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The strain concept in phytoplankton ecology

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Laboratory cultures are important tools for investigating the biology of microalgae, allowing experimentation under controlled conditions. This control is critical for comparative studies, such as those often used to investigate intra-specific variation in properties of interest. By holding the environment constant, the experimentalist can gain insight into the genetic basis of phytoplankton phenotypes and by extension, into the adaptive history of those genotypes. In most cases the adaptations of interest are those that the algae have evolved in response to their natural environment. However, here it is argued that such experiments may instead reveal evolutionary adaptations to, and/or non-adaptive changes induced by, the culture conditions under which the alga is maintained. We present a review of the processes of evolution as they pertain to microalgal culture, and illustrate this discussion with examples of in-culture evolution from both within and outside the field of phycology. With these considerations in mind, recommendations are made for experimental practice focusing on comparative physiology, for which the effects of in-culture evolution are particularly confounding. Finally we argue that, although problematic in some contexts, the evolutionary propensities of phytoplankton cultures actually present an important opportunity for experimental evolutionary research with direct environmental significance.

1. Introduction

“...any change in a clone that is the result of culturing conditions reduces the usefulness of that clone as a laboratory test organism for ecological correlations.”
[Lynda Murphy, 1978]

In their mini-review “*The species concept in phytoplankton ecology*”, Wood and Leatham (1992) made the assertion that *inter-specific* comparisons of phytoplankton should not be made without also considering *intra-specific* data on the characters being compared. They were correctly addressing the issue of sampling as a key facet of any experimental design (Wood and Leatham, 1992).

Intra-specific sampling is most often accomplished in laboratory studies of phytoplankton by using different strains, isolates or clones (Table 1). A measure of the degree of variation present within a species is revealed by the data obtained using strains. To obtain a representative sample of intra-specific variation on the scale being studied, researchers often choose strains because of their different spatial and/or temporal origins. Documentation of

origin is one of the most important aspects of strain designation. Recording and reporting this detail is now *de rigueur* in curating and publishing phytoplankton research.

Given the explicit temporal and spatial origin designated to any given strain, it is tempting to regard that culture as a *living snapshot of a species in time and space*. As such, strains of different origin are often used to study how temporal and geographic patterns of phytoplankton diversity correlate with environmental conditions (e.g., Penno et al., 2000; Marshall and Newman, 2002; de Boer et al., 2005; Degerholm et al., 2006; Ignatiades et al., 2007). Conclusions reached by such comparative studies rely on the implicit assumption that the measured properties of a laboratory culture reflect those of the natural population from which the strain was isolated. Despite a growing body of evidence indicating that key phenotypic and genomic properties of strains may change over time with continued culture (Table 2), this assumption is still often made.

The publication of this special issue of *Harmful Algae* on intra-specific variation presents an opportunity to examine the question of what a strain actually represents, and to examine the processes that cause laboratory cultures to change over time. In the first section of this review we discuss evolutionary principles and the relevance of these principles to the isolation, establishment, and maintenance of phytoplankton cultures. This discussion will be illustrated with empirical observations showing changes in the

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Table 1
Definitions of certain terms as used in this review.

Term	Definition
Strain	A unialgal phytoplankton culture of defined origin which is kept as a distinct, discrete 'lineage' by serial transfer or continuous culture
Isolate	A strain which arises from a single individual
Clone	An isolate which is propagated exclusively through asexual reproduction
Fitness	Unless otherwise described, 'fitness' is a measure of the relative growth rate of phytoplankton.
Homothallism	A mating system in which there are no separate mating types and therefore cells of the same clonal lineage can mate
Heterothallism	A mating system in which only individuals of opposite mating type (e.g., 'male' and 'female') can mate
Isolation	The process by which individual(s) from natural populations are collected, transferred to laboratory conditions and begin to grow successfully
Establishment	The period after sustainable growth is established and during which the new culture is adapting to its new conditions
Maintenance	The period after which the culture has reached a reasonably stable optimum phenotype in its laboratory conditions

properties of such cultures. Examples taken from other kingdoms of life are relevant to the current discussion because the processes and dynamics of evolution are both generalizable (Elena and Lenski, 2003) and scale independent (Leroi, 2000). Therefore additional examples concerning the evolutionary dynamics of other organisms (*Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila* spp. and *Caenorhabditis elegans*) will be presented. Finally we will make some recommendations for experimental practice in light of the issues discussed herein.

2. Processes generating variation

2.1. Mutation

Genetic diversity is a pre-requisite of evolution and is generated through two processes: recombination and mutation. Spontaneous mutation is an inevitable consequence of DNA replication and is thus unavoidable in any growing culture. Spontaneous mutants have been shown to arise at non-trivial rates in cultures of phytoplankton (Reboud and Bell, 1997; Costas et al., 1998; Kassen and Bell, 1998; Goho and Bell, 2000; Colegrave et al., 2002; Collins and Bell, 2004; Lakeman and Cattolico, 2007). Most mutations are

expected to have no effect on the phenotype. However, because of rapid growth rates and high population densities in laboratory cultures, rare mutations that do affect the phenotype are likely to arise. For example, mutations affecting stress tolerance accumulated when sub-clones of a clonal *Heterosigma akashiwo* culture were grown in parallel for approximately a month (Lakeman and Cattolico, 2007). Such spontaneous mutations may have significant impacts on fitness. Experiments with *Alexandrium minutum* illustrate this point (Costas et al., 1998). Distinct *A. minutum* clones, X and Y, were co-cultured for five weeks, after which they were re-isolated into separate X and Y cultures. These 'evolved' clones were then each competed against their parental clones, which in the interim had been maintained as cysts. Results of these experiments demonstrated that the competitive ability of both evolved clones had increased during the co-culture period. The authors interpreted their data as evidence for selection acting on spontaneously arising variability (Costas et al., 1998), although passage through encystment may have introduced an unacknowledged bias in these experiments. The examples cited above demonstrate the continuous production of mutations in laboratory cultures. Given this caveat, some have suggested that the concept of a genetically homogenous 'clone' is a Platonic ideal that does not exist in the real world (Lushai and Loxdale, 2002; Loxdale and Lushai, 2003; Lushai et al., 2003).

Most spontaneous mutations arising through DNA replication error are expected to affect only one or a comparatively small stretch of nucleotides (e.g., point mutations or small-scale insertions/deletions). Larger genome modifications can be made by the movement of mobile genetic elements (transposons). For example, two percent of the genome of the diatom *Thalassiosira pseudonana* was found to be composed of active or relict transposons (Armbrust et al., 2004) and 5.8% of the genome of the diatom *Phaeodactylum tricorutum* was composed of long-terminal-repeat retrotransposons (Bowler et al., 2008). Notably, most classes of transposons were found in the complete genome sequence of the prasinophyte *Ostreococcus tauri* (Derelle et al., 2006). Even larger genomic re-arrangements have been documented to occur in algal cultures. Whole and partial genome duplication (polyploidy and aneuploidy) events were inferred from differences in the cellular DNA content and the ratio of gene copy number to genomic DNA quantity among an assortment of *Thalassiosira weissflogii* strains and sub-clones (von Dassow et al., 2008). Strikingly, the copy number of three separate genes

Table 2
Changes that have been recorded in the properties of phytoplankton cultures.

Change	Taxon	Reference
Life history		
Change in life cycle phase	Prymnesiophyceae	Houdan et al. (2004)
Loss of sexual phase expression	Bacillariophyceae, Chlorophyceae	Montresor and Lewis (2005)
Physiology		
Adaptation to liquid/solid growth medium	Chlorophyceae	Goho and Bell (2000)
Decline in heterotrophic ability	Bacillariophyceae, Cryptophyceae	Lewitus (2005)
Decline in phototrophic efficiency	Chrysophyceae	Jones et al. (1996)
Decreased bioluminescence	Dinophyceae	Sweeney (1986); von Dassow et al. (2005)
Loss of sensitivity to shear	Dinophyceae	von Dassow et al. (2005)
Slower maximum growth rate	Dinophyceae	Sweeney (1986)
Toxicity	Dinophyceae	Martins et al. (2004)
Weaker circadian rhythm	Dinophyceae	Sweeney (1986)
Loss of biomineralization	Prymnesiophyceae	Paasche (2001)
Genome		
Changes in ploidy	Dinophyceae, Bacillariophyceae, Chlorophyceae	Loper et al., 1980; Holt and Pfister, 1982; von Dassow et al., 2008; Hoshaw et al., 1985
Loss of heterozygosity	Bacillariophyceae	Murphy (1978)
Ecology		
Altered associated bacterial community	Raphidophyceae	Connell and Cattolico (1996)

varied in an inconsistent manner among sub-clones of a single strain. This set of sub-clones had been cultured less than three years since being established from a common progenitor clone. Similarly, possible changes in chromosomal number have been observed in both dinoflagellates and green algae (Loper et al., 1980; Holt and Pfister, 1982; Hoshaw et al., 1985). Rapid genetic alteration at the whole-genome level during culturing is of particular concern when selecting laboratory-maintained isolates or clones for physiological, biochemical, molecular or whole genome sequencing studies.

2.2. Recombination

Recombination will contribute genetic variation to any phytoplankton culture that undergoes sexual reproduction. Algal sexuality is well described and most commonly reported in cultures of bacillariophytes, haptophytes, and chlorophytes (Montresor and Lewis, 2005). Although other classes of microalgae are also thought to undergo occasional sexual reproduction, such cases are less well documented. However, even if sexual life history phases have not been observed microscopically, the potential for cryptic sexuality should not be discounted (e.g., Kremp and Parrow, 2006). Cultures of homothallic diatom species are generally expected to manifest sexuality because the gradual decrease in cell size observed during normal asexual division is commonly reversed through an obligatory sexual phase (Chepur-nov et al., 2004). Sexual reproduction will have consequences for the genetic diversity within cultures. For example, *Thalassiosira pseudonana* and *Skeletonema costatum* cultures exhibited marked reduction in heterozygosity only 6 months after they were established (Murphy, 1978). This genetic change was interpreted to have occurred as the result of sexual reproduction, followed by selection for homozygous offspring. Another plausible explanation for this change is homogenization at the observed alleles due to ameiotic recombination (Omilian et al., 2006; see below).

Even completely asexual cultures may not be immune to the effects of recombination. Asexual recombination may alter genetic diversity, either by generating or removing new genotypes. Processes effecting such change are numerous. For example, movement of transposable ‘Ty’ elements may produce new genotypes, as seen in *Saccharomyces cerevisiae* (Wilke and Adams, 1992; Wilke et al., 1992). Additionally, recombinant genotypes

may arise from lateral gene transfer, which in turn could be facilitated by cryptic viral infection of cultures (Lawrence, 2005). Conversely, copy correction may act to reduce diversity by homogenizing alleles in the polyploid chloroplast, mitochondrial or nuclear genomes of many phytoplankton.

3. Processes altering gene frequencies

Although recombination and mutation can lead to genetic variation, it is the processes of genetic drift, inbreeding depression and selection that change the frequencies of genotypes and therefore effect the evolution of cultured cells.

3.1. Genetic drift

Drift occurs when stochastic events or processes alter the frequencies of alleles and/or haplotypes in a population. Evolutionary theory predicts drift will be a significant factor when a population is small or when it passes through a ‘bottleneck’ that reduces population size. At least one of those conditions is met in the maintenance of most phytoplankton cultures. During the serial transfer of phytoplankton batch cultures, usually a relatively small inoculum (consisting of a few hundred to a few tens of thousands of cells) is taken from the parent culture to start a new batch. That inoculum is a random sample of genetic diversity within the parent culture. Unless the volume transferred is large enough to preserve every genotype in exact proportion to what exists in the parent culture, this sampling will introduce genetic drift into the evolutionary history of the strain. Drift can cause non-adaptive, even slightly deleterious mutations to be fixed in the population, a process known as mutation accumulation. Fig. 1 presents the evolutionary consequences of microalgal culture in terms of fitness in laboratory conditions and fitness relative to the natural population. There is a strongly negative contribution of accumulated mutations that are fixed by drift—a significant factor when effective population size (N_e) is small. It should be noted that N_e for serial batch cultures is the harmonic mean of the population size, or roughly the product of the inoculum size and the number of generations needed to reach stationary phase. The specific extent and rate of mutation accumulation in any given microalgal strain will depend on many properties unique to that culture; degree of initial heterozygosity, pre-existing deleterious mutation load, rate

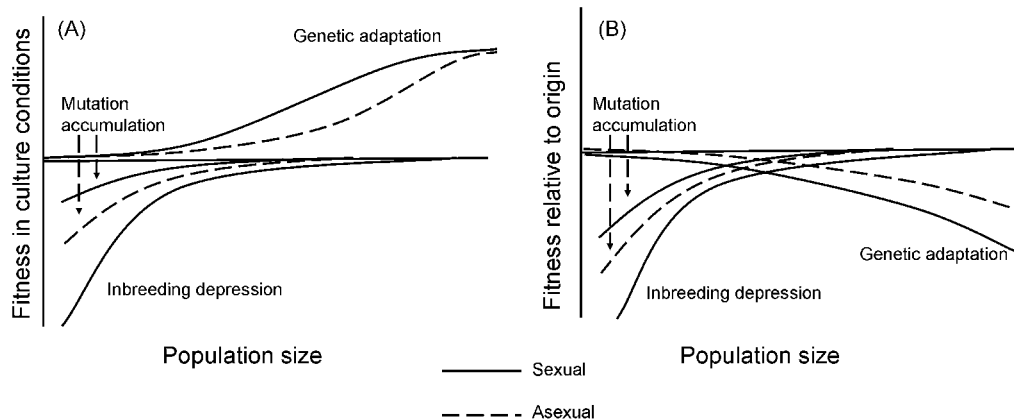


Fig. 1. The expected effects of in-culture evolutionary processes on overall fitness, as a function of population size. (A) Fitness in culture conditions. (B) Fitness of the culture relative to the original population, in the natural environment (i.e., the degree to which a culture is expected to have diverged from its original population). Solid lines indicate expectations for cultures that undergo sexual reproduction, while dashed lines indicate expectations for obligately asexual cultures. The exact shape of these curves will depend on many factors and would be expected to change over time (this plot shows fitness effects that would be reasonably expected at some point during early maintenance). The curves presented here indicate only qualitative, relative measures of the effects of these processes. For the interests of clarity, curves representing the combined effects of these processes have been omitted. However, visual inspection of the right panel shows that intermediate population sizes theoretically should afford less divergence from the original state and that sexual populations should suffer greater negative effects of culture than asexual ones, especially at low population sizes where the effects of inbreeding depression are greatest. Modified after Woodward et al. (2002).

of recombination and strength of selection. Unfortunately this complexity prevents any generalizable statement of when N_e is 'small enough' to allow significant mutation accumulation.

3.2. Inbreeding

Inbreeding depression occurs when sexual reproduction between closely related individuals brings recessive deleterious mutations together into one genotype, which then becomes less fit than either parental genotype (Charlesworth and Charlesworth, 1987). Heterothallic diatoms require mixing of clones for successful mating. If two clones are chosen for mating that are too closely related, then inbreeding depression may result (Chepurnov and Mann, 1999, 2000). This outcome is also likely when single diploid cells give rise to cultures that undergo sexual reproduction.

3.3. Selection

Selection occurs when conditions favor the growth of one genotype over another. Here we define and discuss three main processes in culturing phytoplankton that may exert selective pressure: (a) isolation, (b) establishment and (c) maintenance (Table 1; Fig. 2).

3.3.1. Isolation

The first selective event in the history of any microalgal culture may be the isolation of the targeted organism from its natural environment. As anyone who has attempted to isolate phytoplankton can attest, this process can be very difficult and potentially selective (e.g., Brand, 1982; Shankle et al., 2004). The culture conditions into which newly isolated cells are placed will almost certainly differ from those in the natural environment from which the population was sampled (Roszak and Colwell, 1987). Those individuals whose physiology is better adapted to the new culture conditions will have a selective advantage in the generation of the new population (Service and Rose, 1985).

In any situation in which new isolates are not established with 100% efficiency, selection may have occurred. Of course, the

isolation procedure itself may irreversibly damage or kill cells in a random manner, especially where physical separations such as flow cytometry or single-cell picking are used. Also, less than perfect isolation efficiencies may be the result of sampling from a natural population that contains cells that are simply unhealthy or lacking in viability (Rynearson and Armbrust, 2004). However we may gain insight into selective processes when quantitative measures of isolation success are available. For example, survival of single cell isolates from distinct populations of the diatoms *Ditylum brightwellii* and *Pseudonitzschia pungens* were reported to range from 38% to 96% (Rynearson and Armbrust, 2004) and between 46% and 55% respectively (Evans et al., 2005). Because these two studies were designed to sample the genetic variability within populations, researchers chose quantitative approaches to isolation (single-cell and single-chain picking, respectively). However, in instances where the goal is simply to establish a culture of an alga of interest, methods are often used that offer little quantitative measure of cell recovery efficiency. Enrichment and end-point dilution are commonly used isolation procedures in these circumstances. Severe selective bottlenecks may occur during the application of such methods, yet selection for a small proportion of tolerant individuals would not be apparent, except for a vague qualitative sense of the difficulty in initiating successful cultures.

Any collection from a natural population is a sample. Moreover, if selection occurs, the genotypes that survive in culture are a restricted and non-random sample of the original collection. Selection during culturing introduces a bias in how well those cultures that are established represent their natural populations (Rodríguez-Valera, 2004). The advent of sensitive single-cell, PCR-based genotyping technology should allow further quantitation of the degree to which cultures do or do not represent the populations from which they were isolated (Chandler et al., 1997).

3.3.2. Establishment

When an isolated alga whose phenotype may not be optimal for the imposed culture conditions begins to adapt to its new environment, one or more of the three following mechanisms may be used. At the smallest temporal and biological scales,

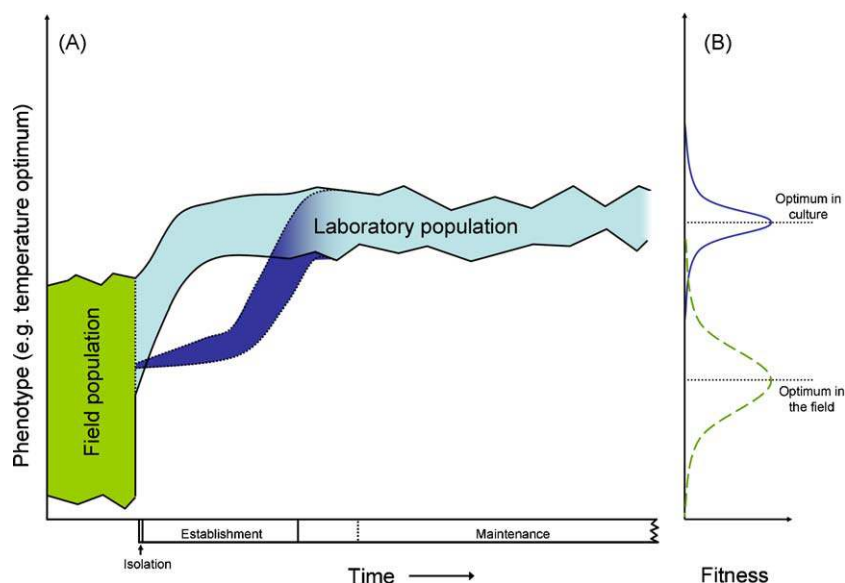


Fig. 2. The three processes of culturing algae can cause evolution of the phenotypic properties of the alga of interest. The vertical axis indicates a quantitative phenotype that is under selection in culture conditions. (A) The distributions of phenotypic values over time in the field (green) and then in the laboratory (blues). Light blue indicate the progress of a culture that is isolated by an enrichment-type approach or bulk isolation, whereas the dark blue indicates a culture established from a single cell. (B) Hypothetical fitness functions for culture conditions (solid blue line) and field conditions (dashed green line). For clarity this figure presents only a single phenotypic trait of interest. In reality the complex phenotype of the organism will consist of many traits occurring in an n -dimensional adaptive landscape (Arnold et al., 2001).

reversible *physiological* change will allow cells to survive altered conditions. Over longer time frames, *epigenetic* adaptation may take place, which allows heritable changes in gene regulation or expression to occur. This modification, though heritable, happens without any change in the sequence of the DNA itself. Lastly, *genetic* adaptation occurs through differential inheritance of genetic variation (i.e., an alteration, over time, of the genetic complement). For the purposes of this review we will concentrate on genetic adaptation. However, a discussion of the overlapping time scales and difficulties in disentangling these processes of adaptation can be found below under the heading 'Acclimation'.

Marked evolutionary change may occur as an algal culture is established, because selection drives the phenotype of the culture towards a new optimum. This process was modeled by Lynch et al. (1991). These authors, using a quantitative genetics approach, predicted (a) an initially slow rate of change, while diversity is established in the new culture and (b) a subsequent period of rapid evolution that significantly alters the phenotypic characteristics of the population. These changes were predicted to occur within a few hundred days of culture initiation (Lynch et al., 1991; see Fig. 1). This model examined evolution in an asexual population. Sexually reproducing cultures are expected to adapt even faster and to a greater extent than asexual cultures. These predictions were confirmed by empirical evidence gathered when sexual and asexual *Chlamydomonas* cultures were subjected to novel environments (Colegrave et al., 2002; Kaltz and Bell, 2002). In these experiments, variance in fitness among sexual lines increased relative to asexual lines immediately after sexual episodes, even though mean fitness of sexuals temporarily dropped below that of asexuals. However, recovery was rapid, and sexual lines adapted at an overall greater rate and to a greater extent in certain environments.

Temperature, irradiance, salinity and nutrients are extrinsic factors which may, in culture, differ significantly from the natural environment and therefore drive directional selection during establishment. Although all four cues can be experimentally manipulated over a reasonably continuous range, discrete choices of these variables must be made for microalgal curation. For example, because of practical considerations, culture collections cannot provide the continuous spectrum of temperatures that are present in nature. The choice of culturing temperature is limited by the number and range of culture chambers available, with the result that the culture may be maintained at a temperature very different from that found in the isolate's natural environment. Aside from this practical limitation, cultures are often grown purposely at temperatures higher than those of their natural environment. These unnatural conditions are chosen to increase growth rate in order to expedite research for which biomass accumulation is rate-limiting. For the same reason, some cultures are grown under constant light, a most unnatural condition for almost any alga. Conversely, for long-term routine maintenance, temperatures and/or light levels may be reduced to slow growth and thereby allow a greater time between transfers (Mann and Chepurnov, 2004). Finally, conditions in nature tend to change continuously whereas conditions in culture are generally held fairly constant. Diatoms are thought to be highly adapted to growth in turbulent, rapidly changing environments (Margalef, 1978). The imposition of constant conditions on diatom cultures may disrupt natural life cycle processes allowing, for example, the formation of unusually small cells (Mann and Chepurnov, 2004; von Dassow et al., 2006).

The phenotypes that determine an optimum temperature, irradiance, nutrient level or salinity needed for growth are 'quantitative traits' (Mackay, 2001), meaning that variation in the traits occurs across a continuous spectrum and has a polygenic basis. Because many genes contribute to each of these phenotypes,

quantitative traits respond readily to selective pressure by incremental change at many loci (Houle, 1992). Such selective pressure may be exerted by culture conditions that do not reflect the natural environment from which the cells were isolated. In an extreme example, volvocalean snow algae that had been curated in a culture collection at 20 °C grew either not at all at 4 °C or very slowly compared to conspecifics maintained in culture at 4 °C (Hoham et al., 2002). This example shows that given strong enough directional selective pressure, laboratory cultures of phytoplankton can reverse tens of thousands of years of evolution in a few short decades.

The process of selection during establishment will occur *every time* culture conditions are altered. This change in environment might be as subtle as a shift in light quality when growth chamber bulbs are replaced or as profound as a modified growth medium when a strain is sent to a new laboratory. Even when the same nominal enrichment medium is made in a new location, final concentrations of key nutrients could differ dependent upon the water source. For example, a *Gymnodinium catenatum* isolate grew at salinities from 15 to 36 PSUs (optimum between 26 and 30 PSU) when seawater from Vineyard Sound, USA was used to make f/2 medium. However, when seawater from Bahía Concepción, Mexico was used to make the same nominal medium, growth occurred at salinities between 25 and 40 PSU (optimum between 28 and 38 PSU; Band-Schmidt et al., 2004). Many media require soil extracts as ingredients, and changes in the source of the soil can lead to culture failure (ECS Duffield, personal communication). Although one may expect seawater and soil from different sources to have unique properties, even subtler differences can lead to 'laboratory dependence'. For example, the outcome of competition experiments between *E. coli* strains was influenced by slight compositional differences between the de-ionized water that was used to make the growth medium in two different laboratories (O'Keefe et al., 2006).

Consideration of subtle changes in culturing conditions is important in comparative physiology studies when strains isolated from distant locations are compared. Those strains may have been maintained in different media and/or environmental conditions. As noted above, even a slight change in culture conditions may drive a directional shift toward better adapted genotypes. Thus, the more times a strain is transferred between laboratories and subjected to novel growth conditions, the greater the potential for it to evolve properties that deviate from those of its original phenotype.

3.3.3. Maintenance

After the potentially rapid evolutionary change of establishment has slowed, and the mean phenotype of the population has equilibrated around its optimum in new culture conditions, significant evolutionary change may still occur. One process that may drive established cultures to evolve is the heterogeneity of selective pressures experienced as the culture progresses through different growth phases (i.e., lag, logarithmic, stationary). Distinct phenotypes may be expected to have relative advantages in these differing growth phases (Dykhuizen, 1990). The ability to grow rapidly under non-limiting conditions would be selected for in exponential growth (r-type or weedy competitors), whereas a slightly better ability to use or acquire whatever factor is limiting during stationary phase, or tolerate the toxic waste products accumulated therein, would be advantageous at that stage (K-type or dominant competitors). Despite theoretical considerations that suggest a generalist type should evolve in such heterogeneous environments (Reboud and Bell, 1997), stable polymorphic populations of specialists have been documented numerous times (e.g., Rosenzweig et al., 1994; Rainey et al., 2000; Rozen and Lenski, 2000; Dykhuizen and Dean, 2004). Whether specialists or a generalist evolve to deal with these heterogeneous selective

pressures may depend on the temporal scale of environmental variation (Kassen and Bell, 1998).

During maintenance of batch cultures, cells periodically enter stationary phase, where growth is saturated and cell density is maximal. These conditions may be abnormally stressful in terms of algal physiology, as few natural populations ever experience such densities for any extended period of time. Cells in stationary phase may be subject to unnaturally high concentrations of secondary metabolites or excreted organic matter (Hellebust, 1965; Lakeman, unpublished observation). Additionally, pH may increase above normal field levels because limited gas exchange is not able to balance the inorganic carbon uptake by the algae (Sunda et al., 2005). These abnormal conditions may lead to selection acting on ecologically relevant traits.

The coccolithophore *Emiliania huxleyi* provides a particularly curious example of unidirectional changes in phenotype that repeatedly arise in long term culture maintenance, potentially as a result of selective pressures applied during stationary phase. Cells are isolated as the calcified diploid form. In culture, calcification continues even after growth stops, resulting in severe drawdown of alkalinity during stationary phase. Because of this effect, archival culture conditions, in which cells are repeatedly exposed to stationary phase, may provide direct selection against calcification. Perhaps as a consequence, non-calcifying “naked” diploid mutant cells frequently arise and take over cultures of *E. huxleyi* (Paasche, 2001). Several studies have attempted to predict the affects of anthropogenic changes in atmospheric CO₂ on coccolithophorids using cultured strains (Riebesell et al., 2000; Zondervan, 2007; Iglesias-Rodriguez et al., 2008). If calcification is indeed subject to rapid selection, care should be taken in extrapolating from short-term experiments with single isolates that have potentially been subjected to severe selective pressures for survival in low alkalinity conditions during long-term culture maintenance.

4. Consequences

The forgoing discussion reviewed overt processes that influence evolution in phytoplankton cultures. The following discussion deals with some less obvious consequences of evolutionary change.

4.1. Correlated responses

One may consider the complexity of genetic interactions within an organism as being represented by a tangled web. One should therefore not be surprised that when selection pulls on one thread, other threads in the web may feel tension. Such threads, or traits, are considered to be genetically correlated. In-culture selection for increased performance on one trait will cause a reduction in performance of a negatively correlated trait. Such a correlated response has been seen in *Drosophila* cultures where laboratory-imposed breeding regimes have selected for greater early fecundity with a concomitant, unintentional decrease in longevity (Linnen et al., 2001). Similarly, a negative correlation was reported to occur between initial growth rates and final cell yield within *E. coli* populations (Novak et al., 2006). Such negative correlations can be the result of antagonistic pleiotropy (where the effects of one gene act on two traits in different directions), linkage disequilibrium between two loci (one of which is strongly selected for), or mutation accumulation (Lande, 1980, 1984; Rose and Charlesworth, 1981; Brooks, 2000).

4.2. Use it or lose it

Just as laboratory conditions may be too stressful to allow cultures to retain fidelity to their origins, paradoxically, they may

also be too benign (Huey and Rosenzweig, 2009). The absence of selection in culture may in fact be more troublesome for the experimental phycoecologist than its presence. In many respects the environmental conditions experienced by an algal culture are considerably more stable and benign than those found in the natural environment. Abiotic factors such as temperature and salinity are practically invariable in cultures, whereas seasonal, daily and, especially in the case of estuarine phytoplankton, tidal cycles are the norm in nature. Laboratory media also differ from waters found in natural environments because they are usually enriched for nutrients and vitamins to levels which are found only in the most eutrophied waters. In culture conditions, biotic interactions are also markedly reduced (or absent) relative to the environment from which they were isolated. Unialgal and axenic cultures lack inter-specific interactions which may be instrumental in driving the evolution and/or stability of certain traits (Kisdi, 2001; Abrams and Chen, 2002). Using benign laboratory conditions may, at first glance, be an effective and appealing approach for culturing an alga of interest. However, like the proverbial child who is spared the rod, these cultures may be spoiled, especially in their usefulness for the study of responses to genuinely stressful conditions.

As mentioned earlier, any growing culture will be expected to have a continuous supply of mutations. Strongly deleterious ones will be selected against and purged rapidly. In contrast weakly deleterious mutations may accumulate because of drift. Many mutations, however, would be expected to be neutral, meaning they have no effect under the current growth conditions. Further, some of these will be *conditionally* neutral, meaning that in routine culture they will have no effect, but in altered or novel conditions they may be deleterious (or in a small minority, beneficial). A good example of this process was demonstrated in experiments that used asexual cultures of *Chlamydomonas reinhardtii*. After replicate clones were grown for 100 generations in either liquid medium or on solid medium, their fitness was measured, relative to each other. On solid medium, the liquid-grown clones exhibited fitness that was significantly lower than that of the solid-grown clones, and *vice versa* (Goho and Bell, 2000). These authors interpreted the data to imply that mutations had accumulated during sustained liquid growth and these negatively affected the cells' ability to grow on solid medium. However, these conditionally neutral mutations acquired during liquid growth were not ‘exposed’ (and therefore not purged by selection) until the clones were subjected to growth on solid plates (Goho and Bell, 2000). A further example of the loss of fitness in one environment after sustained growth in another has been observed, also in *Chlamydomonas*. Parallel cultures of this alga were maintained either phototrophically (in constant light) or heterotrophically (in constant dark on acetate medium). After approximately 100 generations, the light-grown strains performed markedly worse when assayed for growth in the dark, than did the dark-maintained controls, and *vice versa*. This example illustrates a caveat for examining cultures of phytoplankton that exhibit facultative osmo-, hetero- or mixotrophy in the field, but are maintained under strictly phototrophic conditions in the laboratory (Lewitus, 2005 and references therein).

Conditionally neutral mutations in stress response pathways may also accumulate under non-stressing conditions, because selection will not be acting against them. Effects of these mutations will be observed only when the culture is subjected to a chosen stress. Vassilieva et al. (2000) found that laboratory-maintained *Caenorhabditis elegans* lines accumulated mutations that had negative fitness effects when worms were exposed to stressful growth conditions. These mutations had considerably lower net effect on mean fitness under non-stressful conditions.

In addition to mutation accumulation in long-term cultured strains, stress tolerance may also degenerate via other means, such

as antagonistic pleiotropy or linkage disequilibrium between a selected locus and one that impacts stress response. These latter two processes are likely to occur if measurable improvement in a non-stress related trait is observed, due to selection. In this case, degeneration in the stress response could be due to a negatively correlated response to the selected change. However, in most cases it will be difficult to distinguish which process has occurred. That said, the following example illustrates that the temporal dynamics of change can be revealing (Elena and Lenski, 2003). Newly established laboratory strains of *Drosophila* rapidly lost, over a three-year period, the ability to withstand desiccation (Hoffmann et al., 2001). The speed of the deterioration in these flies' stress tolerance was thought to be too great to have occurred solely through mutation accumulation. It was therefore suggested to have occurred as a negatively correlated response to selection for increased fecundity. Given the importance of stress tolerance in

structuring populations (Maltby, 1999), and increasing concerns about anthropogenic environmental perturbation (Boyd and Doney, 2001; Scavia et al., 2002; Parmesan and Yohe, 2003), the analysis of phytoplankton responses to stress represents an important field of research. Data suggest that care should be taken in using long-established strains in determining stress responses (Fig. 3).

Remarkably, some loss-of-function mutations may actually confer an adaptive advantage in culture. A constitutively expressed cellular process that is not maintained by selection in culture, but has some energetic cost, may be lost over time because of 'energy conservation' *sensu* Regal, (1977; Zamenhof and Eichhorn, 1967). Under excessive nutrient supply, characteristic of many growth media, stabilizing selection on certain nutrient uptake mechanisms may be reduced (Lynch et al., 1991). For example, the function of high-affinity transporters may be lost from cells cultured for

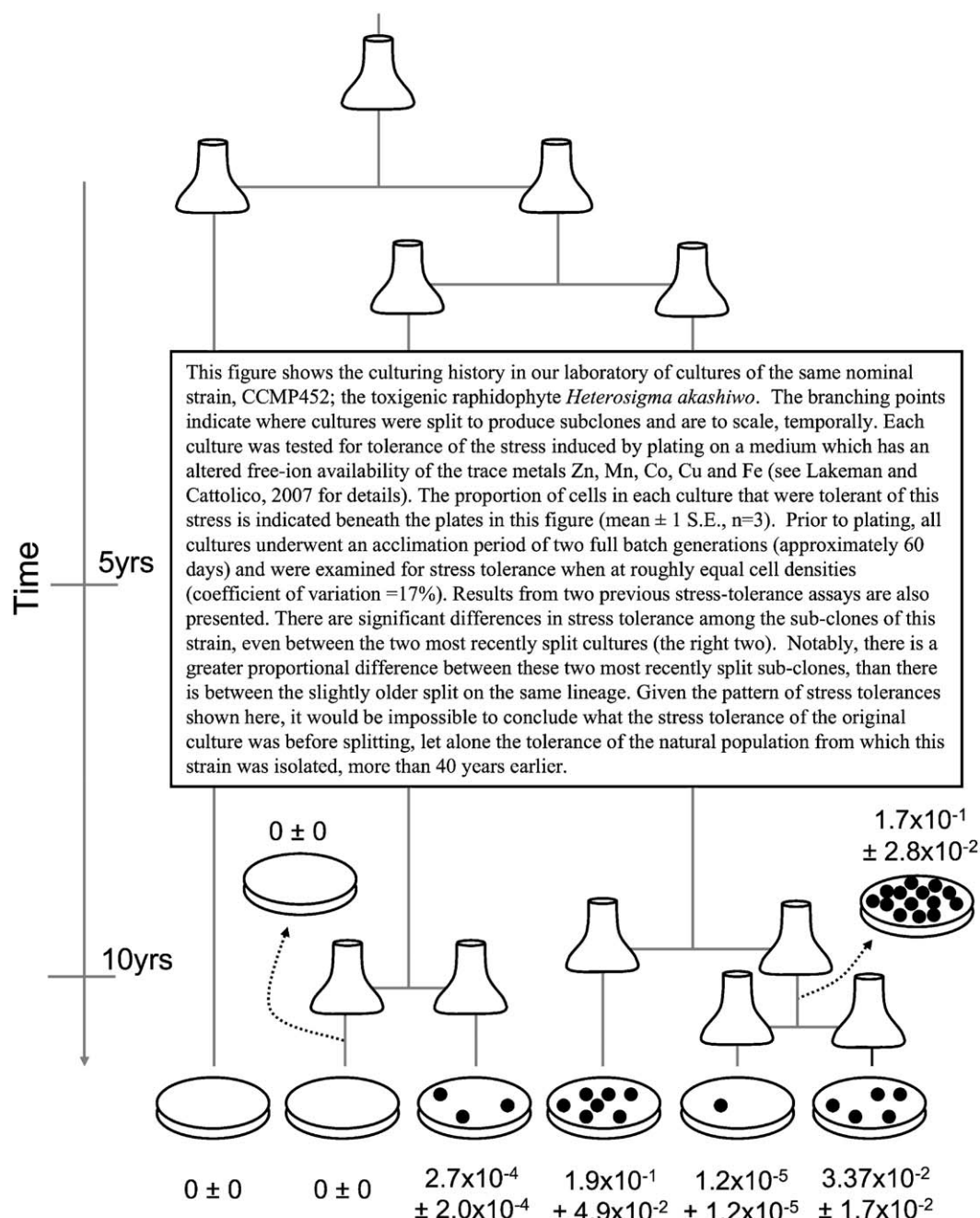


Fig. 3. Stress tolerance is a labile character, varying significantly among sub-clones of a single strain.

extended periods in replete conditions. Additionally, loss of function in biosynthetic pathways can also occur. Consider the biotic environment in culture: a unialgal environment lacks selection for ecological traits such as allelopathic toxicity. A mutation leading to the loss of a constitutive toxin pathway could actually be advantageous. Because cellular resources could be used for growth instead of toxin production, non-toxic mutants would eventually out-compete toxin producers, thereby changing the phenotype of the culture. The following example of evolutionary change in cultures of harmful algae is consistent with a loss of function due to energy conservation. Two sub-clones of a previously toxic *Alexandrium lusitanicum* strain were grown in different laboratories for 12 years, then tested under identical conditions for toxin production. One remained toxic, while the other had lost the ability to produce toxin (Martins et al., 2004). This observation is notable for both demonstrating the loss in culture of an ecologically critical trait and also illustrating the inherently stochastic nature of mutations which underlie evolution.

4.3. Cultural castration

Most microalgae are capable of extended periods of asexual growth, interspersed with sexual reproduction. Both theoretical considerations from life history theory (van Noordwijk and de Jong, 1986; Stearns, 1989) and empirical observations of experimental populations (Ronsheim and Bever, 1987; da Silva and Bell, 1992; Thompson and Eckert, 2004) indicate that these two modes of reproduction have evolved to be in a balanced trade-off. If selection acts to increase the capacity for one form of reproduction, a correlated decline in the performance of the other mode could be expected. Indeed, lines of *Chlamydomonas* selected for increased rate and efficiency of sexual reproduction exhibited decreased vegetative growth rates (da Silva and Bell, 1992). Conversely all-female *Drosophila mercatorum* cultures reproducing for 20 years by parthenogenesis lost their mating capacity (Carson et al., 1982). The implication here for laboratory cultures of phytoplankton is clear. Unless periodic sexuality is induced by routine or experimental culture conditions, life-history characters of these microalgae should be expected to degenerate, possibly to the point where sexual reproduction, and the potential ecologically important morphological and physiological changes that co-occur with sex, may be lost entirely. Evidence for selection against sexual reproduction has been seen in diatoms, which often display reduced sexuality with time in culture (Chepurnov et al., 2004).

4.4. Mutational meltdown

It has long been understood that asexually reproducing populations will gradually accumulate mutations of small deleterious effect via drift, which lower the mean fitness of the population over time (Muller, 1964), a process known as Muller's Ratchet (Felsenstein, 1974). These populations will thus have lower fitness and will tend to become smaller and therefore more subject to the vagaries of drift. Increased drift will lead to accumulation of more deleterious mutations, which will lead to an even smaller population, which leads to even greater drift, etc. This feedback loop will eventually lead to 'mutational meltdown' (Lynch and Gabriel, 1990) and to loss of the culture. Such a process may occur in phytoplankton cultures in which the sexual phase of the organism is no longer expressed—sexual recombination allows slightly deleterious mutations to be combined into the same genotype, compounding their effects and increasing their chances of being purged by selection (Zeyl and Bell, 1997). Using conservative estimates of relevant parameters, theoretical calculations indicate that one deleterious mutation accumulating at each batch transfer of a culture of *Chlamydomonas* is realistic (de Visser et al., 1996). Empirical evidence of mutational

meltdown was observed in populations of yeast kept at effective population sizes of ~250 cells (Zeyl et al., 2001).

For several reasons Muller's Ratchet may progress at a significant rate in laboratory cultures of certain microalgae. First, the sexual phase of some phytoplankton may not be induced in routine culture conditions, especially when sexuality is linked to stress or the requirement of an opposite mating type. Second, serial transfer will introduce drift, increasing the likelihood of fixing slightly deleterious mutations. This concern applies especially to cultures which do not reach high density. Third, the relatively stable environment of the laboratory increases the chances that any given mutation will be only slightly deleterious. In a more dynamic environment the range of mutational effects sampled and purged by selection will be greater.

5. Revisiting Wood and Leatham

In a mini-review that discussed the use of strains as tools to investigate intra-specific algal diversity, Wood and Leatham (1992) compiled a table that presented results of studies comparing multiple strains within species. This table demonstrated nearly ubiquitous intra-specific diversity; in the majority of cases, significant differences in key physiological properties were observed among isolates of the same species. However, the discussion presented above leads us to question whether the differences documented by Wood and Leatham (1992) truly reflect real differences among natural populations, or instead reflect in-culture evolution and divergence. We re-examined the papers cited in Wood and Leatham's Table 1, specifically looking for details regarding the isolation and maintenance of the strains presented. In only eleven out of thirty eight studies were the culture conditions of the compared strains similar enough to suggest that the observed intra-specific variation was probably attributable to differences between natural populations. In the remaining papers culture conditions either were not reported or differed so dramatically for the strains in question that it is impossible to determine whether the observed differences were due to (a) adaptation to the natural environment from which the strain was isolated, or (b) evolutionary changes while in culture. Though we suggest caution in interpreting the analysis in the Wood and Leatham paper, the conclusions presented in that work remain valid. Significant intra-specific diversity certainly exists among the phytoplankton. It is only the attribution of cause for those differences that we wish to question.

Adaptation to the environment from which microalgal cultures have been isolated is almost always invoked to explain their physiological and phenotypic characteristics. Aside from valid criticisms of this adaptationist paradigm (Gould and Lewontin, 1979; Brand, 1984; Lynch, 2007), these discussions all too often ignore adaptation to the culture conditions in which the strains are subsequently maintained. The processes of evolution know no bounds, and do not cease to exert their influence even in our controlled laboratory environments.

6. Recommendations

Given that phytoplankton in culture will inevitably undergo major evolutionary changes, can one meaningfully perform comparative ecophysiological experiments? We believe so, as long as the experimental design and interpretation of data recognize the potential for in-culture evolution. We now consider some of the important facets in designing good comparative physiological studies on phytoplankton (Table 3).

Larry Brand was one of the pioneers of comparative physiological studies of phytoplankton. He conducted large experiments comparing the growth rates (Brand, 1981, 1982, 1985), salinity

Table 3

Evolutionary processes, their impacts phytoplankton and approaches that can be taken to minimize those impacts.

Process	Impact	Approach to minimize
Mutation Recombination	Input of genetic variation Novel combinations of alleles. Increased rate of genetic adaptation	Cryopreservation Avoid sexual reproduction where possible
Genetic drift	Stochastic population dynamics. Fixation of maladaptive alleles. Mutational meltdown	Avoid small population bottlenecks; frequent, large- inoculum transfers
Inbreeding depression	Lowered fitness	Avoid sexual reproduction where possible. Alternatively, establish defined parallel mating lines.
Selection	Genetic adaptation to culture conditions. Correlated changes in linked, but unselected traits	Mimic isolates' 'natural' conditions as closely as possible. Take measurements of properties of interest as soon as possible

tolerances (Brand, 1984) and iron requirements (Brand, 1991) of phytoplankton strains and species. For his intra-specific comparisons (Brand, 1982, 1985), Brand used what we view as the 'gold standard' approach. Single cells were isolated from environmental samples collected as close in time as possible and then placed into identical conditions. With this approach, the selective pressure applied by the culture conditions should be the same on each new isolate. Taking samples as close together in time as possible controls for the effects of incremental evolution in culture. Ideally, if these first two conditions are satisfied, physiological measurements should be taken as soon as sufficient biomass is available, thereby minimizing the chances of in-laboratory divergence from the state of the natural population. This approach was also recommended by Murphy (1978).

In studies where large spatial scales are being investigated, isolates collected from different locations should be collected as close together in time as is practicable and should at least be isolated into the same media and cultured under the same conditions. However, this approach may present a risk that, if the natural environment differs greatly between the regions being studied, using identical conditions for culturing may drive a form of convergent micro-evolution. Such convergence has been observed in adaptation to the laboratory by newly established cultures of *Drosophila* (Matos et al., 2002). In contrast, it must be noted that quantitative genetic models predict that uniform selection acting on isolated populations could actually increase the divergence between samples, especially if the selection is weak (Cohan, 1984). Clearly, care must be taken in interpreting results where samples originate from different environments.

Research into population structure and genetic variability with an explicit temporal component is even more problematic, and the best approach to take is less certain. When collections are made at different time points, the researcher is presented with a difficult choice. Measurements of the traits in question can be made either (a) at the same time on cultures of different age, or (b) at different times when cultures are at the same age. The former approach is most commonly taken, as a 'common garden' allows apparent differences to be ascribed to genetic rather than environmental factors. Most researchers would balk at the latter approach, as inconsistencies in experimental environments from the time of one measurement to the next may confuse results. However, in light of the current discussion, making observations on cultures that have spent different lengths of times in the laboratory should be a concern. Because evolution is a progressive process, older cultures should depart more from their 'natural state' than will newer isolates, especially in terms of traits that are not under selection under regular culture conditions.

With studies on cultures of different ages, concerns of consistency in experimental conditions and uncontrolled evolution are seemingly irreconcilable. The researcher certainly has greater control over the environmental conditions of experimental treatments than over the stochastic events of evolution. We tentatively suggest that when comparisons are to be made between cultures isolated at different times, the measurements

should be taken when the cultures are at the same 'age' in terms of generations. We also strongly suggest that wherever practicable, replicate sub-clones of new isolates should be generated, and maintained in parallel. Such replication would control for divergence due to culturing (see discussion below). If the species of interest is amenable to cryopreservation, concerns about different lengths of time in culture are not an issue. In this case, cultures from earlier collection dates can be frozen at the same age and, once thawed, parallel experiments can be run.

6.1. Acclimation

For comparative physiological studies where new cultures cannot feasibly be collected, established cultures may be the only choice. Such experiments often include a period of 'acclimation' in which cultures to be compared are grown under a consistent set of conditions (the 'common garden') for a length of time that may be arbitrary (e.g., Sullivan and Andersen, 2001; Martins et al., 2004) or until some property of interest has stabilized (e.g., Brand et al., 1981b). The purpose of this acclimation period is an attempt to remove or minimize the effect of the history of previous culture conditions, so that in comparing strains from different sources, any dissimilarity observed after acclimation can be attributed to inherent (read: genetically programmed) responses to the treatment conditions, instead of plastic physiological responses to the immediate history of the cultures.

As discussed above, individual cells can adapt to changing conditions by reversible regulation of their metabolic capabilities (Staehr and Birkeland, 2006; Geider et al., 1998; Finkel et al., 2004). This process appears to be what most researchers are referring to when using the word 'acclimate'. In traditional 'common garden' experiments with sexually reproducing multicellular organisms such as plants and animals, acclimation is clearly confined to changes during the lifetime of any one individual. However, with rapidly reproducing unicells, acclimation can occur on the same time scale as genetic adaptation. In the context of this paper, when the change between previous culture and new acclimation conditions is too great for all cells to adapt physiologically, a population-level adaptation can occur, selecting genetically for only those variants that can tolerate the new conditions.

As scientists we should be precise in our communication. All too often the term acclimation is used without explanation of the adaptive processes that have occurred. We suspect that in most cases this omission is simply due to a lack of awareness. The key data that could help us understand this process are rarely presented; usually the length of the acclimation period is simply reported in the methods, if at all. If growth curves of the cultures being acclimated were presented for this period, bottlenecks in populations could be seen as declines in biomass, thereby illustrating a selective event. Such data were presented by Cifuentes et al. (2001) in a study of the salinity tolerance of nine *Dunaliella* strains. For some strains a steep initial decline in the growth curve was seen during acclimation to the most extreme salinities. This decline was followed by rapid growth,

suggesting selection for well adapted genotypes. Unfortunately the growth curves presented in this case were based on relative *in vivo* chl *a* fluorescence, which may not maintain a tight correlation with cell number during periods of stress (Slovacek and Hannan, 1977; Brand et al., 1981a; Sosik and Mitchell, 1991). Data based on absolute cell number would reveal more clearly the population dynamics of cultures.

Even where there is no obvious decline in population numbers in response to a stressful change of culture conditions, a prolonged 'lag' phase may mask a selective event. A near-constant cell density could result from an equilibrium between the death rate of maladapted cells and the growth of tolerant cells. With longer acclimation periods, genetic adaptation could occur simply through slightly higher growth rates of better adapted sub-populations, even without noticeable die-off of less adapted cells.

If the purpose of acclimation is to minimize the effect of previous environmental conditions so that the power to detect genetically encoded differences among strains is maximized, then any change in those genetic properties that is induced by acclimation will be counterproductive: what is being observed will have been altered by the approach used to observe it.

A partial solution to this conundrum is to include sub-clones or replicate lines in the design of any experiment that requires an acclimation period. This recommendation is based on a considerable body of literature that documents the unpredictability and variability of evolutionary trajectories, even of initially identical populations that are maintained under identical conditions (Harshman and Hoffmann, 2000; Cullum et al., 2001; Elena and Lenski, 2003; Reed et al., 2003). As pointed out by Rose et al. (1996) 'populations tend to differentiate with respect to one another in the absence of any intervention by the experimenter'. When small differences are present among evolving lines at the outset, the divergence of their responses may be even greater. In this case variation would be expected in both the rate and extent of adaptation (Rose et al., 1996). Replicate lines will reveal the contribution of stochastic evolutionary processes to variation in characteristics of interest after the acclimation period.

7. Conclusions

The forgoing discussion has outlined the many processes which, over time, alter the properties of microalgae that are maintained in laboratory culture. It is apparent that all these processes will unavoidably drive a strain to deviate from its condition when isolated from the field. The 'where' and 'when' of a strain's collection are important in distinguishing it from other strains of the same species. But those spatial and temporal data should not have undue weight in the consideration of the properties of an established culture. A strain really represents, over time, just one single trajectory manifested from the vast evolutionary potential possessed by the original isolate. We hope that this review has drawn attention to a new strain concept. A phytoplankton strain is not a static snapshot of a natural algal population, but rather a dynamic, ever-changing laboratory population.

The problems and processes reviewed in this work are in no way unique to phycology. In the health sciences, tissue cultures are routinely used for experimentation. Among researchers in these disciplines, there is an awareness that dramatic changes can occur to the properties of tissue cultures over time (Masters et al., 2000). In fact, the long established HeLa human carcinoma cell-line was even once proposed to be re-classified as an entirely new species because it is now so different from normal *Homo sapiens* cells (van Valen and Maiorana, 1991). Passage number, which indicates how many times a tissue culture has been transferred since being generated, ranks as a critical consideration in experimental design. For newly generated tissue cultures, threshold passage numbers are given, over which

cultures are discarded as useless in generating physiologically or developmentally relevant data (Masters et al., 2000). Microbiologists have also long recognized that bacterial cultures can evolve rapidly within the laboratory (Luria and Delbruck, 1943) and this feature has been exploited to develop model systems for studying the basic principles of evolution itself (Elena and Lenski, 2003).

There are lessons to be learned from these other disciplines. As with tissue culturing in the health sciences, phycologists should develop the critical awareness that the longer an isolate has been in culture, the further removed it will be from its 'natural state'. Long established cultures have their uses (especially as model organisms for research into basic cellular mechanisms) but comparative ecophysiology is not one of them. However we propose that the propensity for evolution to occur in laboratory cultures, although a hurdle for comparative algal physiology, should be used as a tool to develop the currently impoverished discipline of phytoplankton evolutionary dynamics. By acknowledging that evolution can and does occur in laboratory cultures at time scales that allow observation, one can begin to perform manipulative experiments to investigate micro-evolutionary responses of phytoplankton to well controlled changes in culture conditions. This acknowledgment would allow a movement from descriptive science, which permits only correlative interpretations of why algae have the traits they do, towards manipulative science, which allows direct tests of causality.

Two notable recent research efforts highlight the enormous potential in exploiting the ability of microorganisms to evolve in the laboratory. In the first, concerns about the evolutionary impacts of elevated CO₂ levels were addressed by allowing cultures of *Chlamydomonas reinhardtii* to evolve under such conditions. Non-adaptive changes were noted in physiological and phenotypic characteristics (Collins and Bell, 2004; Collins et al., 2006). This approach, which aims to experimentally determine the evolutionary response of algae to altered environments, was validated by comparing the high-CO₂ strains with isolates from naturally occurring CO₂ springs. The range and type of responses seen in the laboratory strains corresponded to those observed in the natural isolates (Collins and Bell, 2006). The second notable example, although not from the field of phycology, illustrates how rapidly advancing technological improvements should allow molecular characterization, down to the base-pair level, of specific evolutionary changes observed in laboratory cultures. An experimental lineage of the co-operative bacterium *Mycococcus xanthus* was observed to evolve through three distinct social phenotypes (social, cheater and dominant social) over time (Fiegna et al., 2006). High-throughput 'sequencing-by-synthesis' allowed accurate characterization of the complete genome sequence of the evolved strain, and identification of 15 single nucleotide polymorphisms responsible for the observed life-history alterations (Velicer et al., 2006). This example points to the potential for combining studies of experimental evolution with '-omic' technologies to greatly advance our understanding of the molecular mechanisms of adaptation and evolution in the phytoplankton.

Systems traditionally used for studying experimental evolution have generally been chosen for expedience and manipulability, rather than for ecological importance. Phytoplankton, and especially those that form harmful blooms, possess all these attributes, and therefore represent a relatively unique opportunity for basic evolutionary research with immediate environmental and societal importance.

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