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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,

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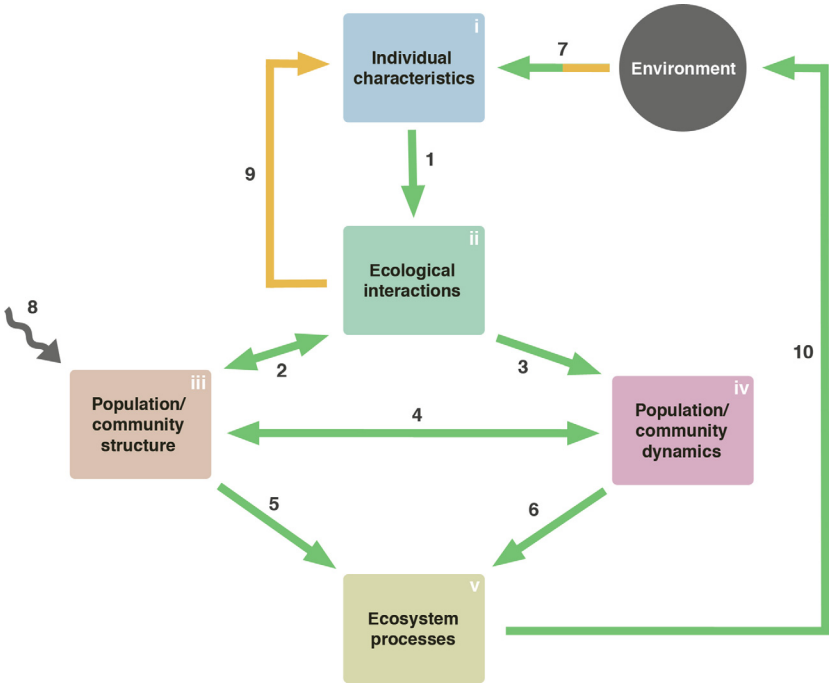

Fig. 1. Causalities between environmental factors, individuals and populations on structure and dynamics across different levels of biological organization (see also Table 1). Green and yellow arrows illustrate ecological and evolutionary causalities, respectively. Individual properties (i), such as 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).

algae and arthropods, have been used as model systems. Protist microcosms (Fig. 2, Lawler 1998; Petchey *et al.* 2002; 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

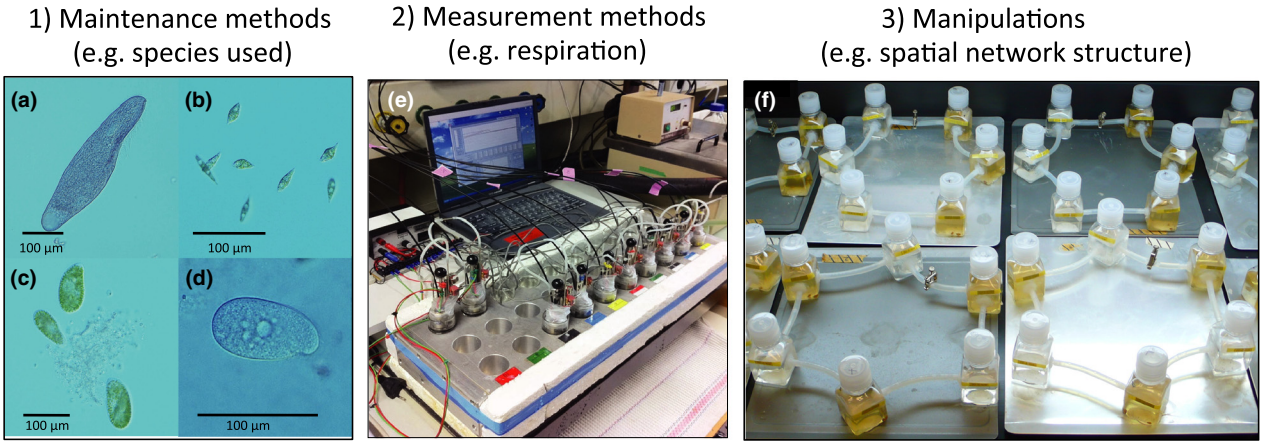

Fig. 2. Experiments with protist microcosms have the advantage that general maintenance methods can be highly standardized (e.g. a–d showing four species which have been commonly used and for which trait data are readily available; a: *Blepharisma* sp., b: *Euglena gracilis*, c: *Paramecium bursaria*, 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

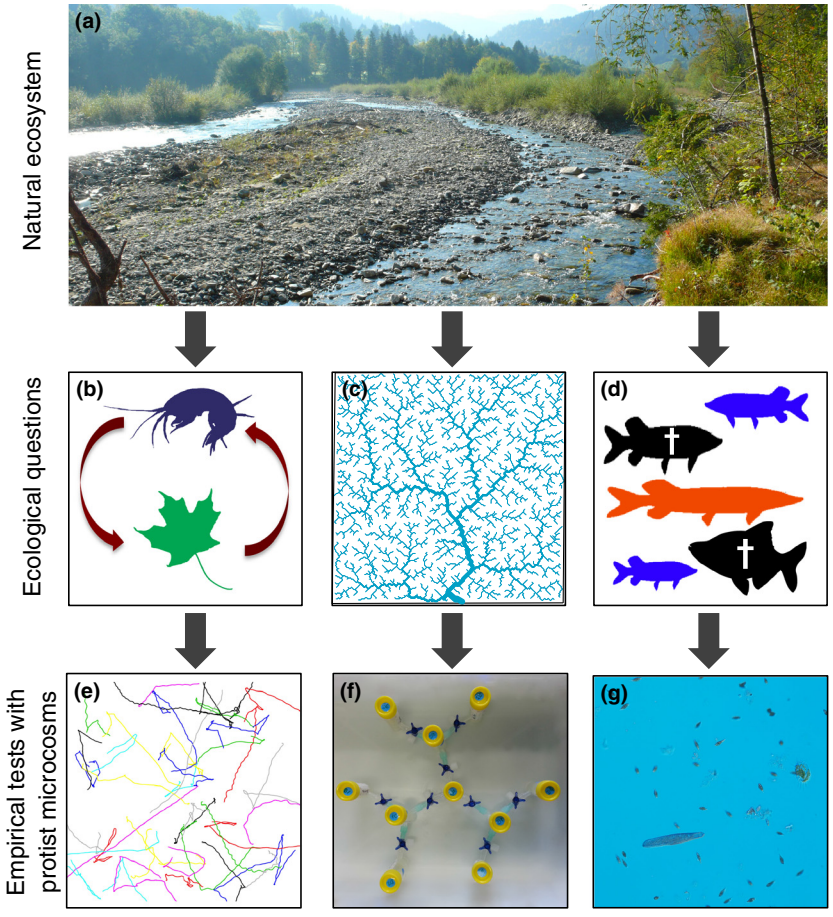

Fig. 3. Protist microcosm experiments are used to address questions in ecology and evolutionary biology derived from natural systems. For example, in a complex natural ecosystem such a river ecosystem (a), questions of interest are how interactions of species with other species or the environment affect 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