific and/or marker-specific (e.g., EnsemblProtists, Tetrahymena Genome Database TGD, Protist Ribosomal Reference Database, Paramecium Database PDB). The bioinformatic treatment of massive data is not trivial for most evolutionary

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ecologists, but Next Generation Sequencing (NGS) platforms and analytic tool developers generally provide help or services to achieve the task.

'Omics' have been proven very informative and powerful in a large number of recent studies, but one can keep in mind that some strong limitations exist to these methods. Troubleshooting may appear at each step of the workflow: sample contaminations, non-reproducible results between techniques, biases during amplification and/or detection of the data, divergent results in function of analytical and bioinformatic tools. To overcome these limitations, users often multiply the number of techniques and analyses and keep only concordant conclusions.

There is a series of general reviews on 'omics' (e.g., Ge, Walhout & Vidal 2003; Quackenbush 2004; Joyce & Palsson 2006; Jex *et al.* 2013)

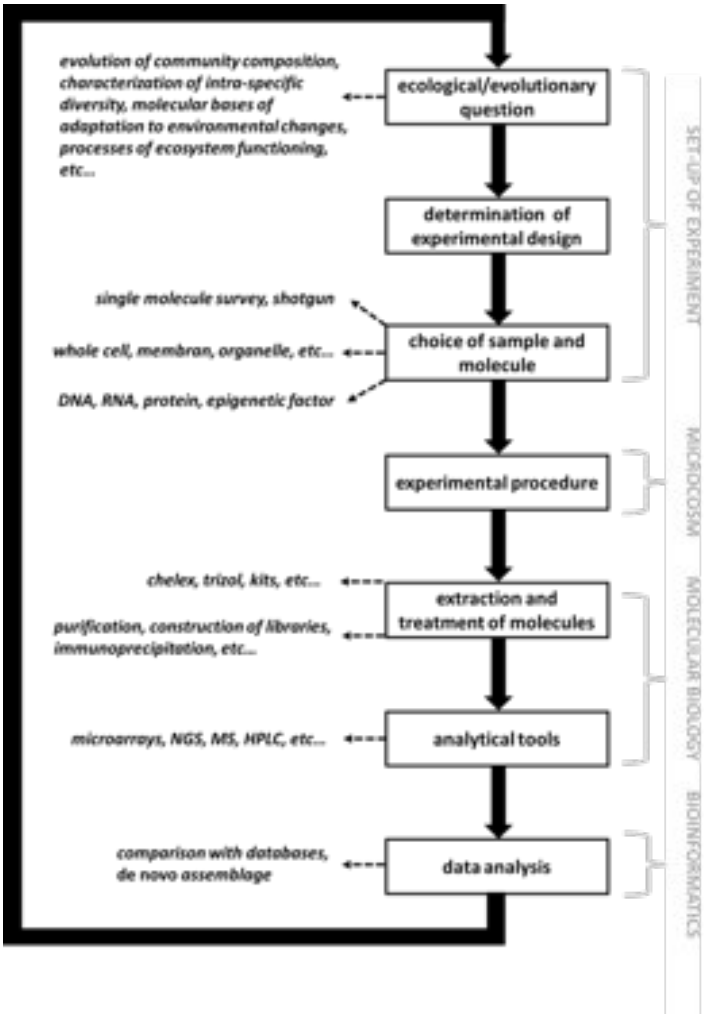

Figure S1: General framework of 'omics' methods applied to protist microcosms. The testing of ecological or evolutionary questions involves numerous steps requiring experimental work, molecular biology competences and most often bioinformatic treatments.

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Example of relevant 'omics' methods to study protist microcosms

The study of global contents of environmental samples allows the characterization of free-living protists, like other unicellular organisms. Surprisingly, however, this has not yet let to many eco-evolutionary 'omics' studies specifically designed for protist micrososms and taxa therein, but we see a high potential for different uses. A notable exception concerns the study of the genome structure and regulation in ciliates (mostly *Tetrahymena thermophila*, *Paramecium* species and *Oxytricha trifallax*). The following examples therefore refer to potential applications of 'omics' methods in protist microcosm studies, for which detailed protocols can be adapted from papers studying other microbial groups, or from papers studying the genome development and cellular physiology in ciliates.

Metagenomics

A metagenomic study refers to the analysis of the genomic DNA from a whole environmental sample. This method can easily be extended to the context of protist microcosms in which experimenters aim at following the species composition of complex communities over situations and time, both qualitatively and quantitatively. In this case, a good strategy is to choose a sufficiently discriminant marker that will allow the clear distinction of each species (see section 2.7, which details the markers of interest in protist barcoding studies).

Major steps are:

- Choose and characterize the DNA marker to be sequenced for each species of the tested community.
- Normalize samples to be extracted.
- Perform total DNA extraction.
- Construct DNA libraries adapted to the sequencer type (Sanger, Next Generation sequencer).
- Sequence libraries.
- Determine the prevalence of each species in the original sample.

There exist both publications on detailed protocols for metagenomic work (e.g., Debroas *et al.* 2009; Ghai *et al.* 2012; Steffen *et al.* 2012) as well as a series of review papers (e.g., Mardis 2008; Dawson & Fritz-Laylin 2009; Gilbert & Dupont 2011; Temperton & Giovannoni 2012).

Single cell genomics

The recently developed single cell genomic approach consists in the sequencing of the whole DNA content in a single cell. Therefore, it allows capturing the entire genome of an individual (nuclear and mitochondrial genomes) but also the genomic content of its endo-parasites or ingested preys. This technique appears promising in the context of protist microcosms because experimenters can directly analyse genome-genome associations of host-parasites or prey-predators interactions.

Major steps are:

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- Isolate the target cell to analyse.
- Perform total DNA extraction.
- Construct whole-genome DNA libraries adapted to the chosen NGS sequencer.
- Sequence libraries.
- Assemble whole-genomes of the isolated cells and the ones of its preys and/or parasites.

There exist detailed published protocols for single-cell genomics (e.g., Raghunathan *et al.* 2005; Hongoh *et al.* 2008; Swan *et al.* 2011; Yoon *et al.* 2011; Mason *et al.* 2012) as well as review papers (e.g., Kalisky, Blainey & Quake 2011; Kalisky & Quake 2011; Lasken 2012; Stepanauskas 2012; Blainey & Quake 2014)

Transcriptomics

The aim of transcription profiling is to develop a complete overview of all the genes in a genome that are up-regulated or down-regulated in response to some factor of interest, in comparison with a designated reference expression (van Straalen & Roelofs 2011). Transcriptomic studies have rapidly spread in ecology and evolution because they allow tackling the first level of the functional response of organisms to environmental changes. The most frequent application, including under the fully controlled conditions imposed within microcosms, is to search for differences in the whole-transcript content of phenotypes that diverge in response to particular environmental conditions.

Major steps are:

- Isolate the divergent phenotypes.
- Perform total RNA extraction of each phenotype.
- Construct cDNA libraries adapted to the chosen NGS sequencer.
- Sequence libraries.
- Assemble transcriptomes and determine both qualitative and quantitative differences in gene expression between the two phenotypes.

There exist detailed published protocols (e.g., Pavey *et al.* 2011; Grant *et al.* 2013) as well as review papers (e.g., Hodgins-Davis & Townsend 2009; Murray, Patterson & Thessen 2012; van Straalen & Roelofs 2012).

Proteomics

To assess the molecular bases of adaptation, evolutionary ecologists commonly use genomic and transcriptomic approaches. The proteomic approach is probably underused because it is more fastidious, requires very good technical skills, and needs expensive measuring equipment. However, proteomes represent the end-result of the adaptive physiological response of organisms to perturbations. Therefore, questions tackled in the transcriptomic paragraph are particularly interesting in a proteomic approach. Another interesting experiment would consist in culturing strains under

stressful conditions and determine the variations in the content of protein classes known to play roles in organisms' stress response, such as heat shock proteins.
Major steps are:

- Isolate stressed individuals.
- Extract total protein content.
- Isolate classes of proteins using 2-dimensional gel electrophoresis.
- Complete protein characterization using Mass Spectrometry.
- Analyse the target classes of proteins and determine their qualitative and quantitative content differences under stressful conditions versus control conditions.

There exist both publications on detailed protocols (e.g., Jacobs *et al.* 2006; Smith *et al.* 2007; Gould *et al.* 2010; Xanthopoulou *et al.* 2010; Le Bihan *et al.* 2011; Oehring *et al.* 2012; Yano *et al.* 2012; Chen *et al.* 2014) as well as review articles (e.g., Tyers & Mann 2003; Yates *et al.* 2005; Beck, Claassen & Aebersold 2011; Dowd 2012; Gotelli, Ellison & Ballif 2012; Armengaud *et al.* 2014; Editorial 2014).

Changes in DNA methylations

Epigenetics refers to stimuli-triggered changes in gene expression due to processes that arise independently of changes in the underlying DNA sequence (Gomez-Diaz *et al.* 2012). Rapid responses to intense environmental changes are supposed to occur most often through epigenetic mechanisms (Flores, Wolschin & Amdam 2013). Among described non-genetic factors there are DNA methylation, histone modification and small non-coding RNAs. Efficient new techniques have been recently developed to determine whole-epigenomes from samples. In experiments aiming at determining the adaptive responses to stressful conditions, the DNA methylation profiles of individuals showing phenotypic adaptations can be compared with profiles of controlled individuals.
Major steps are:

- Choose the appropriate method or the combination of methods to use.
- Isolate DNA from target samples.
- Reveal methylated sites with for example immunoprecipitation or bisulfite sequencing.
- Determine the methylation profiles of selected phenotypes.

There exist both publications on detailed protocols (e.g., Karrer & VanNuland 2002; Bracht, Perlman & Landweber 2012) as well as review articles (e.g., Suzuki & Bird 2008; Nowacki & Landweber 2009; Croken, Nardelli & Kim 2012; Gomez-Diaz *et al.* 2012; Flores, Wolschin & Amdam 2013).

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"Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution"

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2.9 Respirometry

Introduction

Respirometers are devices that measure respiration rates of individual organisms or collections of organisms (e.g., community respiration). They can also be used to measure gross photosynthetic rates when used in conjunction with light-bottle-dark-bottle experiments (e.g., Petchey *et al*. 1999). Respirometers are regularly used for microbial respiration often of environmental soil and water research; food science and preservation; insect respiration; tissue and skin respiration; plant primary production, and a wide range of other applications.

Various technologies exist, though most rely on the consumption of oxygen and or production of carbon dioxide that accompanies respiration, and that rates of consumption are linearly related to rate of respiration. Indeed, respiration rates are usually given in units of amount of oxygen per time (e.g., Fenchel & Finlay 1983).

Technologies for measuring gas concentrations include: oxygen cells, infrared CO_2 sensor, colorimetry, optodes, polargraphic / electrode dissolve oxygen sensors, and manometry. A respirometer is one of these technologies, which embeds a sensor for gas concentration measurement in a sample, containing a culture of organisms. Many such devices exist. For measuring dissolved O_2 concentration with electrochemical sensors see (Pratt & Berkson 1959). For measuring CO_2 concentration within four to six hours based on colorimetric detection, using MicroResp^{TM}, see (Campbell, Chapman & Davidson 2003; Campbell & Chapman 2003).

This document may develop into a list of detailed protocols for each technology and device, in which there would be some overlap with the device's manufacturer manuals. Here, we provide an overview of different available technologies and mention some of the devices that adopt them, listing their advantages and disadvantages. Note that measuring gas concentrations often requires accounting for pressure, temperature, salinity, and pH.

Materials

Equipment

Oxygen cells and infrared CO_2 sensors

2.9 Respirometry

These technologies provide a measurement of the concentration of oxygen or carbon dioxide in a sample of gas. This sample of gas typically comes from the headspace above the liquid in a culture vessel. The gas composition and changes in gas composition of the headspace reflect production and consumption of gases by the organisms in the liquid. Rates of evolution of oxygen are calculated from the rate of change of oxygen in the headspace.

Devices employing this approach need some method of sampling the headspace, and often this must involve the headspace being sealed from the atmosphere. Sealing the headspace for long periods can cause large changes in dissolved oxygen and carbon dioxide concentrations. An example device is the Micro-Oxymax Closed-Circuit Respirometer manufactured by Columbus Instruments. This device has many settings, including the option to refresh the headspace with atmospheric gas, to avoid large deviations in dissolved oxygen and carbon dioxide concentrations. The system automatically compensates for changes in pressure and temperature. It also has the option to multiplex multiple vessels (up to 80) into one respirometer, so that respiration of multiple microcosms can be simultaneously recorded.

Oxygen cells have limited life, must be regularly calibrated, should not be exposed to moist gases. Care must be taken to assure there are no leaks in gas pipes. We have found that a closed circuit respirometer is the type of device that performs best if one lab member has sole responsibility to maintain and operate it, but requires considerable training for each user. Consumables include: oxygen sensors and compounds for extracting moisture from gas.

Fig. S1. A Columbus Instruments Micro-Oxymax Closed Circuit Respirometer. Culture vessels are in the wooden tray (lower left). Yellow tubes take gas from the headspace of the culture vessels through the black guide box to the silver and blue striped pump, dryer, and measurement boxes. The blue gas cylinder contains calibration gas. Photo by Owen Petchey.

2.9 Respirometry

Colorimetry

This technology involves oxygen or carbon dioxide causing a chemical reaction that then results in colour change in a substance. This colour change is quantified and transformed into a measure of respiration rate. Several chemical reactions can be used, and these are embedded into various devices.

An example is the microplate-based respiration MicroResp™ device, which can measure respiration rate in 96 samples simultaneously. The device consists of disposable 96-well plates and a spectrophotometer microplate reader. Samples must be taken and placed in the device, and provide an estimate of the respiration rate of organisms in that sample. Any changes in composition or abundance of organisms during the colorimetry will cause deviation between the respiration in the microcosms and that measured by colorimetry.

Fig. S2. A MicroResp™ starter kit (image from http://www.microresp.com/micro_order.html).

2.9 Respirometry

Optode sensors

Optodes, also called chemical optical sensors, are a relatively new tool measuring environmental variables, such as gas concentration in liquids and gases. The optode is stuck on the inside surface of a culture vessel, and is read by a fibre optic cable placed on the outside of the culture vessel. The fluorescence read by the fibre optic cable is related to the concentration of dissolved gas (e.g., oxygen, carbon dioxide). Measurements are relatively fast (a couple of seconds) and require minimal training of personnel. Apart from the presence of the optode, there need be no disturbance associated with measurements. Calculations are required to transform gas concentrations into measures of rates of gas production / consumption.

Fig. S3. Left: A sensor (optode by PreSens GmbH) glued to the inner surface of a standard culture vessel. Right: A measurement of oxygen saturation being made. Microcosms are fitted with a guide to ensure the fibre optic cable is correctly placed. Photos by Owen Petchey.

Polagraphic / electrode dissolved oxygen sensors can also be used to measure dissolved oxygen concentrations, which could then also be transformed into measures of gas production / consumption. Polargraphic oxygen sensors consist of anode, cathode, and electrolyte solution, separated from the sample liquid by a semi-permeable membrane. These are standard instruments for measuring dissolved oxygen and require that the sensor is dipped into the culture medium, therefore care must be taken to prevent contaminations.

2.9 Respirometry

Manometer based measures

Manometer based measures involve placing the sample in a gas-tight apparatus that include a compound that absorbs carbon dioxide. Because carbon dioxide is absorbed, respiration results in reduced pressure within the apparatus. Therefore, the measurement of pressure changes, for example with a manometer rube, allows measuring respiration. More sophisticated apparatuses include a transducer for converting pressure into an electrical signal that is sent to a computer. As well as providing a digital measure of pressure change, this signal can be used to trigger oxygen production, so that the pressure and oxygen concentration in the apparatus remains constant. One limitation of this method is that organisms that require carbon dioxide will be negatively affected within the apparatus.

Procedure for Optodes

1. Choose culture vessels that are compatible with the optode technology, e.g., pyrex with thin enough walls.
2. Glue the optodes to the inside surface of the culture vessels at a specific position. Ensure that the glue is non-toxic for the organisms.
3. Calibrate individual optodes following manufacturers guides and methods.
4. Autoclave the vessels (optodes are unaffected).
5. Prepare the samples as required.
6. Place the culture vessels inside an incubator, to ensure constant temperature throughout the measurement.
7. Take a measurement as per the manufacturers instructions, ensuring that the microcosms are not moved before a measurement is made. Even small movements can affect measured dissolved oxygen.
8. Perform calculations to transform measures of dissolved oyxgen into measures of oxygen production rate.

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2.10 Nutrient dynamics and litter-bags

Introduction

Most microcosm studies manipulate the food availability by the concentration of the medium. Less frequently is the nutrient composition or elemental balance (i.e., stoichiometry) between carbon, nitrogen and phosphorus taken into account.

Decomposition is a critical ecosystem process due to its influence on nutrient cycling and availability (Ribblett, Palmer & Coats 2005). Microcosm studies of decomposition rate include the effects of biodiversity of non-decomposers affects (McGrady-Steed, Harris & Morin 1997), effects of temperature change (Petchey *et al*. 1999) or spatial habitat structure and composition of leave litter (Davies *et al*. 2009). Decomposition rate is estimated by measuring the weight loss of organic matter (e.g., of a wheat seed or leaf litter) over a specific amount of time, similar to use of leaf-litter bags for measuring decomposition in terrestrial ecosystems.

Individual wheat seeds can be identified, if required, by placing them in small, labelled bags. Since this may rarely be required, the protocol below is for measuring decomposition without identifying individual wheat seeds.

Materials

Equipment

- Microbalance (at least 0.001 g precision)
- Drying oven

Reagents

- Wheat seeds or leaf litter (e.g., *Alnus* sp.)

Procedure

1. Decide how many wheat seeds/leaf litter pieces are required per microcosm and decide the period(s) over which decomposition will be measured (for time estimates, see Ribblett, Palmer & Coats 2005). This will determine the number of wheat seeds required in total, and per microcosm per measurement period.
2. Select wheat seeds that are similar in size and weight, and that are not physically compromised.
3. Dry the wheat seeds at 40 °C until their weight is stable (i.e., all moisture is removed).

2.10 Nutrient dynamics and litter-bags

4. Weigh individual seeds or groups of seeds (depending on the decision made in step 1).
5. Place each seed (or group of seeds) into a foil bag, labelled uniquely.
6. Autoclave all the foil bags.
7. Place the wheat seeds into the microcosms, noting the id of the bag that was put into each microcosm.
8. Remove the wheat seeds from the microcosms, taking care to minimise chance of contamination, and taking care to remove material not part of the wheat seed (e.g., bacterial masses surrounding the wheat seed).
9. Carefully rinse the wheat seeds, again to remove material that was not originally part of the wheat seed.
10. Dry the wheat seeds, taking care to know which microcosm they came from / the unique id of the foil bag they came from.
11. Weigh the wheat seeds over several days at 40 °C, until their weight stabilises.

Timing

Allow up to one week for drying before and after. Allow several hours for initial and final weighing, depending on the number of microcosms in the experiment.

Troubleshooting (Tips and Tricks)

Use preliminary experiments to ensure that treatments do not reach close to 100% weight loss during the experiment. This is to avoid lack of variation among treatments, due to complete decomposition in all treatments.

Anticipated results

Rate of decomposition, measured either as percentage weight loss, or the rate of exponential decline in weight (the latter is likely to be more generally appropriate).

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2.12 Interaction strengths

Introduction

Measuring the strength of competition, predation and host-parasite interactions is often needed. Direct observations can be done in some cases but measuring carrying capacities in individual species and in two-species combinations is usually required to estimate the strength of interspecific competition. Interactions between predators and prey can be quantified via functional response experiments and by fitting a suitable dynamical model to time series of predator and prey population sizes. While this is relatively complex for many systems, protist microcosm are actually a feasible study system to look at predator prey dynamics. In order to fit a suitable predator-prey model to time series in order to estimate the parameters of the functional response, we refer to more specialised literature (e.g., Harrison 1995).

Materials

Equipment

Only standard equipment is required (e.g., that described in sections 1.1, 1.2, 1.3, 1.4, 1.5, and perhaps 2.1, 2.2, 2.3, 2.4)

Reagents

- Lugol's solution can be used to preserve samples

Procedure

Competition

This is a simple procedure to estimate the strength of interspecific competition in a pairwise setting. For a detailed discussion and methodological guidelines on how to measure and calculate competitive interaction in protist communities, see Carrara et al. (Carrara *et al*. 2014a; Carrara *et al*. 2014b). These methods depend on measuring growth rate and carrying capacity of individual species in isolation first. Then species are mixed at half-carrying capacity to measure changes in population density caused by competition.

1. Prepare a bottle of a suitable medium.
2. Set up cultures of individual species at low density to measure growth curves to estimate growth rate (r) and carrying capacity (K). You can skip this step if you already have reliable measurements of these parameters.

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3. Take a sample of the two cultures at carrying capacity and estimate population density in these particular cultures.
4. Take 5 ml of the culture of one species and put it to a suitable bottle (volume at least 20 ml).
5. Add 5 ml of the second species.
6. Create several replicates (at least four, preferably six to eight).
7. Note the time of the beginning of the experiment and the density of the starting cultures (see point 3. above).
8. Keep the mixed culture in a climate chamber with controlled temperature and suitable illumination for at least 10 days.
9. Measure population density of both species at the end of the experiment. You can also do repeated measurements to get a two-species time series (this is not necessary but can decrease uncertainty).
10. Fit a Lotka-Volterra model to the experimental measurements. You need to know r, K, initial density of both species and final density of both species to estimate competition coefficients. See also (Carrara *et al*. 2014a; Carrara *et al*. 2014b).

Predation

The procedures described below apply to predators feeding strictly on other protists and not on bacteria (e.g., *Didinium*). Some species feed on both bacteria and other protists. In such cases, predation rate (as a single parameter) can be estimated by fitting a Lotka-Volterra model described in the section for competition. In such case, one species will have a negative value and the other a positive value of the interaction coefficient. This approach can be also used when screening for potential predators among species whose diet is not well known. On the other hand, in predators feeding only on protists and not on bacteria, conducting functional response measurements is desirable.

Direct measurement of a functional response:

Detailed settings need to be adjusted according to the species used. Here we provide two examples of protocols used previously.

a. An example based on Hammill et al. (2010) using *Paramecium* as a prey and a small flatworm, *Stenostomum*, as a predator.

1. Add a known number of prey individuals from the range of 1 to 60 (can be increased further to make sure that the functional response converges to an asymptote) to 500 microL of protist medium in a well plate. Instead of counting and transferring prey individuals one by one, you can prepare a series of cultures diluted to a varying degree and take a drop from the culture, count the number of prey individuals and use this drop as a source of prey for the experiment.
2. Add one predator individual.

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3. Let the predator feed for 4 hours (the duration must be short enough so that prey reproduction can be neglected).
4. Count the surviving prey individuals, or preserve the sample in Lugols' solution (see section 1.8) and count the protists later.

Stenostomum has a relatively high consumption rate, up to ca. 10 *Paramecium* within four hours (Hammill, Petchey & Anholt 2010), which facilitates the measurements.

b. An example protocol based on Delong and Vasseur (2013) using *Paramecium* as a prey and *Didinium* as a predator.

1. Prepare a series of cultures diluted to a varying degree and place a 50 µl drop from the culture into a Petri dish, count the number of prey individuals (a reasonable range of prey numbers would be ca. 1-20) and use this drop as a source of prey for the experiment.
2. Add one predator individual in a known amount of medium (e.g. 20 µl) so that the total volume of the drop is known (in this case 70 µl).
3. Close the Petri dish to minimise evaporation.
4. Let the predator feed for 4 hours (the duration must be short enough so that prey reproduction can be neglected).
5. Count the surviving prey individuals.

Delong and Vasseur (2013) measured maximum consumption rate by *Didinium* using this setup to be around 5 *Paramecium* consumed during two hours. Based on this, using a slightly longer duration (e.g. 4 hours) of the experiment would be preferable.

Estimating the parameters of a functional response from two-species time series:

Measuring interaction strength this way is more uncertain than measuring the functional response in short-term experiments described above. However it can be used in predators with very low predation rates. As long as one is interested in fitting predator-prey models (e.g., Lotka-Volterra), this method is more precise, because it allows fitting the interaction strength. Thus, the two methods differ in the quantities that they allow to measure.

1. Prepare a bottle of suitable medium (see section 1.2 for details).
2. Set up cultures of the prey species at low density to measure growth curves to estimate growth rate (r) and carrying capacity (K; see section 2.2 for details). You can skip this step if you already have reliable measurements of these parameters.
3. Take a sample of the prey culture at carrying capacity and estimate population density in this particular culture (see section 2.2 or 2.3 for details).
4. Take 10 ml of culture of the prey species and put it to a suitable bottle (volume at least 20 ml). Use larger volume if the predator occurs at low density in

cultures. For example, for *Didinium-Paramecium* species combination, using 100 ml of medium would be preferable to decrease the effect of demographic stochasticity.

5. Add a known number of predator individuals (within the range observed in stock cultures) and close the bottle (do not close the lid firmly to allow exchange of gases between the bottle and the surrounding air).
6. Create several replicates (at least four, preferably six to eight).
7. Note the exact time of the beginning of the experiment and the density of the starting cultures (see points 3. and 5. above).
8. Keep the mixed culture in a climate chamber with controlled temperature and suitable illumination for at least 10 days.
9. Measure population density of both species at regular intervals during the experiment to obtain a two-species time series (see section 2.11 for details).
10. The suitable frequency depends on the generation time of your predator; measuring population density every 24 hours would be suitable for *Didinium*.
11. Fit a suitable predator-prey model to your time series to estimate the parameters of the functional response. As this goes beyond the focus of our work, we recommend looking up the details for doing so in the relevant literature (Jost & Arditi 2001).

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3.2 Density manipulation

Introduction

Density manipulations are an important technique used in microcosm studies using protists to answer questions related to population dynamics (Gause 1934b; Gause 1934a) and density regulation (Luckinbill & Fenton 1978), but also dispersal (Hauzy *et al*. 2007; Fellous *et al*. 2012; Fronhofer & Altermatt 2014; Fronhofer, Kropf & Altermatt 2014; Pennekamp *et al*. 2014), life history evolution (Luckinbill 1979) and cooperative behaviours and sociality in microbes (Chaine *et al*. 2010).

As long as densities are manipulated within the range zero to carrying capacity (K), it is sufficient to grow cultures to K and subsequently dilute them. In case of density manipulations beyond K, or if reaching K takes a long time for slowly growing species, there are two methods to concentrate cells, namely centrifugation and reverse filtration. Centrifugation of cultures is the standard procedure to concentrate cells, if necessary to levels far beyond carrying capacity (orders of magnitude). Luckinbill & Fenton (1978) used hand centrifugation for their tests of population regulation, whereas Warren & Spencer (1996) concentrated cultures of various bacterivorous protists using centrifugation at 1000 rpm for 5 min. Fjerdingstad et al. (2007) used centrifugation to concentrate cultures and remove nutrients from the culture for a starvation experiment. They centrifuged cultures of *T. thermophila* at 2000 rpm for three minutes and repeated this procedure four times. Unfortunately, most studies so far state rotations per minute, which translate however into different g-forces according to the diameter of the rotating axis and the different types of centrifuges (swing-head versus fixed). Reporting g forces is therefore recommended to guarantee comparisons among studies. Centrifugation exposes cells to considerable physical stress. Thus, care has to be taken that the manipulation does not introduce artefacts into the experimental design or has other unwanted side effects that may be confounded with the effect of the density manipulation.

An alternative for concentration is reverse filtration, whereby the medium is filtered out and where the supernatant containing the cells is retained. This method has the advantage that it is less stressful to the cells, but only about 2- to 4-fold concentrations are possible.

3.2 Density manipulation

Material

Equipment for centrifugation

- Appropriate tubes for centrifugation (resisting the physical forces acting on the tubes during the procedure)
- Centrifuge

Equipment for reverse filtration

- Vacuum aspirator or disposable hand held syringes
- Filters with pore sizes smaller than the protists of interest (e.g., ≤1 μm) that can be attached to a vacuum aspirator or to disposable hand held syringes

Reagents

- Medium/water to re-suspend cell pellet

Procedure

Centrifugation

1. Place medium with the protists into the appropriate centrifugation tube.
2. Centrifuge the tubes for 2 minutes at appropriate rpm / g.
3. Quickly remove the supernatant.
4. Re-suspend protist cells in the remainder of medium or some replacement liquid depending on the goal.
5. Quickly proceed with the processing of the cultures, given that a small medium volume with high individual numbers will quickly deplete the remaining oxygen.

Reverse Filtration

1. Place medium with the protists into an appropriate tube, e.g., 50 mL of protist culture.
2. Start removing medium by putting the tip of the filter into the medium and creating a vacuum pressure (either with vacuum pump or with the disposable syringe), such that medium is sucked through the filter out of the protist culture.
3. Importantly, the process of filtration needs to be done carefully and slowly (generally >30 s for removing 50% of the medium in a 50 mL culture), such that protists do not get stuck on the filter but remain in the supernatant.
4. Dispose the filtrate, and keep the supernatant with the protists at a concentrated density.
5. The total volume of medium (of initial culture) divided by volume of the supernatant gives the level of concentration (e.g., 50 mL of initial culture, 12.5 mL of supernatant and 37.5 mL of discarded filtrate give a 4-fold concentration of the culture).

3.2 Density manipulation

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3.3 Disturbance and perturbation manipulations

Introduction

Disturbances can either be a temporary change in the environment that affects the community (i.e., a pulse perturbation), but where eventually the environmental conditions return to the initial state, or be a permanent change in the environment (i.e., a press perturbation), or somewhere on the continuum between pulse and press. Disturbances as persisting changes in the environmental conditions and possible species-specific resistance to the disturbance itself include change in temperature (e.g., to mimic global warming, Petchey *et al*. 1999; Scholes, Warren & Beckerman 2005) and change of the medium with respect to pH or chemical composition (e.g., Jin, Zhang & Yang 1991).

When studying disturbances/perturbations, most interest is on different aspects of the regime (e.g., pulse, press, frequency, magnitude) on population and community dynamics. In principle, disturbances (or perturbations in general) can be achieved through manipulations of many aspects of the abiotic environment (Sousa 1984). For example, this includes temperature, acidity, or toxins. However, manipulations of these are mostly general (e.g., manipulation pH) or not very commonly done with protists (e.g., effect of toxins), and so we do not cover each of them in detail.

The probably most commonly applied disturbance in microcosm experiments is density-independent mortality, where either a part of the community is replaced by autoclaved medium (e.g., Warren 1996; Haddad *et al*. 2008; Altermatt *et al*. 2011), or where a part of the community is killed (by heating or sonication), but the medium retained in the culture, such that chemical and nutritional conditions remain constant (e.g., Jiang & Patel 2008; Violle, Pu & Jiang 2010; Mächler & Altermatt 2012). This type of disturbance is easy to apply but does not allow species-specific resistance to disturbance, but rather reflects different recoveries from disturbances, strongly determined by a species growth rate, and we discuss the different types in the following.

Density-independent mortality via sonication works through a generator providing high voltage pulses of energy (at frequency of about 20 kHz), to piezoelectric converter. The converter transforms the electrical energy to mechanical vibration through the specific characteristics of internal piezoelectric crystals. The vibration is subsequently amplified and then transmitted to the horn (probe). The horn's tip is subsequently expanding and contracting longitudinally. The amplitude is

defined by the distance the tip expands and contracts, and can be set by the user. The energetic waves created by the vibration have disrupting effects on biological membranes and other biological structures (e.g., cell walls, proteins), such that they physically disintegrate.

Materials

Equipment

Replacing medium:

- Pipettes or measuring beakers.

Heat-disturbance:

- Pipettes or measuring beakers.
- Microwave.
- Cooler or box with ice to cool medium after treatment.
- Heat-protecting gloves to hold vessels after microwaving.

Sonication-disturbance:

- Pipettes or measuring beakers.
- Sonicator system, composed of a generator, a converter and a horn (also known as probe).
- Ice-bath (e.g., measuring beaker with crushed ice).

Reagents

No specific reagents beyond what is described in sections 1.2 to 1.4 are needed.

Procedure

Replacing medium:

Depending on the level of disturbance, a large part of the medium (50–99%) (Warren 1996; Fukami 2001; Scholes, Warren & Beckerman 2005; Haddad *et al*. 2008; Altermatt *et al*. 2011; Altermatt, Schreiber & Holyoak 2011; Altermatt & Holyoak 2012; Limberger & Wickham 2012) containing protists is replaced with freshly autoclaved medium. Replacing less than 30% of the medium has generally very little effects on the population and community dynamics of protists, and is sometimes even used as a standard procedure during long-term maintenance. It is very important that all handling procedure (e.g., mixing before disturbance) except the disturbance itself is also applied to the controls.

1. Take the vessel with the protist community to be disturbed.
2. Thoroughly mix it (shaking or with pipette).
3. Remove the proportionate content that should be disturbed. Note: in case of very high disturbance levels (e.g., 99%), it may be easier to remove the content that should be maintained with a pipette, temporarily keep it in the pipette tip, discard all of the rest, and add it back to the vessel.

4. The discarded medium including the protists should be safely disposed, to avoid that protists can escape into the natural environment (autoclaving the disposed medium or by adding bleach).
5. Replace the discarded medium with freshly autoclaved (possibly bacterized, see section 1.2, 1.3) medium.

Heat-disturbance:

The procedure below is for applying density-dependent mortality equally to all species. However, it is possible to cause this mortality in a particular species (the one with the lowest temperature tolerance) only. This requires careful calibration of a temperature disturbance applied to the whole community, so that only this species suffers mortality (e.g., Worsfold, Warren & Petchey 2009).

1. Take the vessel with the protist community to be disturbed.
2. Thoroughly mix it (shaking or with pipette).
3. Remove the proportionate content that should be disturbed. Note: in case of very high disturbance levels (e.g., 99%), it may be easier to remove the content that should be maintained with a pipette, temporarily keep it in the pipette tip, disturb all of the rest, and add it back to the vessel.
4. Place a vessel with the proportion of the medium that should be disturbed in a microwave and heat it up to boiling temperature. The intensity and duration of microwaving needs to be adjusted to the chosen volume. Ideally, the medium is just quickly heated up to 80–90 °C, but does not boil. This kills all protists but minimize evaporation (cover lids, but do not use aluminium foil but glass cover lids) and chemical reactions in the medium due to heat.
5. Let the disturbed (i.e., heated) medium cool down as quickly as possible (using an ice bath) to the exact same temperature as the remaining (i.e., undisturbed) part and put it back.
6. The heating and cooling should be done as quickly as possible (ideally, in less than 1 h), to avoid time-lag effects. For the control treatments, also remove the same part of the medium as being disturbed, store it temporarily at room temperature/conditions the replicates are handled, and only put it back to the replicate after the same time as the disturbed ones are put back.

Sonication-disturbance:

1. Take the vessel with the protist community to be disturbed.
2. Thoroughly mix it (shaking or with pipette).
3. The intensity of disturbance can be set in two-ways: A) a proportion of the medium is sonicated such that all protists die; B) the duration of the sonication process can be varied, such that part of the protists can survive when sonicated for only short periods or at low intensities (usually a few seconds).
4. Remove the content that should be disturbed. We recommend sonicating at maximum amplitude over a short time-span (e.g., 30 to 60 s for a sonicator with 700 W and 20 KHz maximum working power).

5. During sonication, the medium can considerably warm and get hot. To avoid a temperature-effect (e.g., also compared to the control), the sample vial with the medium to be sonicated should be placed in an ice bath.
6. Put the sonicated medium back to the undisturbed fraction of the sample.

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3.4 Nutrient concentration and viscosity of the medium

Introduction

Manipulating the nutrient content of medium

The level and temporal availability of nutrients are parameters that determine ecological conditions such as resource pulses (Yang *et al.* 2008), environmental heterogeneity and autocorrelation (Laakso, Loytynoja & Kaitala 2003). Nutrients interact with intrinsic features of the population or community to create resonance (Orland & Lawler 2004), productivity-diversity relationships (Haddad *et al.* 2008; Altermatt *et al.* 2011) or relationships between productivity and evolutionary responses (Friman & Laakso 2011). Nutrient levels and the temporal availability of nutrients are easily manipulated in microcosms.

In axenic cultures, the nutrient availability is directly manipulated via the concentration of the medium, whereas in bacterized medium, the nutrients available to the bacteria are manipulated, which then feed back into increased bacteria abundance.

Different numbers of protist pellets were used by Holyoak (2000) (1, 2, and 4; each with a weight of 0.57 g, translating to 0.57, 1.14 and 2.28 g per litre for low, intermediate and high concentrations) whereas Orland & Lawler (2004) manipulated the amount (in grams) of the protist pellet directly (low: 0.2 g/l , high: 1 g/l). Cadotte et al. (2006) used levels of 1g, 0.1g and 0.01g of protist pellet per litre for high, intermediate and low nutrient levels respectively, in addition to different vitamin provisions. Haddad et al. (2008) manipulated nutrient levels by replacing part of the medium with nutrient-free sterile spring water, compared to a nutrient treatment that replaced the original medium with fresh medium of the same type.

Luckinbill 1978 and Luckinbill & Fenton (1979) varied the amount of nutrients available directly via changes in bacterial abundance as well as indirectly via nutrient availability. Friman et al. (2008) manipulated low and high nutrient concentrations by two- versus eightfold dilution of the cerophyll medium to study the effects of productivity on the ecological and evolutionary dynamics of a predator-prey interaction.

Besides, seeds that slowly release nutrients are used to manipulate the carbon sources available to bacteria, which in turn feedback to higher abundances of bacteria as protist prey. These are often added to stabilize the dynamics of the communities

(e.g., Haddad *et al.* 2008; Altermatt, Schreiber & Holyoak 2011), but also to manipulate nutrient concentration (e.g., Fox 2007).

Manipulating viscosity of the medium
Methyl cellulose is well-known for increasing the viscosity of liquid media (Sonneborn 1950). A higher viscosity slows down the movement speed/ability of protists, and this is often used to slow down protists for microscopy purposes (Sleigh 1991). However, it can also be used to manipulate the movement behaviour in the context of behavioural experiments (e.g., to affect the outcome of predator-prey dynamics) or the costs of movement/dispersal due to increased drag in liquid medium. According to Beveridge et al. (2010a; 2010b)(and references therein) the most suitable compound for adjusting the viscosity of microcosm media is Ficoll® [GE Healthcare companies] (Winet 1976; Bolton & Havenhand 1998; Abrusán 2004; Loiterton, Sundbom & Vrede 2004). Ficoll has broadly the same effect as methyl cellulose, however, the handling of the substance is easier than that of methyl cellulose. Ficoll dissolves in water regardless of the temperature (methyl cellulose dissolves better at low temperatures), shows Newtonian fluid properties in solution and only requires small quantities to change the viscosity without being toxic.

Materials

Equipment

Manipulating nutrient concentration of the medium:

- Microbalance to weigh specific amounts of protist pellet/seeds

Manipulating viscosity of the medium:

- Microbalance to weigh the amount of methyl cellulose or Ficoll
- Heater or water bath

Reagents

Manipulating nutrient concentration of the medium:

- The same as for the production of the basic medium for dilution.
- Sources of slow nutrient release such as autoclaved and standardized wheat or millet seeds.

Manipulating viscosity of the medium:

- Medium prepared according to section 1.2.
- Methyl cellulose is readily obtained from local pharmacies (often with varying names according to the producer); concentrations of around 3.5 gL^{-1} are reported in the literature (Luckinbill 1973; Veilleux 1979) to manipulate the swimming/movement of *Paramecium aurelia* and *Didinium nasutum*.
- Ficoll (GE Healthcare companies); Ficoll concentrations of 0, 0.5, 0.7, 1.5, 2 and 2.5% (by mass) produce a viscosity range of 1 x 10^{-3} to 1.52 x 10^{-3} Ns m^{-2} at 20 °C, the same as for viscosities expected at temperatures from 20 to 5 °C

3.4 Nutrient concentration and viscosity of the medium

(Beveridge, Petchey & Humphries 2010a; Beveridge, Petchey & Humphries 2010b).

Procedure

Manipulating nutrient concentration of the medium:

- Dilution of the medium to levels of lower nutrient availability.

Manipulating viscosity of the medium:

A) Methyl cellulose:

Because methyl cellulose is a hydrophilic substance and only dissolves in cold water, a special procedure is required to obtain a homogeneous solution:

1. Add half of the powder into warm medium, let it soak for a moment, then add the remainder till particles are well dispersed in the medium.
2. Cool down the medium in ice while stirring leads to a much more rapid dissolution of the particles.

B) Ficoll:

1. Add the selected concentration of Ficoll (by mass) to the medium.
2. Stir and use directly.

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3.5 Spatial structure

Introduction

A large range of theoretical predictions from meta-population (Hanski 1999), meta-community (Leibold *et al*. 2004) and meta-ecosystem ecology (Loreau, Mouquet & Holt 2003) can be tested using protist microcosm landscapes. Furthermore, the temporal dynamics can be used to assess stability and dynamical behaviour of these systems (Giometto *et al*. 2014; Seymour & Altermatt 2014) as well as evolutionary and eco-evolutionary dynamics (Fronhofer & Altermatt 2014). Importantly, microcosm landscapes can be custom built which allows researchers a virtually unlimited flexibility in their experimental design.

The spatial structure can refer both to spatial structure within a patch versus spatial structure between patches. Spatial structure within a patch is often referred to as habitat heterogeneity, and can for example be achieved by adding tiles or glass pearls to microcosms, such that protists can escape/hide from predators. Spatial structure between patches is covered by the metapopulation concept (Levins 1970), which explicitly considers the effects of linking local populations through (rare) dispersal events (Hanski & Gaggiotti 2004).

Using *Didinium nasutum* and *Paramecium caudatum* as a predator-prey system, already Gause (1934) could demonstrate the importance of space for stabilizing predator-prey dynamics. For further examples, including the study of source-sink systems, for instance, see the review by Holyoak & Lawler (2005). More recently, diversity patterns in dendritic networks (Carrara *et al*. 2012; Seymour & Altermatt 2014) as well as the predictability of invasion dynamics (Giometto *et al*. 2014) or evolutionary processes (Fronhofer & Altermatt 2014) during invasions have been studied in protist microcosm landscapes.

Two basic setups exist, one with passive dispersal (dispersal achieved by pipetting small amount of media) and one with active dispersal (patches connected by tubes). While in a passive dispersal setup, connectivity is directly controlled by the experimenter using a predefined pipetting scheme, in an active dispersal setup connectivity patterns are controlled physically by the geometry of the setup, including the number of physical connections between patches and the distances between these patches. Alternatively, instead of manipulating distance, the time a given connection is open can be varied. Both methods are roughly equivalent, yet, varying connection

lengths may lead to complications during the landscape building process, as setups may become highly complex.

While pipetting and passive dispersal allow for a high degree of control and environmental conditions, such as gradient in solutes, can be easily maintained, some experiments may require actively dispersing organisms. Active dispersal may be necessary either because the variable of interest is movement or dispersal behaviour (Fjerdingstad *et al*. 2007; Fellous *et al*. 2012; Giometto *et al*. 2014; Pennekamp *et al*. 2014) or because potential trade-offs, for instance competition-colonization trade-offs (Cadotte *et al*. 2006; Cadotte 2007; Seymour, Fronhofer & Altermatt 2014) should not be disrupted.

Experimenters have a nearly unlimited flexibility in designing microcosm landscapes. The simplest landscapes consist of two connected vials (patches). Evidently, more complex landscapes, either linear setups or (large) networks of patches are possible. A central choice the experimenter has to be aware of is the spatial theoretical framework: should the experiment be carried out in a patch-matrix setup or is continuous space more appropriate. This has important implications for comparisons with theory and potential parametrisation and model fitting. A similar decision has to be made for connectivity: is a continuous-time setup, in which connections are never closed, more suitable, or does the experimenter prefer to impose discrete dispersal and reproduction phases? All approaches have been used so far, reaching from discrete-time patch-matrix to continuous-time continuous-space setups.

Materials

Equipment

Most microcosm landscapes will include building blocks, such as vials, that have already been described elsewhere in detail (see section 2 Methods overview). In order to avoid contamination with fungi or bacteria all materials, including connections between vials (“patches”), must be either autoclavable or previously sterilized and for single use only. As these materials, especially metal and plastic parts, may contain substances that are toxic for protists, all materials have to be thoroughly tested before use. This should happen in a controlled design and involve, for instance, the recording of growth curves to exclude that materials have deleterious effects on growth or on other variables of interest (see section 2.10 time series). Nevertheless, all experiments performed subsequently should include an appropriate number of controls, which reflect the relative amount (and spatial arrangement) of all materials used in the experimental treatments. Previous testing of materials seems especially important if novel techniques such as 3D printing, which imply novel materials are used to build landscapes.

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Equipment for passive dispersal:

- Vials to make the patches. Commonly used vials are polypropylene centrifugation tubes (e.g., 20 or 50 mL size), 125-ml Nalgene square Polycarbonate wide-mouth bottle or multiwall plates (e.g., 10 mL wells).
- Pipettes of varying sizes (10-100 μl, 100-1000 μl, 1-5 mL) to sample as well as to do the dispersal treatment.

Equipment for active dispersal (discrete landscapes):

- Vials to make the patches. Commonly used vials are polypropylene centrifugation tubes (e.g., 20 or 50 mL size) or 125-ml Nalgene square Polycarbonate wide-mouth bottle.
- Drill to make holes in the vials (holes need to be a bit smaller than outer diameter of silicon tubing, such that it tightly fits without leaking).
- Connectors to connect the silicon tubing to the vials (not needed when silicon tubing is directly inserted into the hole).
- Silicon tubing, recommended inner diameter is between 2 to 5 mm.
- Clamps or stopcocks to close connections.
- Pipettes of varying sizes (10-100 μl, 100-1000 μl, 1-5 mL) to sample.

Equipment for active dispersal (continuous landscapes):

- Silicon tubing, recommended inner diameter is between 2 to 5 mm.
- Clamps or stopcocks to close connections.
- Polypropylene T- and Y-connectors.
- Silicon stoppers to close the T-connectors.
- Pipettes of varying sizes (10-100 μl, 100-1000 μl, 1-5 mL) to sample.

Reagents

No specific reagents are required.

Procedure

Passive dispersal

For experiments built on the assumption of passive dispersal (e.g., Warren 1996; Cadotte & Fukami 2005; Altermatt, Schreiber & Holyoak 2011; Carrara *et al*. 2012), in which predefined volumes of cultures are pipetted from the patch of origin to the destination patch, any vials can be used, including wellplates, Eppendorf tubes, Petri dishes or Erlenmeyer flasks, to name just a few (Fig. 1A/C). Connectivity patterns can be determined through pipetting schemes. Especially large and highly replicated setups, or experiments including complex pipetting schemes, may be best performed automatically using robots (for a yeast model system but easily transferable to protists see Bell & Gonzalez 2011).

In order to avoid long-tailed dispersal, one needs to use a mirror-landscape (described in detail by Carrara *et al*. 2012), as otherwise individuals could potentially disperse across more than one patch (distance) in one dispersal step.

3.5 Spatial structure

1. Thoroughly mix the culture (also needs to be done in the no-dispersal control).
2. Sample the proportion of the culture/community that should disperse with a pipette (i.e., "emigration"). For each patch/emigration step, and new and sterile pipette tip must be used.
3. After emigration, migrants can be stored temporarily in a mirror landscape, or be manipulated, or experience a dispersal related treatment (e.g., mortality). Furthermore, the emigrants can be fractioned into a part that is analysed (e.g., diversity and abundance measured.
4. Subsequently, distribute the migrants into the patch(es) of immigration.
5. For the control, the sampled "migrants" are given back into the patch of emigration.

Active dispersal with discrete landscapes

Alternatively, experiments may require active dispersal of protists. For active dispersal patches can be continuously connected or only connected during a certain amount of time in order to control connectivity (Fig. 1B). The connections are most often built of silicone tubes (e.g., Holyoak & Lawler 1996; Cadotte 2007; Altermatt & Holyoak 2012; Fellous *et al*. 2012). Closing connections can be done using clamps or stopcocks, for example.

1. Landscapes are built with vials connected by tubing.
2. Landscapes need to be fixed on a completely horizontal shelf or on a sheet, but can only be moved when all connectors are closed.
3. Holes for fixing the tubing (or connectors) need to be at same height in all vials, as some species stratify in the vial and thus this can affect dispersal.
4. Fill the vials with medium, all connectors open, such that the medium can flow across the whole system and fill it. Filling through one opening/vial prevents formation of air bubbles in the tubing (e.g., happens when filled from two vials simultaneously. Air bubbles need to be removed. This can be done with a syringe and sterile needles, whereby air bubbles are sucked out by inserting the needles into the silicon tubing at the place the air bubble formed.
5. Close all connectors before adding the species. Therefore, remove first the amount of medium that will be replaced by medium containing species. Always make sure that sampling/adding medium happens when all connectors are closed, and make sure that there is a zero net change in medium volume, as otherwise flow among patches occurs.

Active dispersal with continuous landscapes

Finally, experiments may be done in continuous landscapes which do not differentiate between matrix and patch. Active dispersal is continuous through a network of silicone tubes (Fig. 1D, Seymour & Altermatt 2014; Seymour, Fronhofer & Altermatt 2014). Closing connections can be done using clamps or stopcocks, for example.

1. Landscapes are built with silicon tubing only, whereby horizontally installed X-, L- and Y- connectors are used to create different network connectivities.

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2. Make sure that the total amount of connectors, length of tubing and subsequently volume of medium in the landscapes are identical across treatments, and that only the network structure differs.
3. Use vertically placed T-connectors to insert sampling localities. Silicon stoppers are used to close them.
4. Landscapes need to be fixed on a completely horizontal shelf or on a sheet, but can only be moved when all openings are closed.
5. Fill the landscape with medium, all connectors open, such that the medium can flow across the whole system and fill it. Filling through one opening/T-connector prevents formation of air bubbles in the tubing (e.g., happens when filled from two sites simultaneously). Air bubbles need to be removed. This can be done with a syringe and sterile needles, whereby air-bubbles are sucked out by inserting the needles into the silicon tubing at the place the air-bubble formed.
6. Close all T-connector openings for filling in species or sampling. Therefore, remove first the amount of medium that will be replaced by medium containing species. Always make sure that sampling/adding medium happens when all openings except the one being sampled are closed, and make sure that there is a zero net change in medium volume, as otherwise laminar flow occurs.

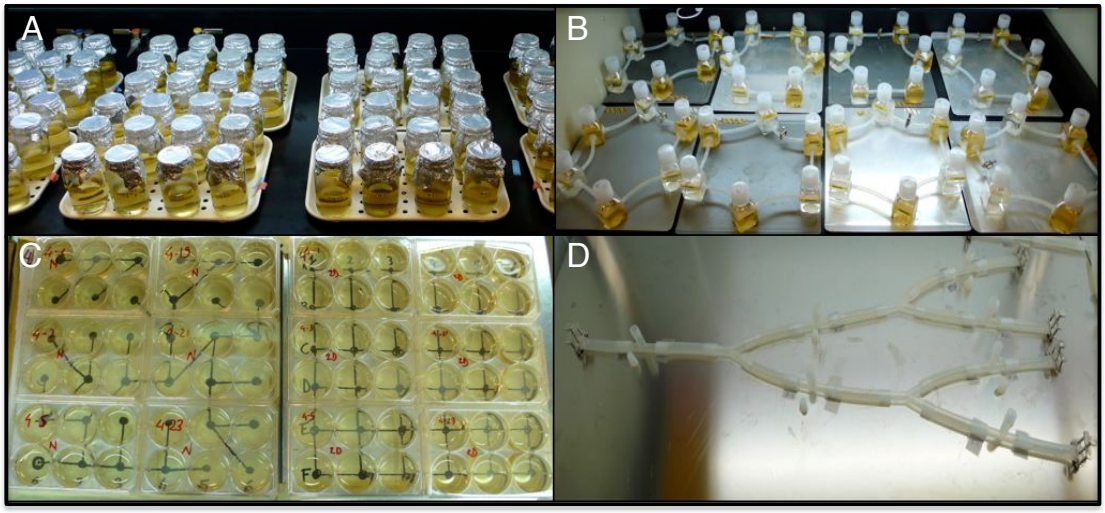

Fig. S1. Examples of protist microcosm landscapes. A) 100 mL glass jar patches connected by passive dispersal (pipetting) along a linear landscape (example from Altermatt *et al*. 2011). B) 100 mL Nalgene vials connected by tubing (i.e., discrete system with active dispersal) in which active dispersal between high and low nutrient patches was manipulated (example from Altermatt & Holyoak 2012). C) 10 mL multi-well plate landscape connected by passive dispersal, comparing dispersal along complex network structures and subsequent effects on diversity (dendritic vs. 2D lattice networks, example from Carrara *et al*. 2012; Carrara *et al*. 2014). D) Continuous landscapes with active dispersal in silicon tubing (example from Seymour & Altermatt 2014; Seymour, Fronhofer & Altermatt 2014).

3.5 Spatial structure

Trouble-shooting (Tips and Tricks)

Two-patch systems, as well as more complex landscapes, may have to be adapted to the specific study organism or set of study organisms. Vial volume may have to be increased for species with very low carrying capacities, for example (see section 2.1). Similarly, for very large species, the diameter of connecting tubes (or the volume pipetted for passive dispersal setups) has to be increased.

Furthermore, different species may stratify characteristically in sufficiently high, unstirred vials. This can be due to oxygen availability, for example. Consequently, the vertical positioning of patch connections can lead to differential dispersal and severe artefacts.

Especially in continuous-time and -space setups the flow of medium has to be exactly controlled and limited to a minimum if the effect is not intended. Often, this can be achieved by constructing microcosm landscapes that are completely air-tight. If landscapes are not moved this reduces the unintended exchange of individuals to a minimum.

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3.6 Temperature manipulation

Introduction

Manipulating the temperature of microcosms is relatively straightforward, with the most important considerations concerning good experimental design. E.g., avoiding or accounting for pseudoreplication, avoiding systematic non-independence of other treatments within controlled temperature environments, choice of appropriate temperature levels and regimes.

Previous studies include effects of temperature on individual metabolic rate (Fenchel & Finlay 1983), movement speed (e.g., Beveridge, Petchey & Humphries 2010), cell size (Atkinson, Ciotti & Montagnes 2003) and competition (Nelson & Kellermann 1965). These individual level effects cause altered population and community dynamics (e.g., Petchey 2000; Leary & Petchey 2009; Fussmann *et al*. 2014) via changes in interaction strengths (Jiang & Kulczycki 2004). Temperature dependent changes in community dynamics can affect ecosystem processes, such as net primary production (Petchey *et al*. 1999).

Materials

Equipment

- Multiple, ideally identical, controlled temperature environments (CTE) such as incubators or water baths.

Reagents

- None

Procedure

1. Design experiment, including exactly where in each CTE each microcosm will be placed.
2. Thoroughly test the temperature control of the CTEs across the range of planned experimental temperatures. Include testing for spatial variation of temperature within CTEs

3.6 Temperature manipulation

3. Ideally, test for difference in ecological dynamics (e.g., single species dynamics) across CTEs that are set at the same temperature (to test for CTE effects).
4. Start the experiment.
5. Remove microcosms from CTEs on when needed and for as short periods as possible (e.g., for sampling).
6. Monitor temperature in the CTEs during the experiment, ideally with an independent probe in a dummy microcosm.
7. Finish the experiment.
8. Check the actual temperatures in the CTE closely match the desired temperatures.

Troubleshooting

Microcosms can experience significant evaporation even with caps on, if these are not tightened. Be aware of and monitor for differential evaporation across temperatures, with higher evaporation rates at higher temperatures. Replace evaporate with distilled or reverse osmosis water. If microcosms are not covered, or if the CTE has strong air circulation, evaporation will be faster.

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"Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution"

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3.7 Manipulation of the biotic environment

Overview

Many aspects of the biotic environment can be manipulated. Thereby, the composition and dynamics of the biotic environment are not only studied as response variables (e.g., number of species, abundances), but are directly changed in order to study the consequences of specific aspects of the biotic environment on ecological dynamics (e.g., productivity, stability of the system). All of these manipulations are directly derived from the ecological question of interest and standardization is thus not directly possible or wanted, such that protocols cannot be given. However, we list below possible manipulations of the biotic environment and give a selection of representative references therefore:

- Manipulation of diversity and identity of species used (Lawler & Morin 1993; McGrady-Steed, Harris & Morin 1997; McGrady-Steed & Morin 2000; Morin & McGrady-Steed 2004; Jiang & Morin 2005; Violle *et al*. 2011; Giometto *et al*. 2013; Carrara *et al*. 2014; Seymour & Altermatt 2014).
- Manipulation of the trophic structure of communities (e.g., Lawler & Morin 1993; Fox, McGrady-Steed & Petchey 2000; Fox 2007; Petchey *et al*. 2008; Worsfold, Warren & Petchey 2009).
- Manipulation of the community assembly history (e.g., Fukami & Morin 2003; Violle *et al*. 2011; Violle *et al*. 2012; Clements *et al*. 2013; Livingston *et al*. 2013).
- Manipulation of invasion dynamics (e.g., Mächler & Altermatt 2012).
- Manipulation of the presence of parasites (e.g., Fellous *et al*. 2012a; Fellous *et al*. 2012b).

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