PhD THESIS

Gomphonema parvulum (Kützing) Kützing: Ecophysiological, Morphometric and Observational Studies of a Species Complex

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ABSTRACT

DAWN TITIANNIA ROSE

Gomphonema parvulum (Kützing) Kützing: Ecophysiological, Morphometric and Observational Studies of a Species Complex.

Perturbations to environmental conditions experienced by any one species, can invoke a physiological and/or behavioural response that may be expressed morphologically and ecologically. This has important implications for diatom taxonomy and the use of diatoms as environmental indicators. The reliable recognition of a taxon with a particular life history, morphology, ecology and physiology may necessitate the adoption of narrower species concepts than those in current floras. This would have the advantage of clarifying the ecological ranges of taxa used in biological monitoring programmes.

Gomphonema parvulum has long been a taxonomic problem. The species exhibit considerable is found over a wide range of environmental conditions. It has also been used as an indicator of "pollution" in diatom indices of water quality.

Clones of *G. parvulum*, exhibiting a range of morphologies, were isolated from different sites into unialgal culture. Experimental investigations on the clones, tested against different environmental variables to determine ecological tolerances of different isolates, occasionally produced auxospores. This rare opportunity allowed the morphology of particular clones to be studied over their full size range (initial cell through to mother cells). Additionally, aspects of diatom behaviour, including sexual reproduction could be observed and recorded.

Results indicate that not only are there differences in cell shape and behaviour with size and environmental condition, but in some *G. parvulum* clones, heteropolarity is not determined in the auxospore or initial cell, but becomes established after a series of vegetative divisions. This increases the likelihood that specimens may have been incorrectly identified as different species or varieties (*G. parvulum* complex encompasses two previously described species, *G. gracile* and *G. hebridense*), underlining the need for experimental studies and culturing. These observations argue strongly against reliance on diatom valve morphology as the sole criterion on which to delimit taxa, and provide a compelling argument for the benefits of algal culturing and observation of live material. There are serious issues for the sampling of waters and the use of diatom indices of water quality. Community analyses, water quality indices and taxonomic studies will be invalid, especially if different parts of the life cycle are shown to be ecologically as well as morphological variable.

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PREFACE

Readers should note that the "Foot & Mouth Crisis" in 2000/2001 and the resulting access bans to many sites affected the ability to collect fresh algal material from a wide geographical area and environmental gradient, although access to urban sites was not affected. This was compounded by the appalling weather conditions with consequent flooding in the autumn of 2000, which also rendered the early acquisition of suitable material for isolation difficult, and delayed the initiation of experimental work.

The absence of light micrographs of live cultures for the light and temperature regime and some stock and experimental cultures was due to late arrival of digital camera equipment for the inverted microscope.

Additionally, in August 2003, the author's home was subject to a flash flood due to torrential rainfall. Consequently, computer equipment, software, data storage devices (hard disk and CDs) and PhD paper notes were damaged. Most of the data was thankfully recovered but inevitably, some data was lost (largely in the light and temperature regime).

Finally, this thesis and all accompanying figures, tables and appendices plus any relevant raw data are available on the CD accompanying this thesis (see pocket at back).

1 INTRODUCTION

Gomphonema parvulum (Kützing) Kützing is a well-known example of a freshwater benthic diatom for which a number of varieties and forms have been described. Morphologically variable, with a seemingly wide distribution (Kelly, 2000; Krammer & Lange-Bertalot, 1986, 1991b; Round, 1993), it is nonetheless used as an indicator of "pollution" in diatom indices of water quality, despite there being no clear link between the various morphologies and particular autecologies. Taxa with wide ecological ranges are usually considered poor biological indicators, so should *G. parvulum* be used in such a way? Alternatively, does the *G. parvulum* species complex actually consist of several discrete taxonomic entities and if so, what are they and how can we identify them?

There are two basic requirements in choosing any taxon as a biological indicator, (i) it must be sensitive to change and (ii) its response must be measurable and predictable to ensure causal inference (Reid *et al.*, 1995). *G. parvulum's* pollution indicator status however, is largely derived from diatom community studies and metals (Ivorra *et al.*, 1999 and 2000 and Oliveira, 1985), or in which it occurs at high abundance under certain, primarily saprobic, conditions (Butcher, 1947; Fairchild *et al.*, 1985). None of these studies recognise the different varieties of *G. parvulum* nor attribute them to a particular habitat, few studies do. There is no evidence of morphological change linked to ecology for *G. parvulum*, and this, along with its wide distribution and morphological variability, raise questions about its taxonomic status and its use in biological monitoring.

The recognition of taxa with narrow ecological ranges can improve the predictive power of diatoms in water quality monitoring (Anderson *et al.*, 1993; Koppen, 1975; Hürlimann &

Straub, 1991). It has been argued that the recognition of "races" with specific habitat requirements would allow finer ecological distinctions to be made within monitoring systems (Hürlimann & Straub, 1991), whereas grouping taxa into complexes (*sensu* Krammer & Lange-Bertalot, 1986, 1988, 1991a, 1991b) may facilitate recognition of taxa, but the cost is loss of ecological information (Cox, 1995) and the production of broad species concepts based on a coarse classification. Mann & Droop (1996) suggest that a very narrow species concept may be necessary based on ultrastructural characters, which would perhaps highlight endemic taxa. A narrow species concept would however result in a number of taxonomic revisions and a large number of species being separated on [sometimes] very slight differences.

1.1 DIATOM DISTRIBUTION

Diatomists have always been interested in the distributional patterns of diatoms. Many appear to be cosmopolitan, some have restricted distributions and fewer still are endemic. Many diatoms such as *G. parvulum* have broad distributional patterns and Kristiansen (1996) remarks, "When discussing distributional patterns of algal species, the species concept is crucial. However, just as important is the stability of the taxa in question ... even if the species are static in morphological characters, their genomes may vary and be able to adapt in various ways throughout the occurrence of the species, in response to environmental factors and geographical isolation". The broad distributional patterns exhibited by many diatom species may have led to the belief that most are cosmopolitan however, the amount of endemism remains unknown. Additionally, the reliance on a few (mostly European and North American) illustrated diatom floras to identify diatoms in the tropics and the southern hemisphere may have inadvertently, through forced fitting, underestimated diatom diversity, distribution and endemism. Global checklists and floras are a long way from being published, though the internet is proving to be a great resource

(e.g. Index Nominum Algarum, AlgaeBase). Add to this a lack of detailed descriptive and photographic evidence, no standard terminology, and ongoing taxonomic arguments (e.g. what constitutes a species and how broad that definition should be) make interpretation and comparison of different studies difficult.

It is clear therefore, that if one is to correctly identify a taxon and associate it with a particular environment, the relationship between diatom morphology and the environment must be understood, e.g. do morphological varieties co-exist in a single habitat or are particular forms only seen at particular times of the year, or in particular water chemistries? If two or more varieties are able to co-exist, it suggests genetic distinctiveness (=gamodemes) (Mann, 1988), if they cannot co-exist then the taxa are probably distinct. As expressed by West-Eberhard (1989), "The environment is not only an agent of selection (arena where different phenotypes achieve different degrees of survival and reproductive success), but also an agent of development, influencing the range of phenotypes that will be produced by a given genotype". Alternatively, in a variable environment a novel morphology can evolve alongside an established morphology without being expressed in the same situation (West-Eberhard, 1989), i.e. even if an established phenotype is more efficient in most situations or individuals, any alternative morphology that happens to be better associated with a particular condition or trait can be positively selected for (facultative expression) (Dawkins, 1980). It is likely that these alternatives or novel individuals can become increasingly divergent and specialised over time, so increasing the number of morphologically distinct taxa. This divergence drives speciation if sufficient to cause reproductive isolation, and if phenotype fixation occurs. Therefore, is the morphological variation so often seen under the microscope, actually a window on the speciation process? How do polymorphisms affect our over reliance on diatom cell wall morphology for identification and classification purposes?

Certainly, there is an inherent danger in assigning new species based on valve morphology alone. As previously suggested, ecology and ontogeny are also important factors, and molecular studies will no doubt add to this information base.

1.2 DIATOM ECOPHYSIOLOGY

Consistently found in extreme conditions, diatoms are excellent bio-indicators. Patrick (1986) however, raised the question of why diatoms might be found in such conditions and suggested that certain species are able to utilise [or neutralise] nutrients that may otherwise be toxic to other taxa.

There are no ecophysiological studies in the literature on the effects of light, temperature, pH or organic pollution on *G. parvulum* and only one metal toxicity study (Ivorra *et al.*, 2002). *Gomphonema parvulum* is often mentioned however, in a number of community studies, suggesting an inherent tolerance to high and low pH, eutrophic conditions and conditions of metal toxicity. For example, Ivorra *et al.*, (1999 and 2000), found *G. parvulum* present in high numbers among benthic algal assemblages subject to zinc and cadmium pollution (on artificially translocated substrata). In the same study, *G. parvulum* abundance ranged between 8 and 19% of the total diatom abundance despite the metal pollution. Additionally, the authors found that the effects of metal exposure varied with the developmental stage of the algal biofilm. Oliveira's (1985) study of phytoplankton communities receiving copper mine effluent, also showed *G. parvulum* as abundant at a mean dissolved copper concentration of between 60 and 390mg/l, with cell densities ranging between 0.08 and 0.34 cells/ml^{-1}.

Butcher (1947), described *G. parvulum* as being resistant to polysaprobic conditions, but relatively uncommon in oligotrophic waters, though also occurring in all other zones from

eutrophic to oligosaprobic, and the dominant diatom taxon in the mesosaprobic zones at the height of the growing period May to October. In another study, Palmer (1969) compiled a list of algae tolerating high organic pollution from 165 authors. The species most often referred to as significantly tolerant were remarkably stable throughout the research. *Gomphonema parvulum* was listed 20th out of 725 species of algae as being particularly tolerant to organic pollution. Later still, Fairchild *et al.*, (1985), used clay pots dosed with specific ions to study the growth responses of algae in a lake. They found *Gomphonema* spp. were NO_3^- limited. Niederhauser & Schanz (1993) found *G. parvulum* to be the dominant species on clay pots dosed with nitrogen, phosphorus and carbon, seemingly confirming their preference for eutrophic waters. These results and comments strongly indicate that *G. parvulum* is a highly adaptive taxon generally tolerant of a wide range of saprobic and metal polluted waters however, authors rarely distinguish between the different forms of *G. parvulum*, nor do they provide micrographs with which to compare different reports. This brings us back to the question raised earlier, i.e. does the species complex *G. parvulum* actually consist of several discrete taxa, each associated with a particular autecology?

In studying the ecophysiological responses of diatoms, growth rate is often used as an analogue for metabolic rate, and according to Finkel & Irwin (2000), "an organism's size is a powerful predictor of its metabolic rate" and this "simple allometric model predicts that small cells should always outcompete large cells". One would therefore expect there to be metabolic differences related to size even within the same taxon, e.g. between vegetative parent cells and initial cells produced after sexual reproduction. Other factors, e.g. intracellular pigment concentration, can however complicate this model.

Although each diatom taxon has specific growth requirements, generalisations can be made. Generally, it is the chemical content of the water together with light and temperature that determines where particular taxa are found, and in what numbers. Studies have shown that in addition to sodium, calcium, iron, silicon, nitrates, phosphates, sulphates, oxygen, carbon dioxide and humic acid (Patrick, 1948), copper, zinc, and organic substances are also important (Button & Hostetter, 1977; Ellwood & Hunter, 2000; Gerringa *et al.*, 1995; Joux-Arab *et al.*, 1998; Lee & Morel, 1995). In one case, even cadmium has been shown to have a biological function (Lane & Morel, 2000).

1.3 MORPHOLOGY, SPECIES CONCEPTS AND TAXONOMY

Morphological variation occurs in a number of diatom taxa, e.g. *Caloneis amphibaena* (Bory) Cleve, *Anomoeoneis sphaerophora* (Ehrenberg) Pfitzer, *Craticula cuspidata* (Kützing) D. G. Mann, *Cymbella amphicephala* Naegeli in Kützing, *Navicula gregaria* Donkin, *Sellaphora pupula* Mereschkowski. In some cases, different morphologies in a single taxon have been linked to different environmental conditions, whereas in other taxa, e.g. *Sellaphora pupula*, morphological variation has been shown to be a manifestation of discrete breeding populations (Mann, 2001; Mann *et al.*, 1999, 2004). Taxa can also be morphologically similar but occur in different habitats.

The aim of the systematist is to provide a natural hierarchy of classification for all living and fossil organisms. This necessitates a terminology to describe units of variation and change (Walters, 1989). Turesson (1922) as cited in Walters (1989) was the first to propose the term "ecotype", having discovered via transplantation experiments that many of the morphological variants of species were genetically distinct. Later, Gilmour & Heslop-Harrison's (1954) paper defined the term "deme" with the appropriate prefixes to

denote particular kinds of demes (now used by modern taxonomists such and Mann, 1990) to describe "... any specified assemblage of taxonomically closely related individuals". Walters (1989), paper goes on to describe the misuse of this terminology, which continues to exists in its incorrect form today, e.g. workers using the term deme without prefixes and to describe what should be a gamodeme. Unfortunately, the term "ecotype" has not escaped erroneous use either. Perhaps this is partly down to the original and subsequent authors not being clear in their definitions or clarifying appropriate use, and it may be why none of these terms is in widespread use today. On the other hand, perhaps it is an unwillingness to change from what is comfortable and familiar. As Mann (1990) has said, "It is discourteous to dismiss an idea merely because it disagrees with the current orthodoxy".

The role of authority is also important in terms of defined criteria for application. In Williams & Round's (1994) paper, the role of authority in systematics and the need for a set of principles is discussed. The real question however is what principles? Who is going to choose them and will they be widely accepted and incorporated into the Botanical Code? It is likely that they will change as science moves forwards, but this should not preclude open discussion. The authors also caution against the overzealous use of authorities simply because they are named, suggesting instead that judgment should be made on all data available and the methodology behind it, echoing Mann's (1999) "total evidence approach".

What perhaps needs to be stated is that taxonomy, at the level of species and below at least, is a fluid process, i.e. taxa are continuously evolving. What was once described as a variety or form, may eventually become either distinct enough to allow upgrade to

species or become extinct. This means that varieties and forms cannot be easily defined and the point at which a new species forms is unknowable. The development of a clonal nomenclature has been suggested (Round, 1997a, 1997b) as a suitable alternative but has not been developed further. For a thorough evaluation of species concepts in diatoms however, Mann's (1999) detailed review is required reading.

When referring to species, biologists generally have two objectives in mind, one practical and concerned with the description and cataloguing of individuals (grouped into "species" for convenient identification) and the second, to facilitate the study of processes such as speciation and evolution (Endler, 1989; Rines, 1994). Yet there is much debate regarding species concepts, largely because these concepts depend on the questions being asked and the personal preferences and preconceptions of the researcher. Consequently, species concepts can often be viewed as clashing rather than complementary, e.g. taxonomic vs. evolutionary (does one want to catalogue or study processes) or reproductive vs. cohesive (species defined by their ability to interbreed or by whatever cohesive forces maintain the integrity of the taxon) (Endler, 1989).

Although most contemporary diatomists recognise the existence of genotypic and ecophenotypic variation in morphology, the requirement of the International Code of Botanical Nomenclature (ICBN) to designate a nomenclatural type, sometimes leads to a typological species concept being followed (Rines, 1994). This concept is no longer in use, and whilst at one time a type specimen was considered a perfect example of the species in botany, today its purpose is nomenclatural not taxonomical Rines (1994). This begs the question of why have type specimens at all? Fixing a name to a single specimen sitting on a slide in a darkened drawer only perpetuates the typological species

concept whether intentional or not. A species consists of a group of organisms evolving in time and space. In 100 years, will *G. parvulum* still be *G. parvulum*? In terms of its name, probably yes. In terms of the features that determine it taxonomically as *G. parvulum*, possibly no. The concept of species is of fundamental importance in any taxonomic work, yet a stable and universally acceptable definition remains elusive.

What of the infraspecific separation of taxa? According to Round (1996, 1997b), the use of variety has been almost exclusively based on morphological data and there are several instances of upgrading a variety to species status, e.g. *Sellaphora pupula* (Mann, 1989b). Droop's (1994) paper on the morphological variability of the valve in the diatoms *Diploneis smithii* and *D. fusca* highlights some of the problems involved in identifying and naming parent varieties. However, one must be careful to include as many discriminating characters as possible to ensure complete separation of the groupings, and if these are well defined and clearly described, there should be no need to weight them. Failure to follow this course of action may result in incomplete separation due to similarity in characters chosen whereas they may in fact be clearly recognised by eye as distinct entities (see Round 1996, 1997a, 1997b). Droop (1994), however also points out that this is not always possible because some characters, e.g. valve shape and shape of raphe endings, are so difficult to measure quantitatively. Cryptic factors such as the presence of certain proteins or chemicals also need to be considered. Based on the biochemical and physiological factors of the taxon under study, cryptic characters can be determined by experimental means and are capable of separating taxa into ecologically important entities (Round 1996, 1997a, 1997b). Relating such cryptic features to morphology, especially where significant inter-clonal variability is found, will not only increase the possible number of species but also provide precision in biomonitoring.

Size diminution is also an important aspect of morphology. The overall size diminution over the diatom vegetative life cycle, with full size only being restored through sexual reproduction (Crawford, 1981; Geitler, 1932), is known as the MacDonald-Pfitzer rule (MacDonald, 1869; Pfitzer, 1871), the first authors to give detailed accounts of cell size change during the diatom life cycle. The process of size diminution during vegetative growth in most diatoms will additionally cause changes in overall valve shape, symmetry, volume of nucleus, cytoplasm and vacuoles, position of chloroplasts and loss and/or distortion of some morphological characters such as striae and raphes. Size reduction is also accompanied by a change in relative proportions, e.g. longitudinal axis of the pennate diatoms shortens faster than the transverse axis. This leads to changes in valve outline, e.g. increasing heteropolarity, and loss of pole detail, which in turn can lead to misidentification unless the full size range of a particular species is known. Maximum cell size is only restored by meiosis and pairing to produce an auxospore (= zygote). Any disturbance during cell division however can result in abrupt changes to cell dimensions and valve morphology and may lead to death of a population.

1.4 THE USE OF LIVE MATERIAL IN DIATOM TAXONOMY

The identification and classification of diatoms relies heavily on the morphological features of the silica cell wall, including symmetry, shape and ultrastructural features. Little attention has been given to cell contents, physiology, biochemistry, colony form, reproductive behaviour or the production of different forms of extracellular polysaccharide substances (EPS) as taxonomic criteria. Whereas earlier diatomists and non-diatom phycologists regularly use such features in their diagnoses, most contemporary diatomists do not. Attempts to introduce taxonomic characters based on the features of live cells, e.g. use of chloroplasts, pyrenoids and reproductive behaviour (e.g.Cox 1987, 1996; Mann, 1984a, 1984b, 1984c, 1984d), are however on the increase. These studies

are reassessing taxa previously established using light microsopical studies of the frustule (Mann, 1988, 1989b, 1990; Mann & Stickle, 1988, 1995).

There is additionally, a need for studies evaluating the sensitivity of taxa to various environmental conditions, particularly if these are also linked to a stable morphology. Compared to the data available for marine and freshwater planktonic diatoms however (e.g. Gensemer *et al.*, 1993; Morel *et al.*, 1978), information on the sensitivity of freshwater benthic diatoms to, e.g. metal pollution (Takamura *et al.*, 1989), is sparse. The formation of morphologically abnormal diatom valves in the presence of heavy metals or extreme pH is well known (Barber & Carter, 1981; Cox, 1981a; Dickman, 1998); however, cytological cell deformities such as increases or decreases in cell volume, chloroplasts and vacuole number and size, and the production of exopolymeric substances are rarely investigated. Yet they can indicate the overall state of health and hint at ultrastructural deformities and ecological preferences. Together with other cytological features, such as lipids, polyphosphates and mitochondria, these features can indicate disruption to, e.g. photosynthesis and ATP production.

Additionally, the culturing of diatoms and the induction of sexual reproduction allows the full size range and life history to be observed and measured, and can clarify species identifications. Workers are now recognising that in order to maximise the benefits of using diatoms as bio-indicators, taxonomy needs to be clarified and there is a need for a synthesis of ideas, techniques and information from different areas of phycological research (and beyond).

1.4.1 Diatom Organelles as Taxonomic Characters

Chloroplasts are the most obvious organelle in diatoms, the shape and orientation being taxon specific. The number, shape and ultrastructure of the diatom chloroplasts was reviewed by Duke & Reiman (1977), and Cox (1981b, 1996), who has shown that there are consistent differences between species in their chloroplast arrangement and that this can facilitate and clarify species recognition. Chloroplast morphology, movement and inheritance were reviewed by Mann (1996).

Data on vacuole morphology is contained within Raven's (1987) comprehensive review, which provides the background to the role of the vacuoles in plants such as increasing surface areas for the acquisition, storage and transport of resources and regulating cytoplasmic volume. In particular, Ravens' analysis suggests that the specific growth rate of a cell under optimal growth conditions is decreased if it is vacuolated, yet resource acquisition under limiting conditions may be enhanced by vacuolisation. It is therefore a balancing act, and the flexible nature of the vacuole facilitates this and may be largely taxon specific.

Large oil bodies are also highly visible in diatom cells. They are a food reserve, occur frequently in both cultured and wild diatoms, and diminish if diatoms are kept in the dark for extended lengths of time. Lipids in the chloroplasts are assumed to have the same density as the larger oil bodies, but the chemical make-up and function of the two types is not known (Drum, 1963). However, no studies have been carried out to discover whether the production of oil bodies increases or decreases in relation to cell cycle or environmental conditions. How useful, variable or visible other organelles are in live cells

is not clear and does not appear to have been researched to any great extent, though a variety of stains are available to show up various organelles within the diatom cell. It is likely that most other organelles only become visible, and or mobile, during cell division, e.g. the Golgi bodies (Duke & Reiman, 1977).

1.4.2 Diatom Growth Mode and EPS Production

An important adaptation of diatoms to their environment is the production of extracellular polysaccharide substances (EPS) in the form of cell or colony coatings, tubes, stalks, apical pads, and adhering films or fibrils. Although diatoms function as single entities either freely motile or attached to substrata, they are also often associated in colonies, and as Round *et al.*, (1990) point out, "There is no reason why sytematists should not use colony form, and the means diatoms employ to achieve this, as sources of taxonomic information".

The production of EPS has been linked to changes in environmental conditions, and different growth stages of diatoms, indicating a high level of genetic control (Abdullahi *et al.*, 2006; Underwood & Paterson, 2003). EPS are associated with both planktonic and sessile diatoms, and have a wide varieties of forms, e.g. crystalline, fibrous or mucilaginous (Hoagland *et al.*, 1993). Whilst it is generally known that EPS comprise of polysaccharides together with small amounts of protein and glycoproteins and are involved in gliding motility and adhesion (Chiovitti *et al.*, 2003; Staats *et al.*, 1999), much remains unknown in relation to their function and synthesis. The structure, biochemistry and biosynthesis of EPS are beyond the scope of this study (see reviews of Hoagland *et al.*, 1993 and Wetherbee *et al.*, 1998) however, the morphology and ecological

significance of the types of EPS produced by *G. parvulum* clones under various culture conditions will be examined.

G. parvulum was first reported as producing mucilaginous stalks or mucous coverings in dense aggregations by Kützing, (1849), and modern diatom floras (e.g. Krammmer & Lange-Bertalot, 1986) do not depart from these descriptions. However, EPS in the form of a mucilaginous matrix is considered rare, and there is no description in the literature defining a matrix. The literature points to two colony forms involving EPS for *G. parvulum*, (i) when free-living, it forms mass aggregations encapsulated in a mucilaginous covering or matrix, and (ii) when sessile, it forms stalks, possibly dichotomously branched. It is not clear whether these are related to different ecotypes or ecomorphs. If the different modes of attachment remain stable, they would provide additional information for taxonomic and ecological diagnoses.

1.4.3 Sexual Reproduction

Diatoms are presumed to be primarily asexual because this is the stage in which they are invariably observed however, the significance of asexuality in taxonomy differs depending on the species concept used. It would be highly relevant to the biological species concept for example. The presumption of asexuality may simply be a result of not looking in the right places at the right time of year/day, or perhaps the cell cycle is spread over much longer time spans as in some higher plants (e.g. such as those that only flower once every 50 years, or only flower when an exceptional event such as fire occurs). This is one reason why repetitive field sampling and exploratory experimental assays are so important in diatom taxonomy.

Only a small number of pennate diatom life histories have been studied to date, but according to Chepurnov *et al.*, (2004) they are better documented than for other major diatom groups. Two of the earliest researchers were Smith (1856), and Geitler (1927, 1932, 1939, 1951a, 1951b, 1952a and1952b, 1953, 1957, 1969a and1968b, 1977, 1979, 1984, 1985), who studied sexual reproduction in representative taxa from several genera including *Cymbella, Synedra, Eunotia, Navicula, Nitzschia* and *Gomphonema*. Few other researchers took the time or interest to study this area of diatom taxonomy until the early 1980's and 1990's, when interest began to resurface and earlier works were rediscovered (e.g. Chepurnov & Mann, 1997; 1999, 2000, 2003; Cohn *et al.*, 1989; Cox, 1985; Davidovich & Bates, 1998; Edlund & Stoermer, 1991, 1997; Mann 1982a, and 1982b, 1984a, 1984b, 1984c and 1984d, 1993a and 1993b). Edlund & Stoermer's (1997) review suggests only a dozen or so researchers have contributed to nearly all the knowledge on diatom life histories to date.

Sexual reproduction in diatoms has been reviewed by Drebes (1977); Edlund & Stoermer (1997); Mann (1993a); Patrick (1954) and Round *et al.*, (1990), and more recently, Chepurnov *et al.*, (2004) provide an historical and "next steps" review of experimental studies on sexual reproduction in diatoms. There remains however, a lack of information on individual species. Primarily this is due lack of knowledge about their culture requirements, the environmental cues stimulating sexual reproduction, and the low incidence of diatom sexual reproduction in nature and culture.

The only cytological and life history works (as opposed to valve morphology or experimental studies) conducted on *Gomphonema* spp. are those by Dawson (1973a, 1973b), Drum & Pankratz (1964b); Geitler (1973) and Hohn and Patrick (1959). The

literature tells us that most raphid diatoms are isogamous, having male and female gametes that are morphologically and/or behaviourally different (Drebes 1977; Geitler, 1932; Round *et al.*, 1990). We also know through these studies that, in raphid diatoms, sexual pairing is an active process due to or because of, the motile nature of diatoms.

Whilst a number of factors are shared by several taxa, the process of pairing, gametogenesis, fertilisation, mucilage production, auxospore and initial cell development however, are very diverse (Chepurnov *et al.*, 2004) and probably species-specific. The cytological ultrastructure of sexual reproduction in raphid diatoms has been studied in only a few taxa, e.g. *Gomphonema parvulum* and *Neidium affine* (Ehrenberg) Pfitzer (Drum *et al.*, 1966). The arrangement of cell organelles, especially chloroplasts, can change in a predictable and taxon specific manner during the cell cycle, e.g. the pyrenoids may temporarily disappear (Cox, 1996). In *G. parvulum*, the single chloroplast moves to the girdle and divides longitudinally prior to cytokinesis, so that each offspring receives one chloroplast.

A rational train of thought might lead one to consider that inducement of sexual reproduction is more likely under favourable conditions. Waite & Harrison (1992) have shown in the much-studied marine diatom *Ditylum brightwelli* (T. West) Grunow in Van Heurck however, that sexual development can also be a stress response. They studied natural populations of this diatom, and found that a portion of the population sexualised at a time of nitrogen limitation. Culture studies indicated that sexual induction increased sinking rates up until the post auxospore stage, when it then became positively buoyant. It was suggested that this allowed the diatom to procure the additional nitrate required at an early stage in sexual development, allowing it to complete sexual reproduction prior to

re-colonisation of surface waters. It would be interesting to know if this was an aberrant occurrence, due to unusual environmental conditions, or a regular, perhaps seasonal pattern.

Studies have also shown that auxospore formation can occasionally occur within a single unpaired mother cell via automixis. Automixis involves either fusion of two normally differentiated gametes (=paedogamy), or meiosis is suppressed and two of the four nuclei fuse (=autogamy). Autogamy within the raphid diatoms has been shown to occur only in *Denticula tenuis* Kützing (Geitler, 1953) and *Cymbella ventricosa* Kützing (Geitler, 1953) (now *Encyonema minutum* (Hilse ex Rabenhorst) Mann) (Geitler, 1985). All reports of paedogamy occur in the raphid diatoms, and include representatives of *Gomphonema* (Round *et al.*, 1990). This phenomenon suggests that, in normal allogamous sexual reproduction, there must be a mechanism that prevents sister gametes fusing (Chepurnov *et al.*, 2004), though no study to date has discovered what that mechanism is or might be.

It is also possible for size restoration to come about via asexual auxosporulation (vegetative apomixis) (Drebes, 1977; Geitler, 1973; Nagai *et al.*, 1994; Sabbe *et al.*, 2004), in which meiosis and fertilisation are by-passed, and a single mitotic division occurs to form the auxospore. True vegetative size restoration in which no auxospore is formed also occurs, and it is believed such populations are permanently asexual, e.g. Mann (1989a) and Mann *et al.*, (2004) have shown that populations of *Sellaphora lanceolata* have a narrow size range that has remained unchanged over many years of observation with no sign of sexulisation. In the absence of sexual cell enlargement, size

restoration is instead brought about by "vegetative cell enlargement" as detailed by von Stosch (1965).

Observations on auxospore formation in heteropolar diatoms like *G. parvulum* have revealed that gomphonemoid initial cells are almost isopolar (Passy-Tolar & Lowe, 1995). The restoration of a marked heteropolarity, only after several vegetative divisions suggests that daughter valves are not simply moulded by the parent walls as suggested by Mann (1994), but perhaps also cytoplasmic factors in some situations (Cox & Kennaway, 2004).

The ecology of diatom sexuality has also been overlooked until fairly recently with Edlund & Stoermer's (1997) review of diatom life history strategies. One of the strategies, "Synchronous sexuality under favourable growth conditions", is utilised by most diatoms and describes a population in which a number of cells undergo sexual reproduction at the same time. The authors suggest that this is the favoured method for spring blooms of *Cymbella* spp. and *Gomphonema* spp. and may therefore be tied to seasonal signals such as light and/or temperature, together with favourable nutrient conditions, but again few other diatomists have studied this aspect.

The inclusion of cytological and life history research in taxonomy alongside traditional morphological studies can only enhance our knowledge about diatoms and their ecology. As Edlund & Stoermer (1997) suggest, this can lead to an organismal approach to diatom classification and a more natural classification system. It has already had an impact on

diatom classifications, with several genera having been resurrected and others re-designated, e.g. *Biremis* (Round *et al.*, 1990), *Craticula* (Mann & Stickle, 1991) and *Placoneis* (Cox, 1987; Mann & Stickle, 1995). There is clearly a need for, and diatomists are now working towards, a more holistic approach in the study of diatoms.

1.5 *G. PARVULUM*: AN HISTORICAL PERSPECTIVE

Gomphonema parvulum exhibits considerable morphological variability **(Figure 1.1)**. In considering the circumscription, nomenclature and typification of *G. parvulum*, two questions arise: "What was Kützing's original definition of *G. parvulum?* and "Does Kützing's original definition comply with modern taxonomists views of what constitutes *G. parvulum*?" In other words, has Kützing's original concept been maintained? Clarifying the taxonomic and nomenclatural issues associated with the different morphologies within the Kützing material is important because, any new species or ecotypes revealed by experimental study will need to be formally identified and named. Similarly, experimentally defined ecological tolerances must be linked to defined taxa. Accordingly, Kützing material housed at the Natural History Museum (NHM), in London was examined alongside copies of Kützings original descriptions and notes.

The current International Code for Botanical Nomenclature (ICBN) (St. Louis Code 2000), outlines the rules for naming type material however, prior to the current and some earlier codes, different species concepts prevailed and the concept of type and type material was not generally accepted. If it was, it did not conform to the modern interpretation. Not until 1930 did taxonomists agree to the first ICBN, and the first definition of a type specimen did not come into force until 1958. Thus, when Kützing first identified *G. parvulum*, he was not adhering to current rules. Early authors often considered the most

common species as "typical" of a genus (not to be confused with the contemporary meaning for type in taxonomy), but this has caused confusion in that several "typical" specimens represented one species.

NB: question marks after species names in the following sections are deliberate, appearing in this manner in Kützing's original material.

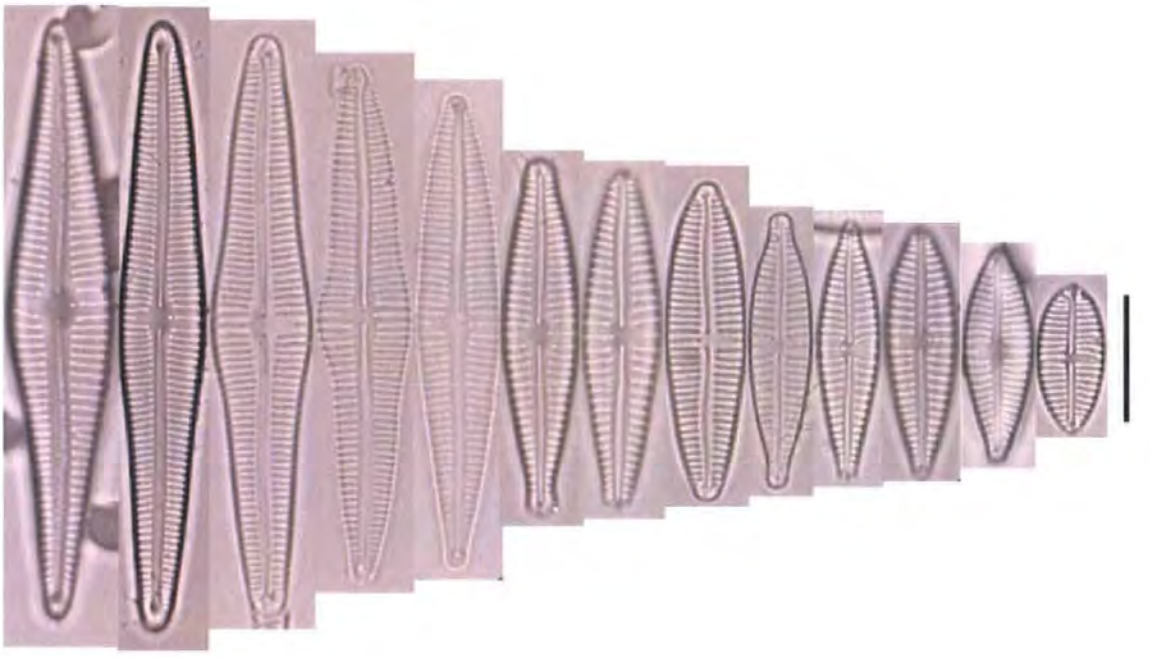


Figure 1.1
An example of the range of morphologies exhibited by G. parvulum *in this thesis.* Scale bar = 10μm.

1.5.1 Kützing's Material

In the 1840s, gomphonemoid diatoms were split into two genera, *Sphenella* and *Gomphonema*. The former genus was composed of free-living forms and the latter of attached and stipitate forms. In all other respects, the species had numerous other characters in common. Kützing's original description for what is currently known as *G. parvulum*, was originally described as two separate species (1) *Sphenella parvula* (free-living form) (Kützing, 1844) and (2) *Gomphonema parvulum* (stipitate form) (Kützing, 1844 and 1849).

Examination of the Kützing Collection Catalogue (originals held by the Zoological Society of Antwerp in the van Heurck collection) **(Figure 1.2a-b)** reveals four packets of diatom material associated with the collection, and copies of Kützing's original description and drawing **(Figure 1.3a-b)** confirmed the material as originating in Falaise, a small town in Normandy, northern France. Kützing's catalogue cites two packets of material from which he identified *S. parvula,* and two packets from which he identified *G. parvulum* **(Table 1.1)**. All four packets were donated by de Brébisson under his manuscript names *Gomphonema minutissimum*? and later, *Sphenella parvula*? from which Kützing identified *Sphenella? parvula* and *Gomphonema parvulum* respectively **(Table 1.1)**.

Additionally, four slides **(Table 1.1)** were located that corresponded to each of the four packets identified. As was consistent with the times, no single packet or specimen was identified as the type by Kützing. However, Dawson (1972) had labelled two slides as the type slides for *G. parvulum* and *S. parvula*. Dawson also wrote notes on the back of these

"type" slides in which she stated that each slide was identical to the other. However, as all four slides originate from four different but corresponding packets of material, and may have been collected at different times, this is incorrect.

Kützing's description (1844) **(Figure 1.3b)** of *S. parvula* is extremely brief and his drawings very small **(Figure 1.3a)** and perhaps a little exaggerated. Nonetheless, together they depict a diatom that is small, raphid, almost lanceolate-like with tapered but expanded apices that are subcapitate or sub-rostrate, and a shape that is heteropolar and asymmetrical about the transapical axis. In his description, Kützing also recognised that the species could easily belong to the genus *Gomphonema*. This may be why he maintained the question mark after the genus name *Sphenella* in his 1844 publication. Kützing's description of *G. parvulum* came four years later in 1849, together with a description for *Sphenella parvula* as follows:

> Sphenella parvula
> Sphenella minuta, in stratum, aggregate, laevissima, latre secundario lanceolato, apice acuminato, basi producto subdilatato
> Long 1/80
> Gomphonema minutissimum Bréb.
> Inter. Diatomeas prope.
> Falaise legit cl. De Bréb (v.s.)
>
> Gomphonema parvulum
> Gomphonema baciliis magnitudine et habitu Sphenellae parvulae, sed stipatum et in stratum mucosum dense aggregatum – In aqua dulci Germaniae et Galliae.

There are no drawings for either taxon in the 1849 publication, but Kützing does specify in his description that *G. parvulum* has very similar characters to *S. parvula*, and was only separated because it was stipitate, often with a dense mucous covering when aggregated. In his description for the genus in 1844, Kützing suggests it is probable that

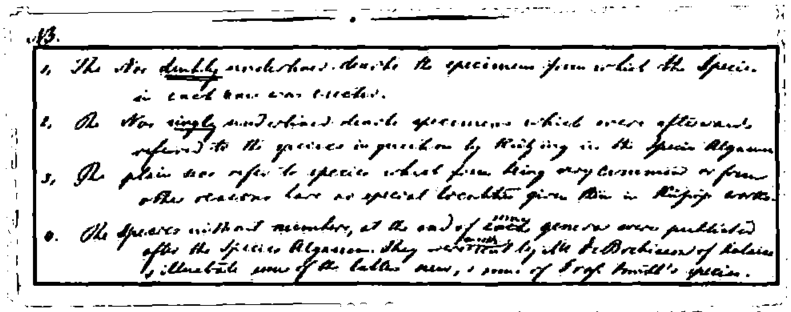
most *Gomphonema* live freely and only later settle and develop a stalk. Thus, we have two taxa that were initially separated because of their distinctly different modes of attachment, but that in all other respects were the same. This could mean that Kützing was looking at two different forms of the same species, two different species or simply that he did not observe attachment in some samples.

Table 1.1

Packet numbers, manuscript names and slide numbers of material used to diagnose *S. parvula* and *G. parvulum*. Catalogue of the Kützing Collection held at the Natural History Museum, London. BM = British Museum.

Packet number	De Brébisson's manuscript name	Kutzing's manuscript name	BM Slide Number	Dawson's type material
1262	*Gomphonema minutissimum?*	*Sphenella parvula*	BM18587	*Sphenella parvula*
1269	*Gomphonema minutissimum?*	*Sphenella parvula*	BM18588	
1260	*Sphenella parvula?*	*Gomphonema parvulum*	BM18696	*Gomphonema parvulum*
1648	*Sphenella parvula?*	*Gomphonema parvulum*	BM18695	

a



b

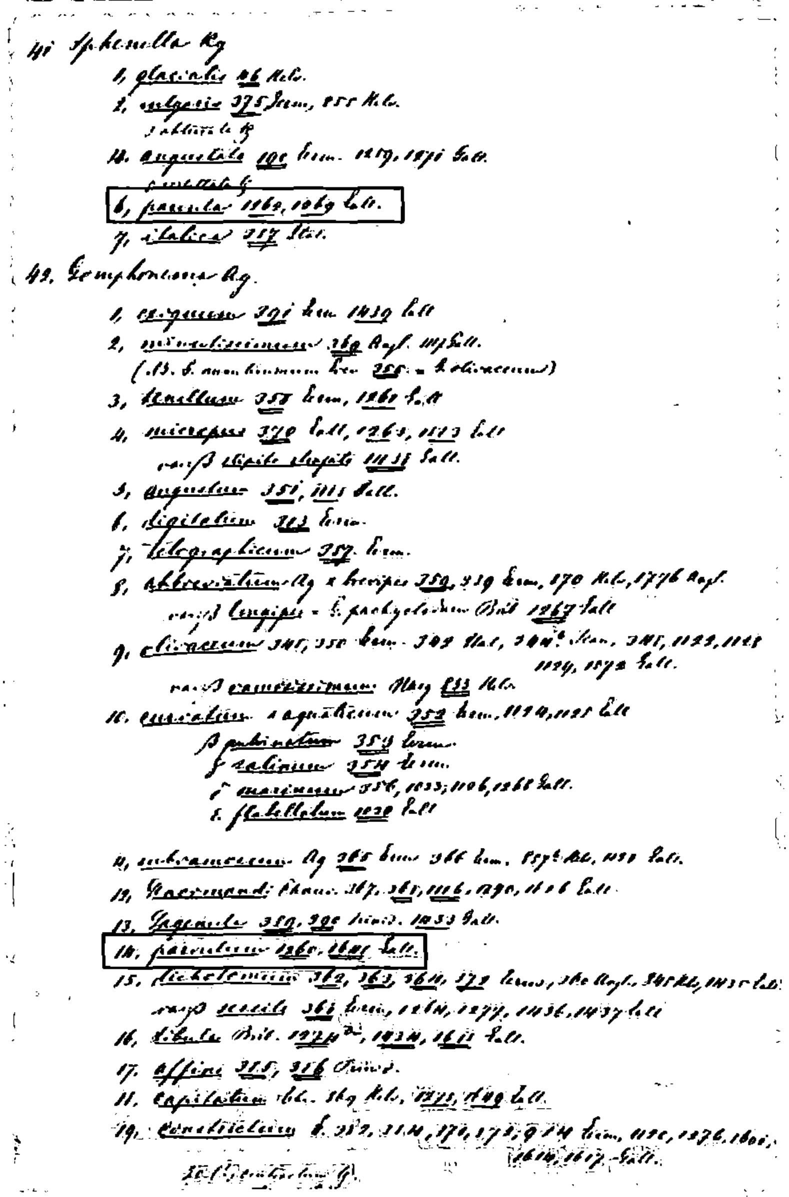
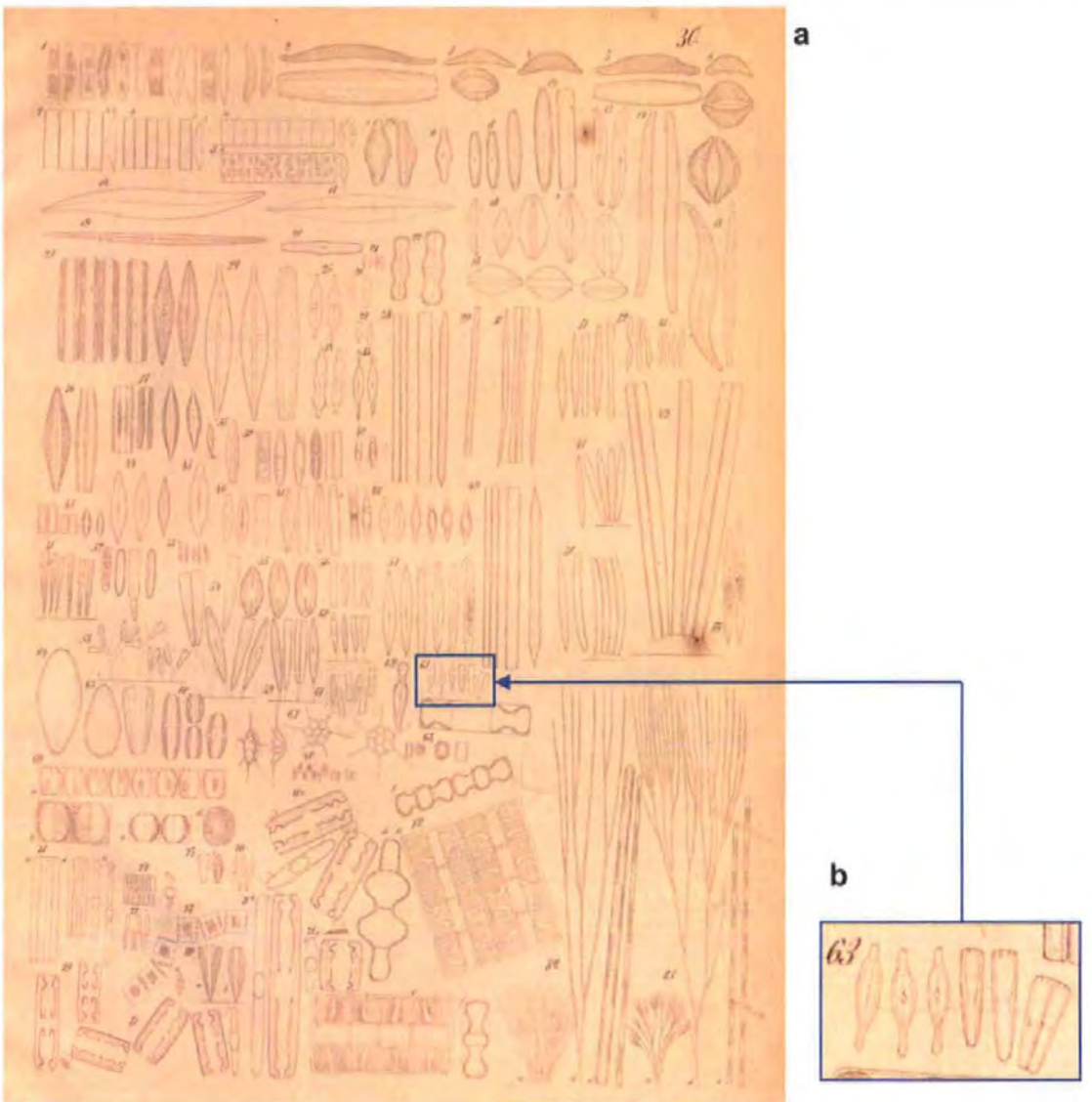


Figure 1.2: a - b

Scanned copies of the relevant pages from the Catalogue of the Kützing Collection held at the Natural History Museum, London, showing **(a)** the notation used by Kützing and **(b)** the numbered packets of material used to diagnose *S. parvula* (packets 1262 and 1269) and *G. parvulum* (packets 1260 and 1648).



($\frac{4}{1}\frac{2}{0}$). S. minuta, laevissima, latere secundario lanceolato, apice acuminato, basi producto subdilatato.

Gomphonema minutissimum *De Bréb.* ex specim.

Unter Diatomeen von Falaise: Herb. Binder! — Länge $\frac{1}{80}'''$.

Kann leicht zu Gomphonema gehören.

Figure 1.3: a-b

Scanned extract from Kützings 1844 publication "Die Kieselschaligen Bacillarien order Diatomeen" (Page 83-84). **(a)** Kützings illustration and **(b)** describing *Sphenella*? *parvula* for the first time.

1.5.2 Taxonomic Treatment post Kützing

Table 1.2 summarises the taxonomic treatment of *G. parvulum* and its varieties post Kützing. Heiberg (1863) recognised that *Sphenella* and *Gomphonema* could no longer be maintained as separate genera because they were founded on the same characters occurring in the same species. Attitudes were also beginning to change with respect to the separation of genera based on habitat, e.g. Rabenhorst (1864) considered *G. parvulum* and *S. parvula* as the same species regardless of mode of attachment, as did Grunow (1878). Consequently, *Sphenella* was rejected as a genus and all gomphonemoid taxa appear to have been subsumed under the genus *Gomphonema*. Whether this was because *Gomphonema* had priority or was simply the preferred name is not clear. Certainly, *Sphenella* as a genus was erected after *Gomphonema* (1843 and 1844 respectively). Either way *Sphenella* as a genus fell out of use. Thus, the correct circumscription for the species is *Gomphonema parvulum* (Kützing) Kützing (1849).

Grunow (1878) attempted to arrange the known species of the *Gomphonema* systematically, taking heed of Heibergs reasoning for subsuming *Sphenella*. Grunow additionally separated *Gomphonema* into those with, and those without stigmata, and consequently *G. parvulum* was placed within the Stigmatica. Cleve (1894-1895) supported Heiberg, but excluded some of the American forms of *Gomphonema*, putting them instead in the genus *Gomphoneis*.

Cleve (1894-1895) noticed there were similarities between the *Gomphonema* and *Cymbella*, noting that *Gomphonema* spp. are often asymmetrical about the longitudinal

axis as well as the transapical axis and that all forms of *Gomphonema* were highly variable. Cleve (1894-1895) however did not recognise Van Heurck's varieties, which he instead subsumed as synonyms for the species, stating that too much emphasis had been placed on valve outline. He did however recognise *G. parvulum* var. *micropus* as a variety rather than a species as Kützing (1844) had originally stated. It was therefore apparent early on, that *G. parvulum* was a variable taxon, although up to this point descriptions had been very brief and representative figures lacking.

At the turn of the 20th Century, more detail was beginning to emerge and additional varieties were established. Mayer (1917) was the first to record *G. parvulum* in Bavaria and two new varieties *G. parvulum* var. *curta* and *G. parvulum* var. *lagenula* were described by Frenguelli (1923), the latter of which Kützing had considered a distinct species, whilst the former was new for Argentina. Mayer (1928) also noted that larger forms of *G. parvulum* could easily be confused with *G. angustatum* in outline, although the latter has stronger and more widely spaced striae. Mayer (1928) listed three new *G. parvulum* varieties and appears to have been the first to recognise and describe a form, *G. parvulum* var. *genuinum* fo. *semiaperta*. Hustedt (1930) noted that *G. parvulum* var. *subelliptica* was difficult to differentiate from *G. parvulum* var. *micropus*.

In 1932 came the seminal and important work of Geitler (1932). His paper was the first of its kind, and in it, he carefully described the changing morphology and sexual reproduction of *G. parvulum* var. *micropus* (amongst other diatoms). Geitler described in detail the processes of sexual and vegetative reproduction and noted how growth rates changed with age. His experimental approach helped highlight changing morphology within diatoms. This morphological variability was further examined in relation to size

diminution (the loss of stria and pole detail) and auxospore formation by Wallace & Patrick (1950) and Hohn & Patrick (1959).

Carter (1960) was the first to recognise formally that the nomenclature of the *Gomphonema* was in a "chaotic state" and to consider valve outline an invalid character for diagnosis due to the morphological plasticity exhibited both within the genus and within species. In agreement with his predecessors, he considered *G. parvulum* to be a common species, but added that it was not always seen in any quantity. Carter (1960) also suggested that *G. micropus* may be a variety of *G. parvulum* and that *G. parvulum* var. *lanceolata* and *G. parvulum* var. *subcapitata* differed little from the "type".

In 1971 van Landingham published the Catalogue of Fossil and Recent Genera and Species of Diatoms and their Synonyms. This has proven very useful in validating names to the 1970's, listing 12 varieties and 4 forms for *G. parvulum*. In this publication the varieties *G. parvulum* var. *exilissima*, *G. parvulum* var. *tergestina* and *G. parvulum* var. *genuinum* were declared invalid, but *G. parvulum* var. *lanceolata* was maintained. Meanwhile Dawson (1972, 1973a, 1973b, 1974) was the first to undertake an SEM study of *Gomphonema* spp. and observed that *G. gracile* closely resembled *G. parvulum* in all respects except valve outline and the presence of pits on the valve face. Dawson also claimed that *G. gracile* had mucilage pores at both poles (Dawson 1974). Dawson acknowledges that this had not been previously recorded in the genus and certainly, no subsequent flora's have described *G. gracile* as having mucilage pores at both apices. It is therefore possible that what Dawson recorded was initial cell morphology, which is known to differ considerably in most sexually reproducing diatoms, and not the morphology of *G. gracile sensu stricto*. Certainly, her SEMs are of very large cells.

Lowe (1974) also produced a useful reference guide in which he reviewed a large number of papers and tabulated the environmental requirements and tolerances of various species. The review highlighted how little was, and still is, known about the ecological tolerances and preferences of *G. parvulum* or other diatom taxa. Similarly, Patrick & Reimer's (1966-1975) account of *G. parvulum* recognised that if the "extremes of the gradients of forms that exist" are found, then certain varieties, e.g. *G. parvulum* var. *exilis* and *G. parvulum* var. *micropus* might be considered distinct from *G. parvulum*.

Relatively modern floras, e.g. Krammer and Lange-Bertalot, Süßwasserflora von Mitteleuropa (1986-1991), have provided more detailed descriptions of the variability within species, in addition to known details on ecology, morphology and reproduction (including the larger, morphologically different post auxospore cells that are rarely seen). Krammer & Lange-Bertalot have recognised and commented on the difficulty in the demarcation of the numerous varieties and forms of *G. parvulum*. Similarities in pore structure/stria arrangement between *G. parvulum* and *G. gracile* for example, were highlighted by Krammer & Lange-Bertalot (1986, 1991b) and Reichardt (1999) as well as Dawson (1972 and 1973a and 1973b), but without a series of intermediate forms, no taxonomic link could be deduced. Auxosporulation is still not recorded in most diatom taxa and consequently, the full range of sizes, shapes and morphological characters at different stages remains unknown. Krammer & Lange-Bertalot (1986), suggest that genetically isolated populations are likely to exist, and note that the smaller forms do not correspond to Kützing's type material despite claims that they are representative examples by Geitler (1932); Germain (1981) and Hustedt (1930). However, their comments take no account of the different species concepts held at those times.

Table 1.2: part 1

Taxonomic treatment of *G. parvulum* and its varieties post Kützing. Text in red indicates authority for that taxon.

Row ID	Ehrenberg	de Brébisson	Kützing
A			*Gomphonema parvula* (1844)
B		*Gomphonema minutissimum?*	*Sphenella parvula* (1844)
C		*Sphenella parvula?*	*Gomphonema parvulum* (1844)
D			
E			
F			*G. parvulum var. subelliptica* Cleve
G			*G. parvulum* var. *exilis* Grunow
H			*G. parvulum* var. *exilissima* Grunow
I		*G. angustatum*	*G. parvulum* var. *micropus* (1844)
J			
K			
L			
M			*G. parvulum* var. *tergestina* Grunow
N			
O	*G. parvulum* var. *lanceolata* (?)		
P			
Q			
R			
S			
T			*G. parvulum* var. *lagenula* (?)
U			
V			

Table 1.2: part 2

Taxonomic treatment of *G. parvulum* and its varieties post Kützing. Text in red indicates authority for that taxon.

Row ID	Grunow	Héribaud	Van Heurck
A			
B			
C	*Gomphonema parvulum* Kützing		*G. parvulum* (1880)
D			*G. parvulum var.subcapitata (1880)*
E			*G. parvulum* var. *lanceolata* (1880)
F			
G	*G. parvulum* var. *exilis* (1878)		
H	*G. parvulum* var. *exilissima* (1878)		
I	*G. angustatum var. intermedia* (1878)		
J	*G. micropus* fo. *major* (1880)	*G. micropus* var. *major* (1903)	
K	*G. micropus* var. *minor* (1880)		
L	*G. micropus* var. *exilis* (1880)		
M	*G. parvulum* var. *tergestina* (1880)		*G. parvulum* var. *tergestina* Grunow
N	*G. parvulum* var. *subcapitata* (1880)	*G. parvulum* var. *subcapitata* van Heurck	*G. parvulum* var. *subcapitata* Grunow
O	*G. parvulum* var. *lanceolata* (1880)	*G. parvulum* var. *lanceolata* Ehrenberg	*G. parvulum* var. *lanceolata* Ehrenberg
P			
Q			
R			
S			
T			*G. lagenula* (1880)
U			
V			

Table 1.2: part 3

Taxonomic treatment of *G. parvulum* and its varieties post Kützing. Text in red indicates authority for that taxon.

Row ID	Cleve	Dippel	Mayer
A			
B			
C	*Gomphonema parvulum* Kützing	*G. parvulum* van Heurck	*Gomphonema parvulum* (Kützing) Kützing
D	*Gomphonema parvulum* Kützing		*G. parvulum var.subcapitata* van Heurck
E	*Gomphonema parvulum* Kützing		
F	*G. parvulum var. subelliptica* (1894)		*G. parvulum var. subellipticum* Cleve
G			*G. parvulum* var. *exilis* Grunow
H			*G. parvulum* var. *exilissimum* Grunow
I	*G. parvulum* var. *micropus* (1894)	*G. parvulum* var. *micropus* Cleve	*G. parvulum* var. *micropus* (Kützing) Cleve
J			
K			
L			
M	*Gomphonema parvulum* Kützing		
N	*Gomphonema parvulum* Kützing		
O	*Gomphonema parvulum* Kützing		
P			*G. parvulum* var. *genuinum* (1928)
Q			*G. parvulum* var. *genuinum* fo. *semiaperta* (1928)
R			*G. parvulum* var. *aequalis* (1928)
S			
T	*Gomphonema parvulum* Kützing		
U			
V			

Table 1.2: part 4

Taxonomic treatment of *G. parvulum* and its varieties post Kützing. Text in red indicates authority for that taxon.

Row ID	Frenguelli	Hustedt	Pascher
A			
B			
C	*Gomphonema parvulum* Kützing	*Gomphonema parvulum* (Kützing) Grunow	*Gomphonema parvulum* (Kützing) Grunow
D			
E			
F			*G. parvulum var. subelliptica* Cleve
G			*G. parvulum* var. *exilis* Grunow
H			*G. parvulum* var. *exilissima* Grunow
I			*G. parvulum* var. *micropus* (Kützing) Cleve
J			
K			
L			
M			
N			
O			
P			
Q			
R			
S	*G. parvulum* var. *curta* Rochoux d' Aubert		
T	*G. parvulum* var. *lagenula* Kützing		*G. parvulum* var. *lagenula (*Kützing? Grunow) Hustedt
U			
V			

Table 1.2: part 5

Taxonomic treatment of *G. parvulum* and its varieties post Kützing. Text in red indicates authority for that taxon.

Row ID	Lowe	Patrick & Reimer	Dawson
A		*G. parvulum* (Kützing) var. *parvulum*	
B		*G. parvulum* (Kützing) var. *parvulum*	
C	*Gomphonema parvulum* (Kützing) Kützing	*G. parvulum* (Kützing) var. *parvulum*	
D			
E			
F	*G. parvulum var. subelliptica* Cleve	*G. parvulum* (Kützing) var. *parvulum*	*G. parvulum* var. *subellipticum* Cleve
G		*G. parvulum* (Kützing) var. *parvulum*	
H		*G. parvulum* (Kützing) var. *parvulum*	*G. parvulum* var. *exilissimum* Grunow
I	*G. parvulum* var. *micropus* (Kützing) Cleve	*G. parvulum* (Kützing) var. *parvulum*	
J		*G. parvulum* (Kützing) var. *parvulum*	
K		*G. parvulum* (Kützing) var. *parvulum*	
L		*G. parvulum* (Kützing) var. *parvulum*	
M			
N		*G. parvulum* (Kützing) var. *parvulum*	
O		*G. parvulum* (Kützing) var. *parvulum*	
P			
Q		*G. parvulum* (Kützing) var. *parvulum*	*G. parvulum* var. *genuinum* Mayer
R			*G. parvulum* var. *aequalis Mayer*
S			
T		*G. parvulum* (Kützing) var. *parvulum*	
U			
V			

Table 1.2: part 6

Taxonomic treatment of *G. parvulum* and its varieties post Kützing. Text in red indicates authority for that taxon.

Row ID	Germain	KRAMMER & LANGE-BERTALOT
A		
B		*Gomphonema parvulum* (Kützing) Kützing 1849
C	*G. parvulum* (Kützing) Grunow	*Gomphonema parvulum* (Kützing) Kützing 1849
D		
E		
F		
G		*Gomphonema parvulum* (Kützing) Kützing 1849
H		
I	*G. parvulum* var. *micropus* (Kützing) Cleve	*Gomphonema parvulum* (Kützing) Kützing 1849
J		*Gomphonema parvulum* (Kützing) Kützing 1849
K		*Gomphonema parvulum* (Kützing) Kützing 1849
L		
M		
N		
O		
P		
Q		
R		
S		
T		*Gomphonema parvulum* (Kützing) Kützing 1849
U		*Gomphonema parvulum* fo. *saprophilum* (1993)
V		*Gomphonema parvulum* var. *parvulius* (1993)

McBride & Edgar, (1998), were the first to note that *G. parvulum* was capable of producing a morphological oddity, the Janus cell **(Figure 1.4),** occurring when two morphologically distinct valves occur in a single diatom frustule, often seen in the contrasting stria arrangements on the valve faces. This phenomenon is considered rare (but presumably inducible?), and indicates that *G. parvulum's* various phenotypes could be produced from the same genotype, i.e. the developmental pathway can be modified. Mann (1999) suggests that because the two halves of a diatom frustule form at different times, each may be influenced by different environmental conditions and hence exhibit two different morphological forms within the same species. Experimental work might therefore be beneficial in revealing the true nature of *G. parvulum's* phenotypic plasticity, as well as its ecological tolerances. The advantage of this type of environmental interaction is that it may allow a population to maintain a narrow tolerance range (lower cost in energy terms) but still exhibit some plasticity in environmental adaptation.

Modern diatom floras and keys continued to be published, e.g. Hartley (1986), Kelly (2000) and Prygiel & Coste (2000), and a recent review of Krammer and Lange-Bertalot's diatom combinations (Metzeltin & Kusber, 2001) indicates that *G. parvulum* var. *undulatum* Cleve, has been raised to a species, and the name changed to *Gomphonema astridae* E. Reichardt *et* Lange-Bertalot (in Reichardt, 1990).

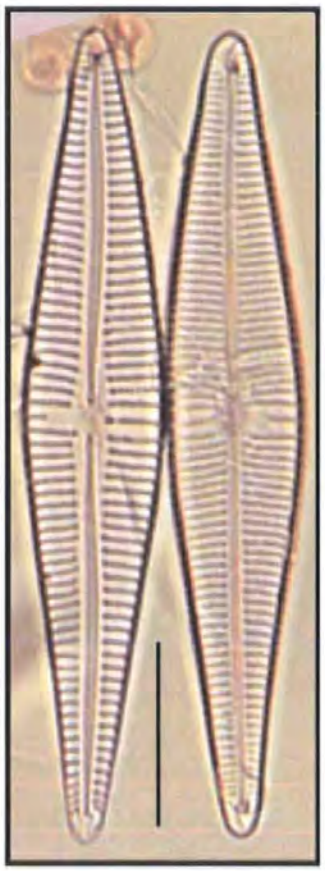


Figure 1.4

Light micrograph example of Janus valves in *Gomphonema* sp. One valve has more striae in 10µm than the other. Scale bar = 10µm.

1.5.3 Identifying the Type Specimen

As Kützing did not specify a type specimen in any of his material and Dawson (1972, 1973a and 1973b) did not publish specific material or specimens as type (this was not a requirement of the Botanical Code at the time), her labelling of particular slides and by association, material as type, is questioned. To this end, the material on all four slides was re-examined in an attempt to identify a specimen that most closely resembles Kützings original description and drawings. On initial examination of the four slides (BM18696, 18695, 18587, 18588) representing a sub-sample of each of Kützings four packets of material from Falaise, it was apparent that more than one form of *Gomphonema* was present on each of the slides, raising the question "Which form was Kützing was referring to?" Kützing's descriptions of *G. parvulum* were minimal. There is no mention of stria density or form, limited no doubt by *G. parvulum's* small size and the resolving power of Kützing's microscope. Therefore, one is reliant on the small drawing to get a feel for valve and apical pole shape.

Figure 1.5 illustrates representative specimens of the different forms present on each slide, and their respective labels of either *S. parvula* or *G. parvulum*. Referring back to Kützing's original, but somewhat brief descriptions and drawing for *S. parvula/G. parvulum*, the specimens relating most closely to those in Kützing's original publication (1844 and 1849) are indicated by green arrows. Both these forms have pronounced rostrate to capitate poles as indicated in Kützing's descriptions and illustration. **Figures 1.6 to 1.9**, further illustrate the different forms for each slide and using modern floras (Krammer and Lange-Bertalot, 1986 and 1991b), the forms were identified as either *G. parvulum*, varieties of *G. parvulum* or some other *Gomphonema* species. This therefore presents a dilemma, which of the two specimens identified in **Figure 1.5**. should be

nominated as the type? As slide BM18587 and thus packet 1262 represent the earliest material from which Kützing described what we now know to be *G. parvulum*, the principle of priority would seem to apply and thus this specimen should be considered the species type, Falaise the type location, packet 1262 the type material and slide BM18587 the type slide.

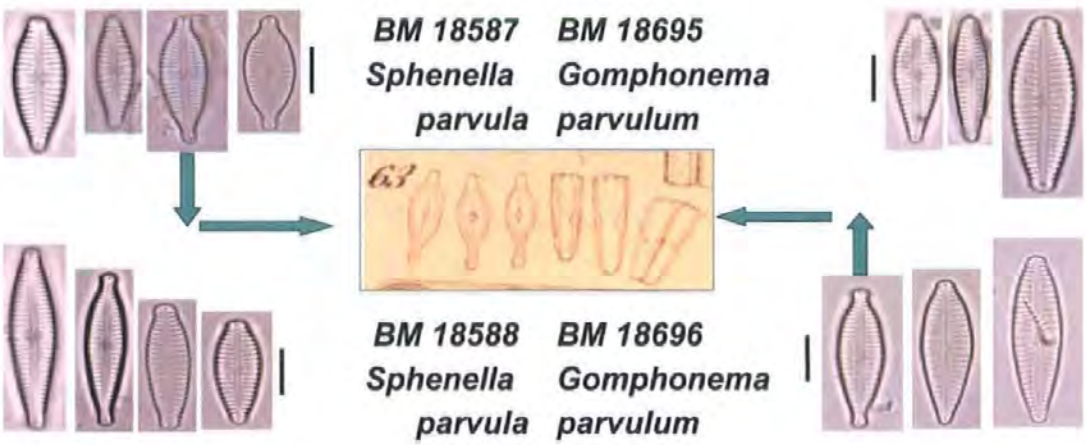


Figure 1.5

Diagram showing representative specimens of the different forms of *Gomphonema* spp. present in each packet of Falaise material. The specimens most closely relating to Kützings original descriptions and drawings (centre) are indicated by green arrows. All scale bars = 10µm.

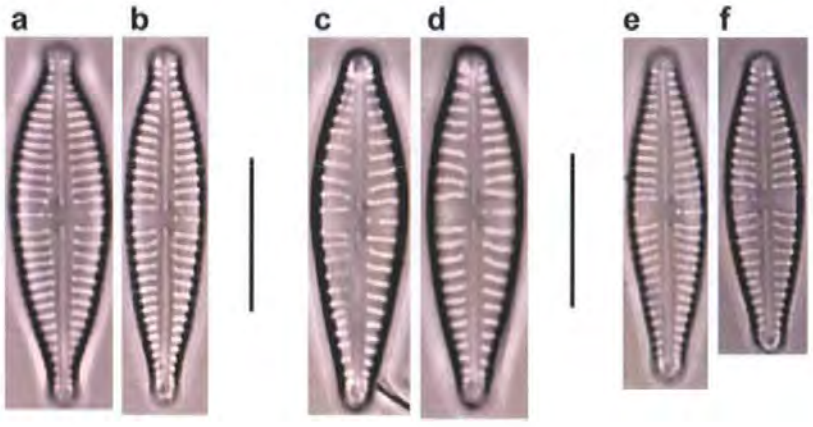


Figure 1.6: a - f

Three forms of *Gomphonema* recognized in Kützing's Falaise material, packet 1262, slide BM 18587, and identified as, **(a-b)** *G. parvulum* var. *parvulum* fo. #1; **(c-d)** *Gomphonema* cf. *angustatum* # 1 and **(e-f)** *Gomphonema* cf. *angustatum/parvulum*? All scale bars = 10µm.

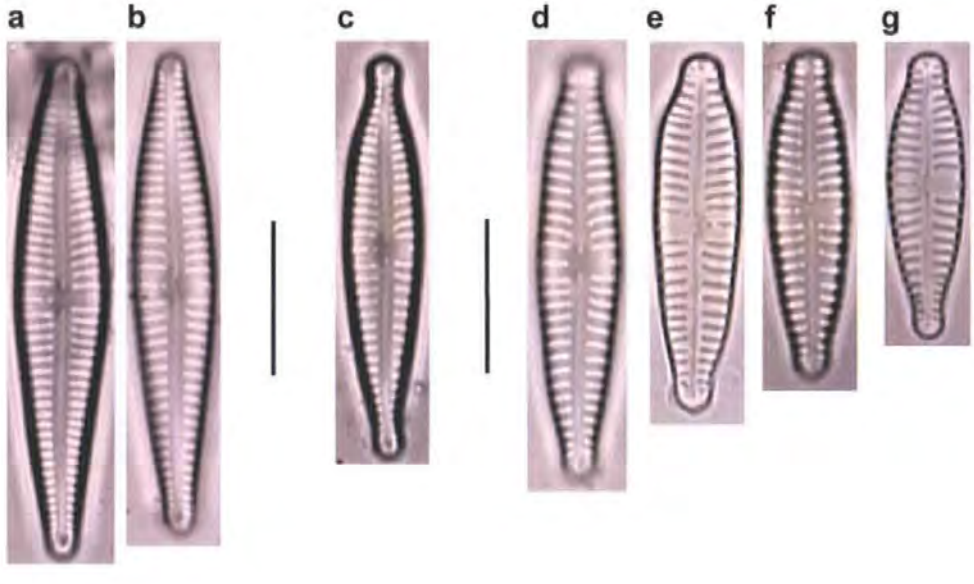


Figure 1.7: a – g

Three forms of *Gomphonema* recognized in Kützing's Falaise material, packet 1269, slide BM 18588, and identified as, **(a-b)** *G. gracile* **(c)** *G. parvulum* var. # 3 and **(d-g)** *G.* cf. *angustatum/sarcophagus/micropus*. All scale bars = 10µm.

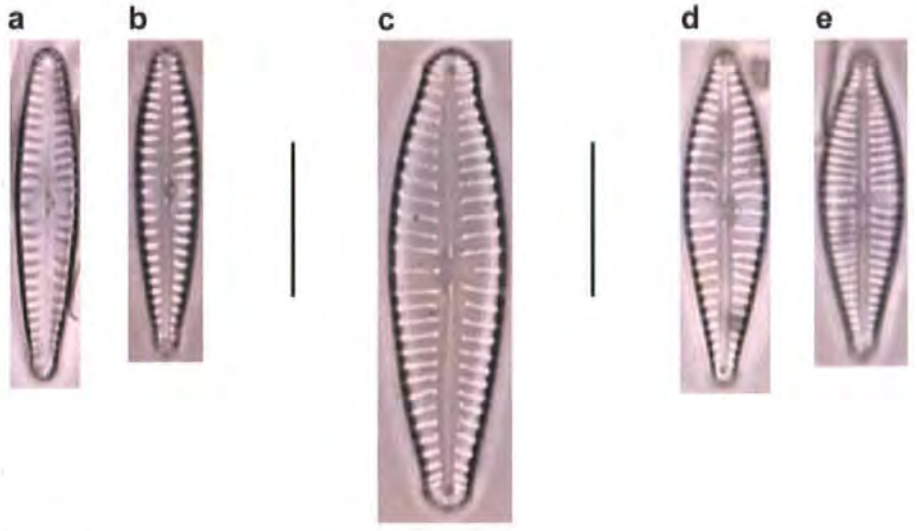


Figure 1.8: a - e

Three forms of *Gomphonema* recognized in Kützing's Falaise material (BM 18695) and identified as, **(a-b)** *G. gracile* **(c)** *G. angustatum* #3 and **(d-e)** *G. angustatum* # 4. All scale bars 10µm.

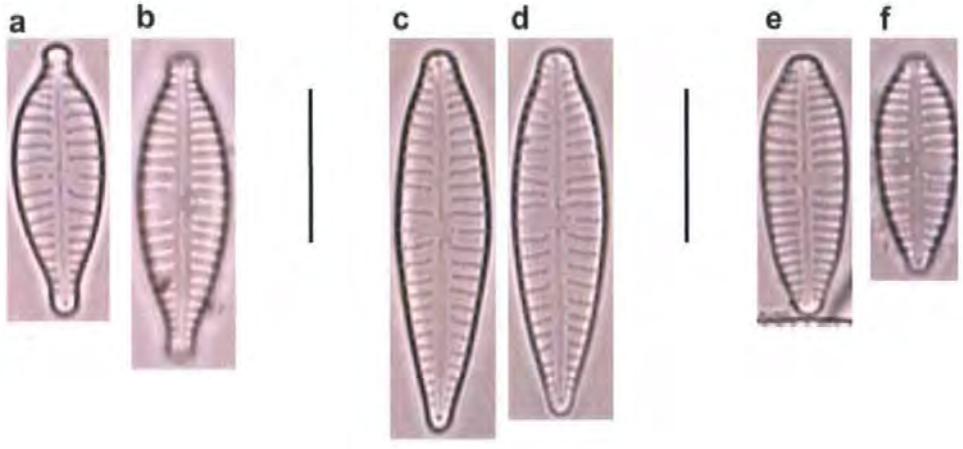


Figure 1.9: a - f

Three forms of *Gomphonema* recognized in Kützing's Falaise material (BM 18696) and identified as, (**a-b)** *G. parvulum* var. *parvulum* fo. #2, **(c-d)** *Gomphonema* cf. *angustatum* # 2 and **(e-f)** *G. parvulum*. All scale bars 10µm.

1.6 RATIONALE

- *G. parvulum* is widely distributed, morphologically variable and used in diatom indices of water quality despite there being no clear link between various morphologies and particular autecologies.

- Taxa with apparently wide ecological distributions are usually considered poor biological indicators.

- There is no evidence in the literature that *G. parvulum's* morphology changes in a predictable manner.

- Few diatom studies have investigated the autecology and taxonomy of freshwater benthic diatoms.

In order to refine the use of *G. parvulum* in water quality monitoring, it is desirable to develop a better understanding of the effects of specific environmental variables such as light and temperature, pH, organic pollution and heavy metals on valve and protoplast morphology and diatom colony formation.

1.7 AIMS AND OBJECTIVES

The collection of material for laboratory studies will enable the relationship between morphology and ecology to be investigated, the taxonomic relationship of populations from different sites evaluated and the use of *G. parvulum* in biological monitoring assessed.

Variation in distribution and morphology will be documented for wild populations against a range of environmental parameters. In particular, organic pollutants, heavy metals and pH. Clones will be isolated into unialgal cultures from a range of sites representing

different environmental conditions and compared against environmental data. The clones will be tested against different environmental parameters to establish clonal autecology and to assess any morphological modification. Additionally, clonal performance will be monitored using growth rate.

Preliminary results will indicate whether different morphological races have particular autecologies, or whether morphology is a response to environment, prompting a taxonomic revision of the species and the recognition of ecotypes or ecomorphs.

2 GENERAL METHODS

This chapter outlines the general laboratory, field collection, material preparation and statistical methods used throughout this study.

2.1 EQUIPMENT & MEDIA

Aseptic techniques were used throughout the study. Field collecting equipment was always rinsed in local water prior to collection to minimise cross contamination, and algal samples were always placed in clean plastic vials, petri-dishes or glass bottles. Water samples were placed in clean plastic bottles and labelled.

All re-useable glass and plastic-ware were soaked for at least 24 hours in 5-10% hydrochloric acid, thoroughly rinsed in distilled water, with a final rinse in de-ionised water to remove any ions adhering to the vessel surface. Glass and plastic-ware used for media containment and filter sterilisation were autoclaved at 120°C for 15 min (Midas 56 Priorclave). Medium transfers were always performed in a laminar flow cabinet. Diatom cells were transferred between culture dishes using a flame-sterilized micropipette. All petri and multi-well dishes (Nunclon™) were sterile. Distilled water was obtained via Millipore ROs™ and Elix™ water purification system (reverse osmosis & electro-deionisation respectively). Deionised water was obtained via Milli-Q™ Gradient System using UV filtration and ultrafiltration.

All stock diatom cultures were maintained in the liquid growth medium Woods Hole MBL, pH 7.2. All experiments used MBL as the base medium with the appropriate variable or

nutrient adjusted or added, and filter sterilised in the manner described above. All media preparations can be found in **Appendix I**.

2.2 COLLECTION AND INITIAL PREPARATION OF WATER SAMPLES

Two 50 ml samples of filtered water (Nalgene Syringe Water Filter 0.45 µm pore) were collected at every sampling site. Within 12 hours of collection, one of each pair of water samples had 2 ml of Nitric acid (AnalaR 60%) added to preserve the chemical integrity of the sample and to stop precipitation of cations and their adsorption onto the container wall. All samples were stored at -25°C until such time that water chemistry analyses could be performed. Water chemistry analyses consisted of ion chromatography (non-acidified samples) and Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) (acidified samples), with a resolution to parts per million (ppm/10^{-6} or mg/l).

2.3 COLLECTION AND PREPARATION OF DIATOM SAMPLES

Muddy sediments were sampled to a 2cm depth using a simple corer made from a section of plastic drainpipe approximately 5cm in diameter. Field cores were placed in petri-dishes with a little local water. In the laboratory, distilled water was added and stirred into the core sample and then allowed to settle for 10 minutes. Two layers of lens tissue were placed over the mud cores allowed diatoms to migrate to the surface overnight. On the following day, the lens tissue was removed and placed into clean glass vials with a little MBL medium. Unconsolidated sediments were sampled by means of a Pyrex tube approximately 1m long and 0.7cm in diameter and placed in clean vials. Algae growing on man-made surfaces were scraped with a small penknife and placed in vials with local water, whilst algae attached to rocks, gravels and cobbles were collected whole with a little local water for separate scrapings in the laboratory. At least three rocks of similar size were collected from a site and the material combined.

Sub-samples of algal material were placed into petri-dishes with fresh MBL and maintained in a culture cabinet under a light and temperature regime of 16hrs light: 8hrs dark, 20°C and an average light level of 15cd/m^2/sec^{-1}. These "rough" cultures of algae were allowed to settle for 5 -7 days prior to examination for and isolation of, single *G. parvulum* cells. All remaining field samples were transferred to small glass vials, preserved with Lugol's iodine and stored in a fridge until they could be prepared for light and scanning electron microscopy (LM and SEM).

2.3.1 Isolation and Culturing of *G. parvulum*

"Rough" cultures established from field material were examined under an inverted binocular microscope (Olympus CK2), to determine the sample comprised primarily live, healthy cells, and to isolate single *G. parvulum* cells. Several individual drops of prepared MBL medium were placed in sterile petri-dishes (one dish for each sample site) using a sterile, disposable plastic pipette and covered. Several *G. parvulum* cells from each "rough" culture for each sample site were isolated and transferred into the one of several prepared drops of medium by micropipette and allowed to settle for a few minutes. Each drop was examined, and individual cells located and micropipetted into a neighbouring drop of MBL. This process was repeated at least 5 times per single cell, per sample to clean each cell of any bacteria, fungi and smaller green algae. After a final wash, a single cell was inoculated into a second batch of prepared sterile petri-dishes (two dishes per site), each containing a single drop of MBL. Each drop was checked to ensure it contained a single undamaged *G. parvulum* cell before each petri-dish was topped up with MBL. Petri cultures were allowed to grow into uniclonal cultures for 3-5 weeks. If these initial inoculations were successful, single cells were removed and inoculated into fresh media, using the same isolation method every 4-6 weeks. These clonal stock cultures were used for all assays, as well as providing material for morphological analysis

of cell diminution, and when it occurred, sexual reproduction. Material was harvested every 4-6 weeks, digested and permanent slides prepared for light microscopy (LM). All stock cultures were maintained at 20°C with a 16hr light: 8hr dark regime and an average light level of 15cd/m^2/sec^{-1}.

2.3.1.1 Sexual Reproduction in Uniclonal Culture

Four of the ten uniclonal stock cultures underwent sexual reproduction, producing auxospores and subsequently, a second generation of uniclonal cultures. Both the original parent culture and their offspring were studied. The original parent culture was subsequently denoted as generation 1 (G_1) and their offspring as generation 2 (G_2). Only uniclonal cultures established from Kings Mere, Ham Gate Pond, Llyn Idwal and Parys Mountain underwent sexual reproduction. The denotation is used throughout the thesis.

2.3.2 Light and Electron Microscopy

All live observations utilised two digital cameras. One attached to an inverted microscope (Olympus CK2) with a maximum magnification of x40, and one to a Zeiss Axioplan with a maximum magnification of x1000 (+ oil immersion).

For cell wall studies, all cells were digested using 48 hours cold acid treatment (Nitric Acid, 60%) for low yield samples, or for dense samples, boiling in nitric acid to rid cells of their organic content. In both cases, the supernatant was pipetted off and replaced with distilled water. Samples were then centrifuged at least three times, more often five times (Sigma 3-10 Howe) for 10 min at speeds of 3000 revolutions per minute (rpm). After each centrifugation, the supernatant was pipetted off leaving behind a diatom pellet that was

then re-suspended in distilled water. This process ensured displacement of any remaining acid. After final centrifugation and pipetting, the diatom pellet was placed in a clean, clip-top glass vial with equal proportions of distilled water and 90% alcohol, to deter fungal and bacterial growth, and stored in a covered box in a cool room.

Drops of preserved material were placed onto glass coverslips and allowed to dry naturally over a period of 24 hours. Once dried, coverslips were examined to ensure sufficient material had been deposited. Where insufficient material was present, an additional drop of preserved diatom material was added and allowed to dry. Once dried, coverslips were inverted onto mounting medium (Naphrax or Zrax) and gently heated on a hotplate to release the solvent before cooling and allowing the medium to harden.

For SEM's, a coverslip was glued onto an SEM stub prior to the samples being prepared as described above. Stubs were then sputter-coated with gold-palladium, and examined under a Philips XL30 SEM. Air dried samples of non-digested material were also examined under SEM to examine and illustrate any extracellular polymeric substances (EPS) present.

2.4 ASSAY METHODS

All experiments were conducted on two illuminated water-filled, temperature controlled metal gradient plates. Both light and temperature could be varied and controlled by the positioning of an overhead strip light (raising and lowering and moving back and forth) and a water pump/heater maintained by a timer switch respectively.

Sterile, multi-welled dishes (= square Nunclon™ dishes, each with 4 round micro-wells per dish each of which was able to hold a maximum of 2ml of medium) were used for all experiments. Gradient plates were subject to a 16hr light: 8hr dark cycle for all experiments. Experimental temperature was maintained within ±2°C of the target and monitored daily. Four replicates of each treatment were checked every other day for 6 days and the number of diatom cells counted. Six experimental gradient regimes were undertaken: light & temperature, pH, artificial sewage, copper, zinc and cadmium.

For the Light & Temperature Regime, the metal gradient plates were divided into nine equal sections each accommodating 9 Nunclon™ dishes. Three bands of light (High, Medium, Low) and six grades of temperature (5°C, 10°C, 15°C, 20°C, 25°C, 30°C) were established **(Figure 2.1)**. For all other experimental regimes, light and temperature were held at 22°C (±2°C) and 38cd/m^{-2}/sec^{-1} (±3cd/m^{-2}/sec^{-1}, due to variability in power supply and shading) across the two plates.

For the pH regime, the pH of standard MBL medium was adjusted by the addition of 32% HCl or 10% sodium hydroxide (NaOH) prior to filtration to within ± 0.2 pH units. The pH series ran from pH 3.5 to pH 11.0 at 0.5 unit intervals, providing 16 parameters per clone.

An artificial sewage was produced following the recipe and protocol described by OECD (1981) (recipe: **Appendix I**). The artificial sewage series consisted of five strengths of artificial sewage, i.e. full strength, 1/2 strength, 1/5 strength, 1/10 strength and 1/25 strength, plus a control of standard MBL without any artificial sewage added.

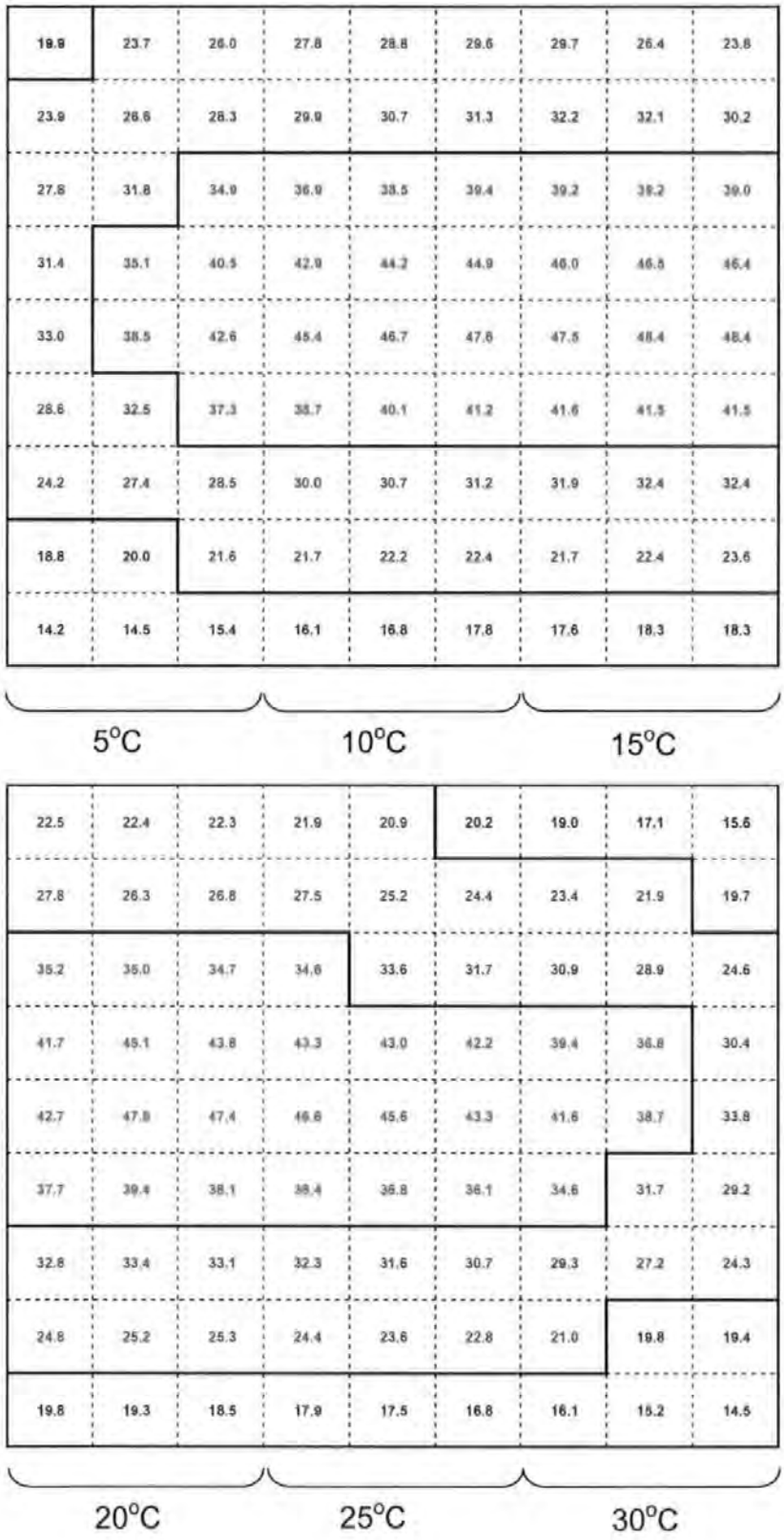


Figure 2.1.

Gradient plate map depicting low, medium and high irradiance levels (4–20: blue; 21–34: green and 35-48: red, $cd/m^{-2}/sec^{-1}$ respectively), and temperature range from left to right (5°C to 30°C).

Copper and Zinc are micronutrients required for growth by diatoms and were present in the standard MBL medium as $CuSO_4.5H_2O$ and $ZnSO_4.5H_2O$ respectively. Concentrations of $CuSO_4.5H_2O$ and $ZnSO_4.5H_2O$ were adjusted to provide nine elemental Cu and Zn concentrations. This was the "initial run".

Preliminary examination of cell counts on the initial run indicated increased growth at the higher concentrations for all clones. The Zn and Cu experiments were subsequently extended to provide a further eight elemental metal concentrations. This was the "extended run". The assays in this study exceeded the maximum allowable concentrations of Cu and Zn in drinking water per WHO, EU and UK legislation. All medium recipes can be found in **Appendix I**.

Duplicate treatments at 0.05 and 12.50µg/l metal concentration, were also tested without the addition of sodium ethylenediamunetetra-acetic acid (Na_2 EDTA) (each denoted with the letter 'E'). EDTA is normally included in media to prevent the precipitation of salts. Although trace metals are essential for microalgal growth, little is known about the relationship between different metal species and biological availability. Non-EDTA treatments would give an indication as to how influential EDTA was in binding Cu and Zn at low and high concentrations.

2.5 GROWTH RATE ANALYSIS

Diatom cell numbers were transformed into growth rates using the equation:

$K_e = \log (N_1/N_0) (3.322/t)$ (Stein, 1973) **Equation 1**

Where K_e the growth constant, is the number of logarithm-to-base 10 units increase per day, N_1 and N_0 are the number of cells present at times t_1 and t_0 respectively, and where $t = t_1 - t_0$ days. Growth rate (k) was calculated from successive counts made every other day (day 0, 2, 4 and 6) to identify the period of mean exponential growth rate (MEGR). If mean growth rate fell after, e.g. day 4, the period of exponential growth reflecting experimental conditions was taken to extend from day 0-4 and the growth rate was re-calculated to reflect this four day period and denoted k_{0-4}. Averaging of the separate values of k for days 0-2 and 2-4 would not have yielded the same value as k_{0-4} (Stein, 1973).

2.6 MORPHOMETRIC ANALYSIS

Diatom material was collected from different habitats and 10 clonal isolates established. Preparations of acid cleaned samples enabled morphological examination under LM. Selected subcultures were also examined under SEM. Initial identification and naming of each clone was based on a synthesis of the morphological characters detailed in the literature. (Cox, 1996; Dawson, 1972; Geitler, 1932; Krammer & Lange-Bertalot, 1986, 1991b and Kützing, 1844), thus all clones utilised in this study had the taxonomic features

of *G. parvulum sensu stricto*. Clones were then assigned to variety or form where possible.

The terminology for the main elements of the siliceous cell wall and protoplast, and descriptions of morphogenesis, reproduction and modes of growth in the general pennate diatom cell, as well as *G. parvulum*, can be found in the following publications: Barber & Haworth (1981); Cox, (1981b); Cox, (1996); Drum and Pankratz, (1964a); Mann, (1996) and Round *et al.*, (1990). A full account of sexual reproduction in *G. parvulum* can be found in Chepurnov *et al.*, 2004 and Geitler (1932). Readers should also take note of the description and formation of the Voigt discontinuity in Voigt, (1943).

Additionally, Cox (2004, unpub.) has shown that under the electron microscope, the various pores that comprise the striae are often seen to be occluded by delicate flaps or plates of silica, however the terminology for these flaps is somewhat confused. Cox (2004, unpub.) has also shown that different terms are being used for similar structures in centrics and pennates and conversely, identical terms are being used to describe dissimilar structures in the pennates. It is clear from the literature (Mann, 1981; Ross *et al.*, 1979) that six types of pore occlusion have been identified. These are cribra, hymen, volae, rotae, foriculae and tectulum and they describe finely or coarsely perforate pores or flap-like closures. This thesis will uses the terminology of Cox (2004, unpub.) for the partially occluded areolae, with flap-like unilateral foricula, previously termed volate areola in the Gomphonemataceae.

Clones were cultured according to **section 2.3**. and 14 morphological characters were examined **(Table 2.1)** for each subculture, six of which were measured quantitatively. These characters were chosen either because they are standard diatom measures (e.g. length, breadth, striae density), and/or because in *G. parvulum,* the character is observed to vary. Additionally, **Figures 2.2a-c**, define what is meant by inserted, broken and short striae; characters which although mentioned in the literature, have not previously been systematically recorded, enumerated or analysed. Up to 50 valves per stock sub-culture, and 10 valves per assay level for each clone were morphologically examined.

Whilst in stock culture conditions, four clones auxosporulated (from Kings Mere, Ham Gate Pond, Llyn Idwal and Parys Mountain). This enabled the full size and shape continuum to be recorded for these clones. Three clones also auxosporulated whilst in experimental medium (Llyn Ogwen at pH 6.5, Ham Gate Pond at pH 8.5 and 9.0 and at 1/5th sewage concentration, and the Scion Pond clone at a copper concentration 0.00016μg/l).

Table 2.1: part 1

Fourteen morphological characters examined under LM and SEM and subject to statistical analysis.

Character Code	Character		Character Description
L	Valve length		Length is measured along the apical axis of the valve face, apex to apex.
B	Valve breadth		Width is measured transapically at the widest point.
R1	Raphe length	Upper raphe	Measured from central nodule to apical nodule of head pole (head pole = that half of the valve face that is often wider and shorter than the other half)
R2		Lower raphe	Measured from central nodule to apical nodule of foot pole (foot pole = that half of the valve face that is often narrower and longer than the other half)
S1	Number of central striae in 10μm.	Stigma side (primary) of valve	Measurements were taken 5µm either side of the mid-point between the central raphe endings.
S2		Non-Stigma side (secondary) of valve	
SD1	Striae direction	Radiate	Radiate = when the striae radiate outwards from the central area
SD2		Convergent	Convergent = when the stria are all laying in the same direction, converging towards one of the apices
CSS	Number of central short striae opposite stigma		There is often, but not always a short stria opposite the stigma
OSS	Other short stria	Present (1)	The presence or absence of short stria other than that opposite the stigma (see Figure 4.1a)
		Absent (0)	
IS	Inserted striae	Present (1)	The presence or absence of 'inserted' striae refers to stria that do not extend to the valve margin (see Figure 4.1b)
		Absent (0)	
BS	Broken/interrupted striae	Present (1)	The presence or absence of 'broken' or 'interrupted' stria refers to stria that are not uniform across the valve face (se Figure 4.1c)
		Absent (0)	
STIG	Number of stigma		The number of stigma were counted and recorded for each valve in a sample.

Table 2.1: part 2

Fourteen morphological characters examined under LM and SEM and subject to statistical analysis.

Character Code		Character	Character Description
SHP1	Valve shape	Narrow lanceolate	Valve shape was determined according to Cox, 1996 and Barber, 1981.
SHP2		Lanceolate	
SHP3		Lanceolate-clavate	
SHP4		Ovate	
SHP5		Ovate-clavate	
SHP6		Elliptic	
HPS1	Head Pole shape	Rounded	Pole shape was determined according to Cox, 1996 and Barber, 1981.
HPS2		Rounded-subrostrate	
HPS3		Subrostrate	
HPS4		Subrostrate-rostrate	
HPS5		Rostrate	
HPS6		Rostrate-subcapitate	
HPS7		Subcapitate	
FPS1	Foot Pole Shape	Rounded	Pole shape was determined according to Cox, 1996 and Barber, 1981.
FPS2		Rounded-subrostrate	
FPS3		Subrostrate	
FPS4		Subrostrate-rostrate	
FPS5		Rostrate	
FPS6		Rostrate-subcapitate	
FPS7		Subcapitate	
ABN	Abnormalities	Present (1)	Gross abnormalities included bulges and dents to valve, major disruptions to striae or raphe systems, or any atypical morphology. An abnormality was recorded as present or absent for each cell in a sample.
		Absent (0)	

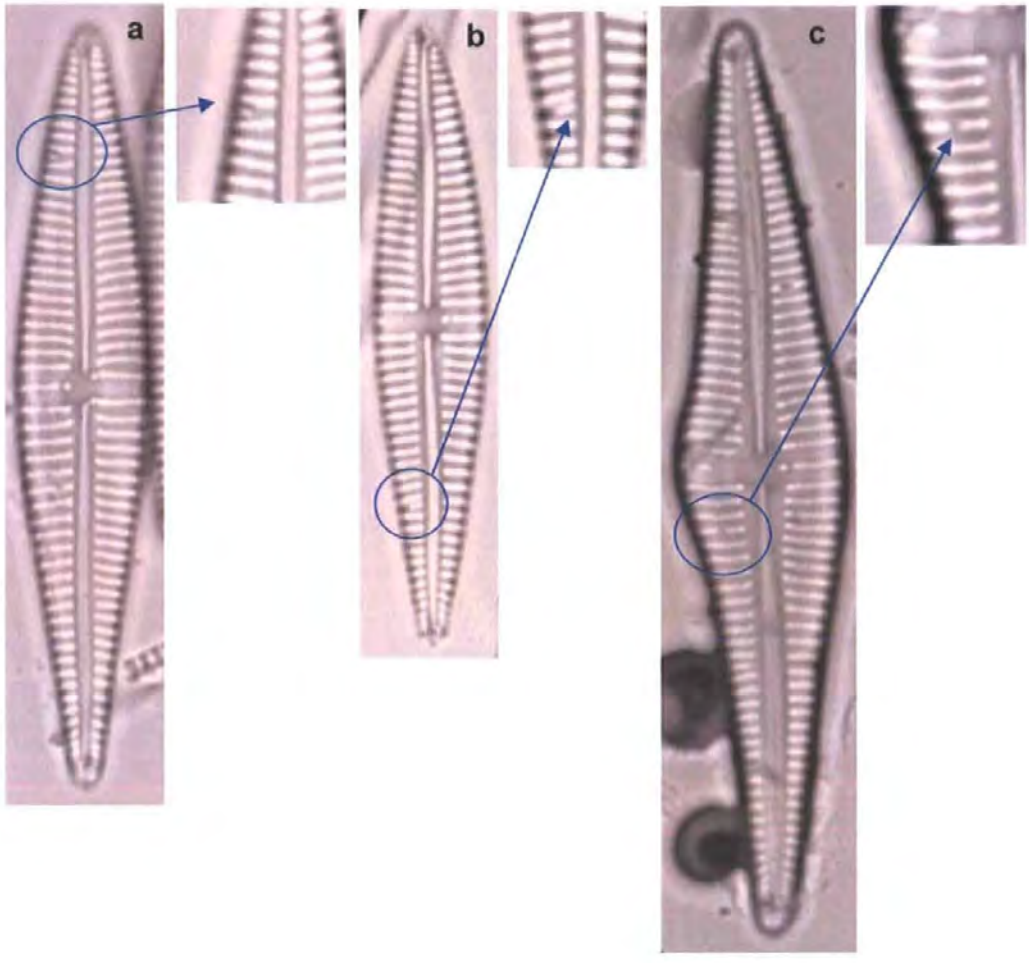


Figure 2.2:a-c

Morphometric characters not previously described **(a)** other short striae (OSS) **(b)** inserted stria (IS) **(c)** broken/interrupted striae (BS).

2.7 ANALYSIS OF LIVE MATERIAL

All stock cultures were observed in their live state on a regular basis over a period lasting up to two years. Data were obtained from observations on seven morphological or behavioural characters that might be related to size, sexual stage or culture regime **(Table 2.1)**. These characters were chosen as the ones most easily identifiable in LM. For the experimental regimes, a distinction was made between observations on days when cell counts were made, i.e. days 0-6 (denoted $K_{0\text{-}6}$) and observations post day 6 (denoted K_{6+}). This allowed for any differences in observations during and post exponential growth phase to be highlighted. LM examples of each character state are provided in **Figures 2.3 to 2.18**.

All observations were summarised in a simple binary coded presence/absence table for each clone, under each growth regime and time period (= Operational Unit, where data were available). The presence of a character was indicated by 1 and its absence by 0. The binary coded table formed the basis of several cluster analyses as described in section 2.9.

Table 2.1

The morphological character states and descriptions used for observations of live *G. parvulum* cultures.

Character Code	Character Description
Attachment Mode	
ATT1	Non attached cells, primarily suspended in the water column
ATT2	Cells primarily attached in valve view
ATT3	Cells primarily attached in girdle view
ATT4	Cells primarily attached by mucilage pad at foot pole
ATT5	Cells primarily attached by mucilage stalk at foot pole
Colony Type	
COL1	Primarily single cells
COL2	Primarily paired cells
COL3	Cells forming short chains (valve face to valve face)
COL4	Cells forming short chains (girdle to girdle)
COL5	Cells forming tufts of cells
COL6	Cells forming an amorphous mucilaginous matrix
COL7	Cells forming dense mass aggregations
Chloroplast Colour	
CHL1	Dark brown chloroplast with darker patches/peripheries
CHL2	Golden brown chloroplast with darker patches/peripheries
CHL3	Pale golden brown chloroplast with darker patches/peripheries
CHL4	Yellow chloroplast
CHL5	Green chloroplast
CHL6	Colourless chloroplast
Chloroplast Size	
CHL7	Chloroplast normal H-shaped
CHL8	H-shaped but chloroplast lobes extended into apical pole space
CHL9	Chloroplast appears to occupy entire cellular space
CHL10	Chloroplast constricted to medial area
CHL11	Chloroplast constricted to cell walls (usually by enlarged vacuoles)
Pyrenoid	
PYR1	Visible and triangular
PYR2	Visible and rounded
Vacuoles	
VAC1	Visible, 2 polar (normal)
VAC2	Visible, 1 polar (abnormal)
Other	
OIL1	Lipids
GRAN1	Granules
OTH1	Unidentified dark bodies, slightly larger than granules
OTH2	Cell wall teratology observed in some cells
OTH3	Culture auxosporulated

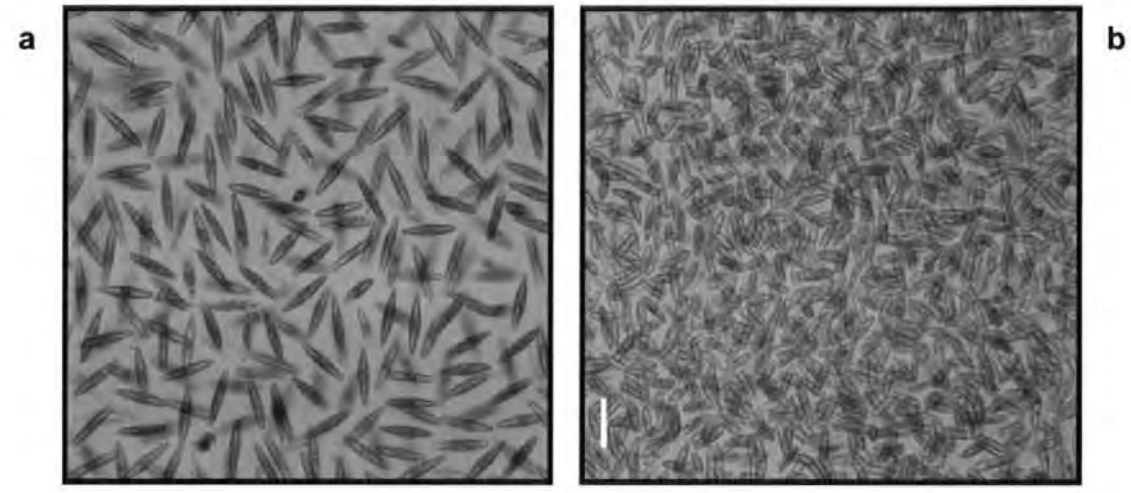


Figure 2.3: a-b

Examples of observational character state: Single (Code: COL1) Suspended Cells (Code: ATT1). Scored as present when the majority of the cells are not attached to the culture vessel but suspended in the water column. Scale bars = 40μm.

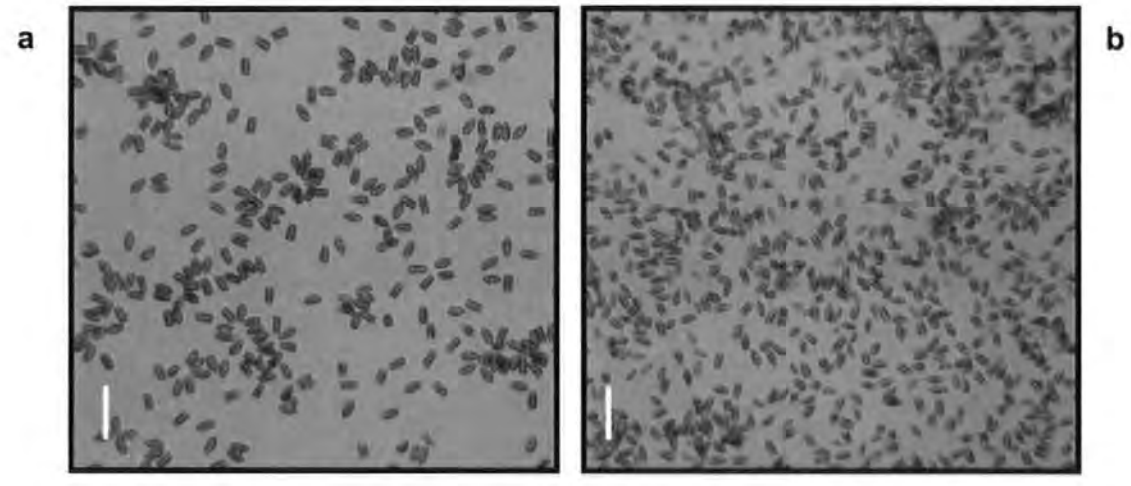


Figure 2.4: a-b

Examples of observational character state: Single (Code: COL1) attached in valve and/or girdle view (Codes: ATT3 and ATT4). Scored as present when the majority of the cells are attached to the culture vessel in valve and / or girdle view. Scale bars = 40μm.

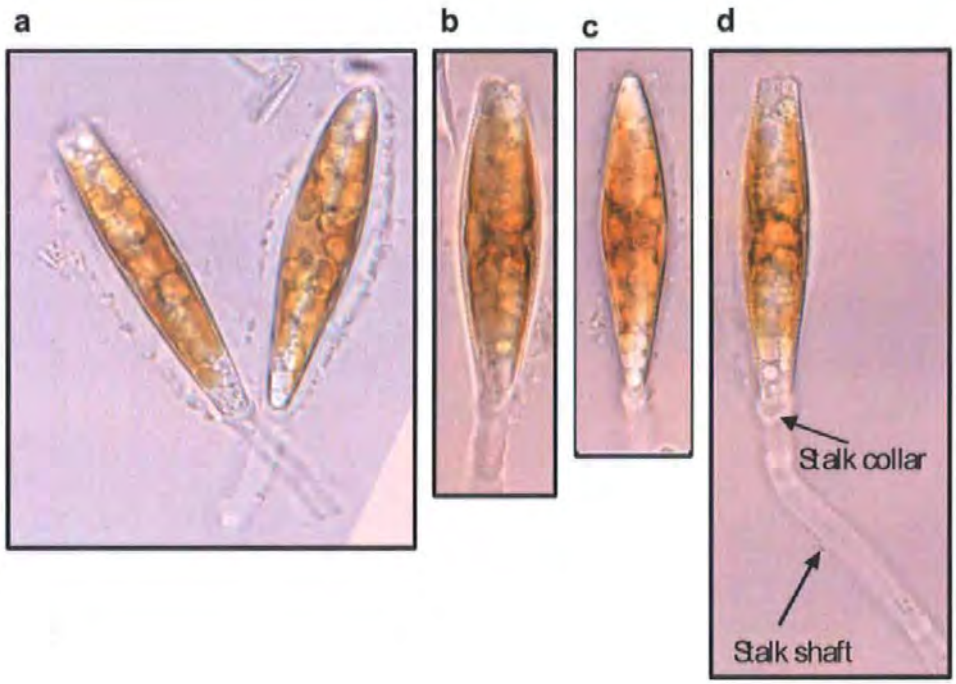


Figure 2.5: a-d

Examples of observational character state: Stalked cells (Code: ATT4). Scored as present when cells are attached to the culture vessel by a mucilaginous stalk at the foot pole. Note the mucilage surrounding individual cells in figure a. Scale bars = 10μm.

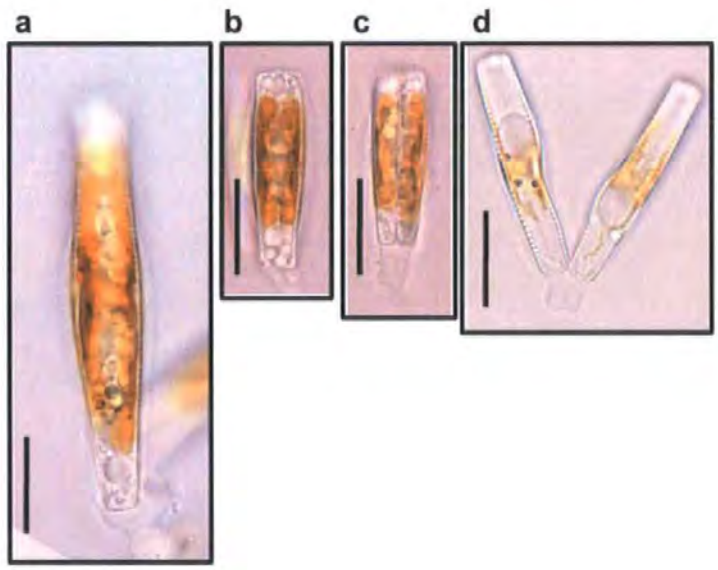


Figure 2.6: a-d

Examples of observational character state: Mucilage pad (Code: ATT5). Scored as present when cells are attached to the culture vessel by a mucilaginous pad at the foot pole. Scale bars = 10μm.

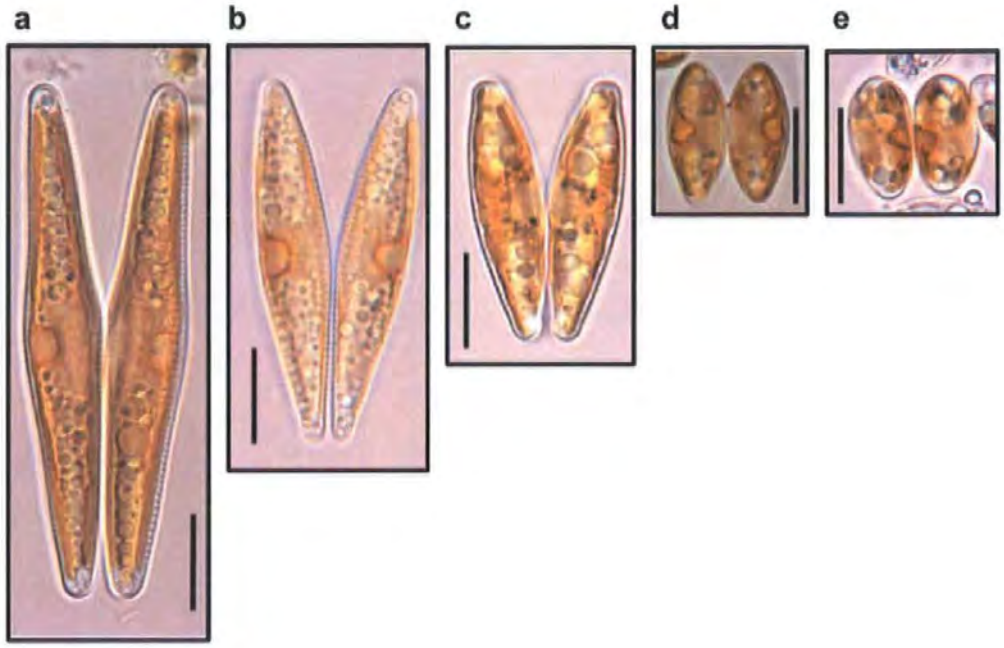


Figure 2.7: a-e

Examples of observational character state: paired cells (Code: COL2). Scale bars = 10μm.

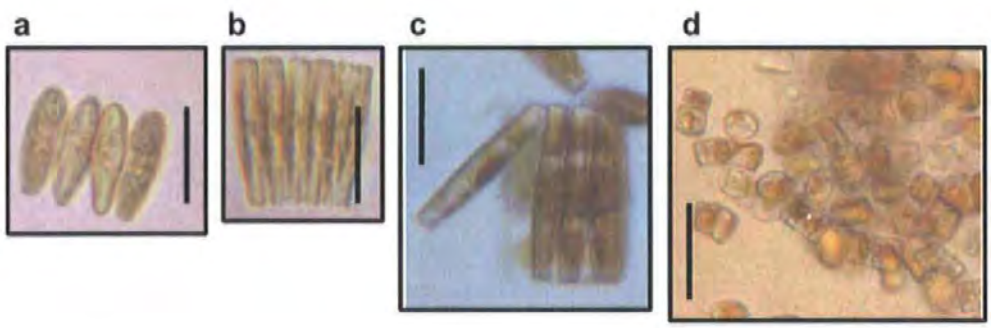


Figure 2.8: a-d

Examples of observational character state: chains formed valve face to valve face (CODE: COL3) or girdle to girdle (CODE: COL4). Scale bars = 10μm.

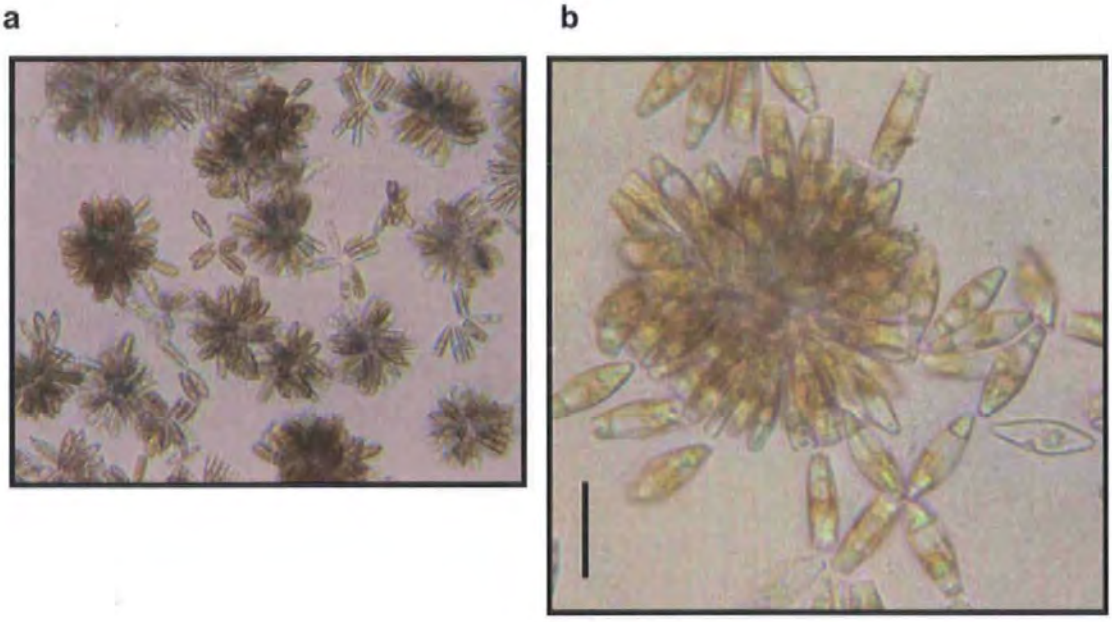


Figure 2.9: a-b

Examples of observational character state: tufted colonies (CODE: COL5). Scale bars = **(a)** = 40 μm **(b)** = 20μm.

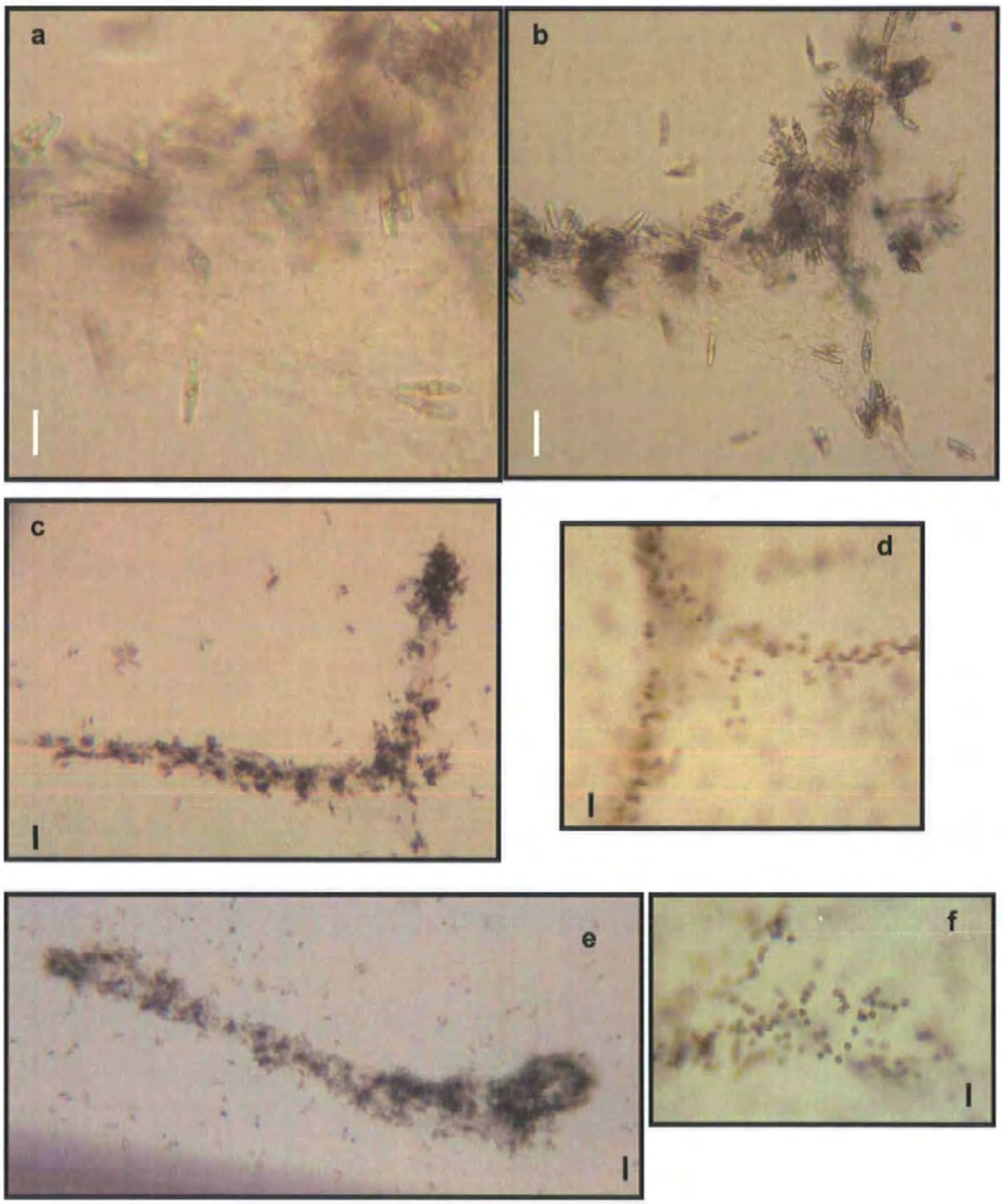


Figure 2.10: a-f

Examples of observational character state: EPS Matrix (CODE: COL6). Scale bars = **(a, c-f)** 20μm **(b)** 40μm.

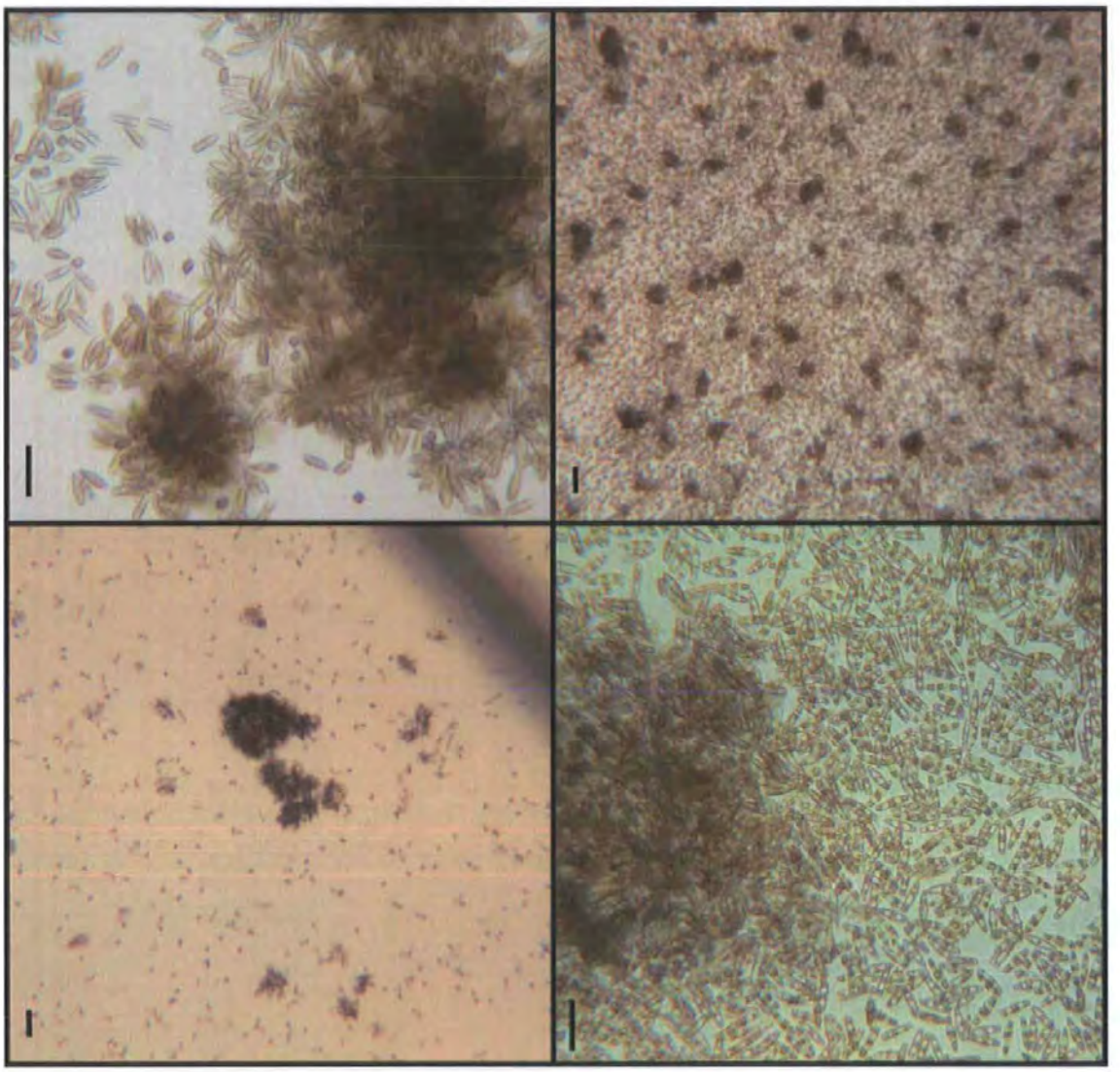


Figure 2.11: a-d

Examples of observational character state: Mass aggregations of cells, often forming clumps (CODE: COL7). Scale bars = **(a and d)** 40μm **(b and c)** 20μm.

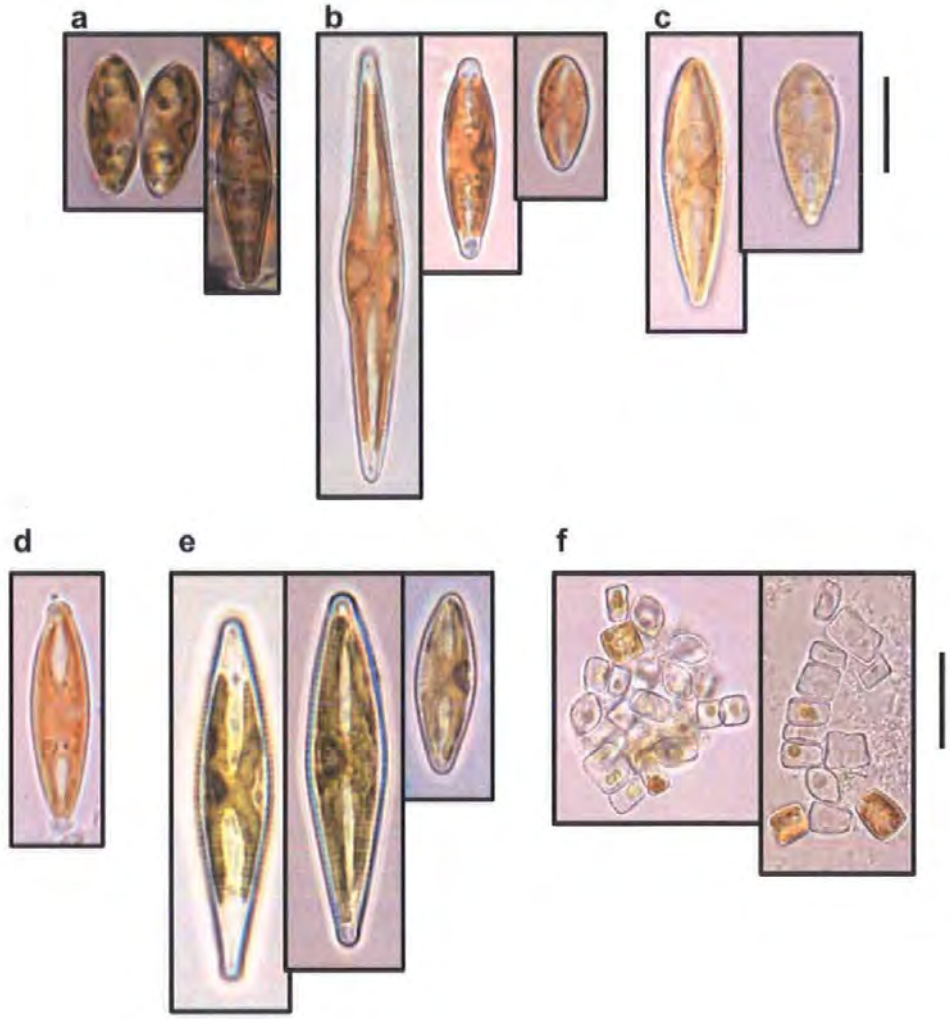


Figure 2.12: a-c

Examples of observational character state: **(a)** dark brown chloroplasts (CODE: CHL1), **(b)** golden brown chloroplasts (CODE: CHL2), **(c)** pale golden chloroplasts (CODE: CHL3), **(d)** Pale yellow chloroplasts (CODE: CHL4), **(e)** green chloroplasts (CODE: CHL5) and **(f)** colourless chloroplasts (CODE: CHL6) . Scale bars for all = 10μm.

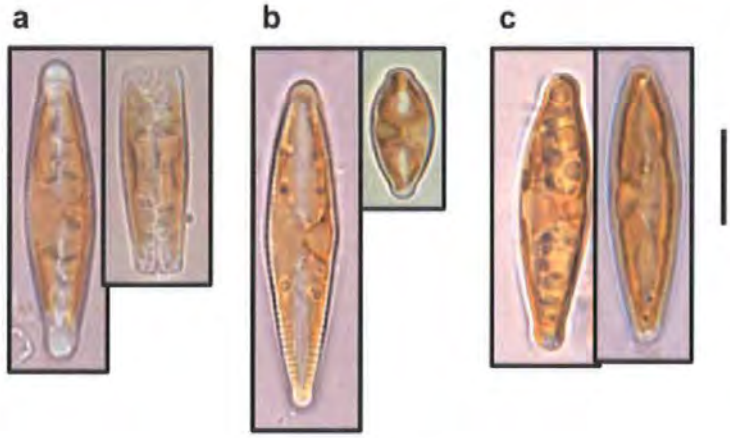


Figure 2.13: a-c

Examples of observational character state: **(a)** normal H-shaped chloroplasts (CODE: CHL7), **(b)** chloroplast lobes extend to poles (CODE: CHL8) and **(c)** chloroplasts occupies full cell volume (CODE: CHL9). Scale bar for all = 10μm.

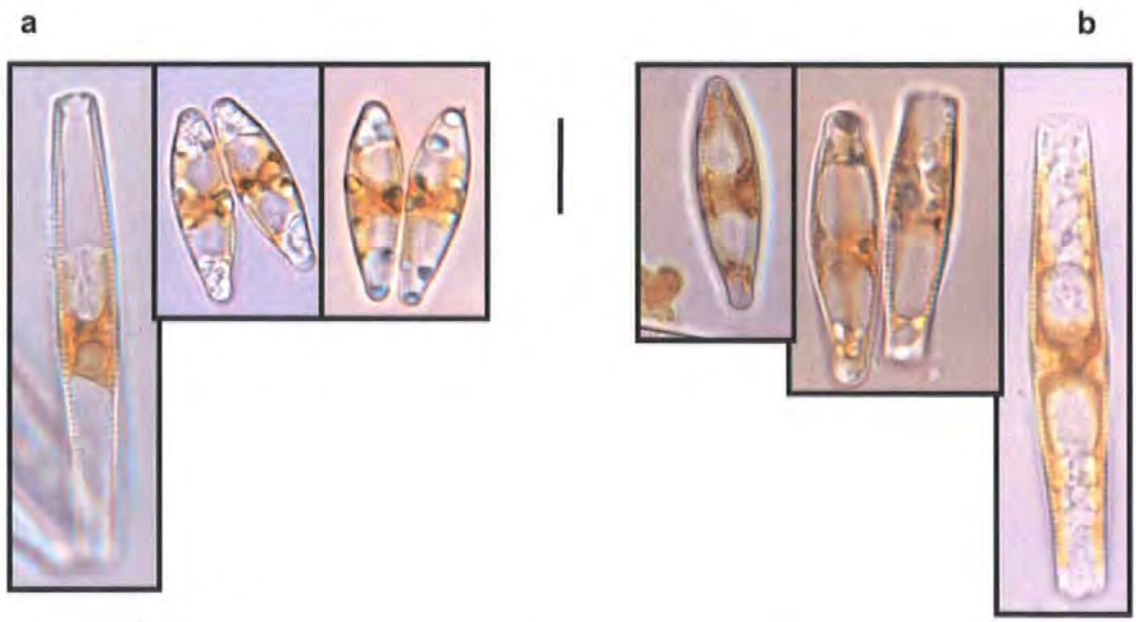


Figure 2.14: a-b

Examples of observational character state: **(a)** chloroplasts contracted to the medial area of the cell (CODE: CHL10), **(b)** chloroplast constricted to the cell wall, usually by one or two enlarged polar vacuoles (CODE: CHL11). Scale bar for all = 10μm.

Note also the large vacuoles (CODE: VAC1 and VAC2).

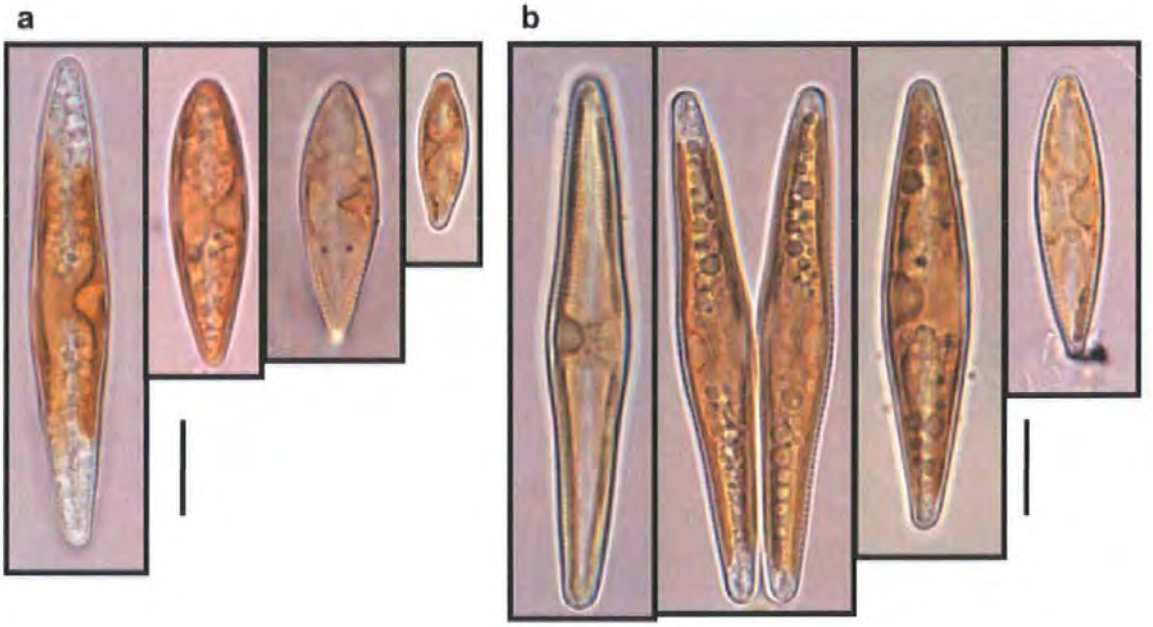


Figure 2.15: a-b

Examples of observational character: **(a)** triangular pyrenoids (CODE: PYR1), **(b)** and rounded pyrenoids (CODE: PYR2). Scale bars for all = 10µm.

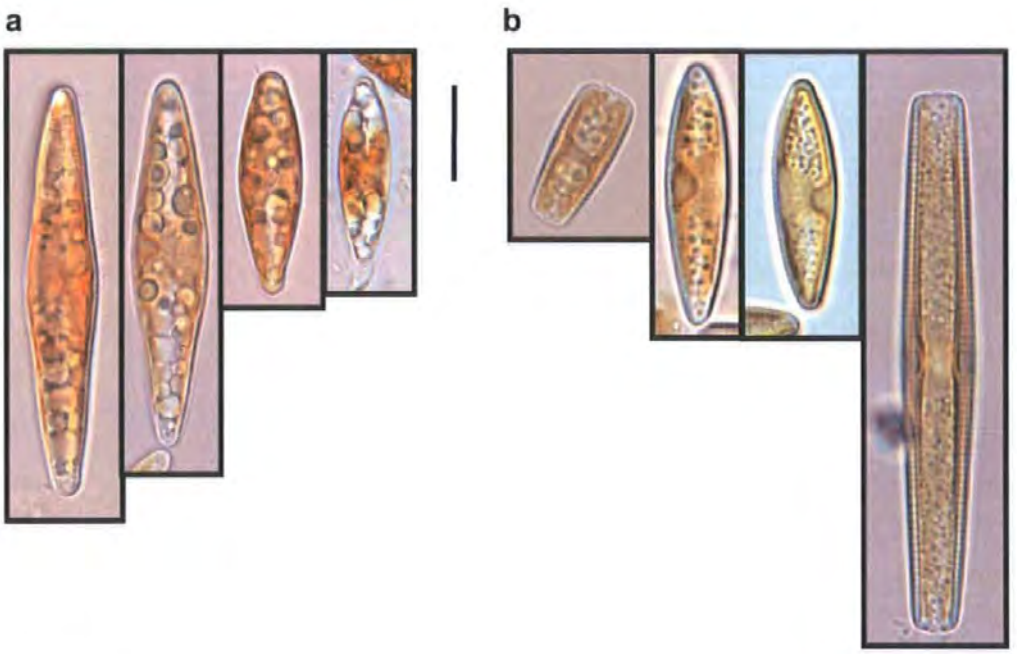


Figure 2.16: a-b

Examples and description of observational character: **(a)** Lipids (CODE: OIL1) and presented as small or large globular droplets within the live cell, **(b)** Granules (CODE: GRAN1), small, dark round bodies, usually present in quantity within a cell. Scale bar = 10μm.

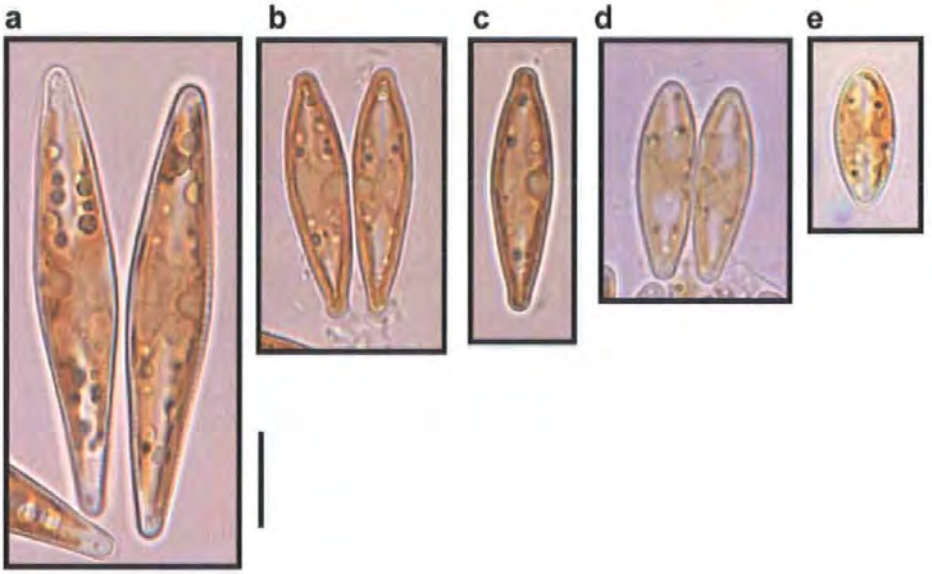


Figure 2.17: a-e

Examples of observational character: dark bodies (CODE: OTH1), larger dark spherical bodies, usually only 2-4 per cell. Scale bar = 10μm.

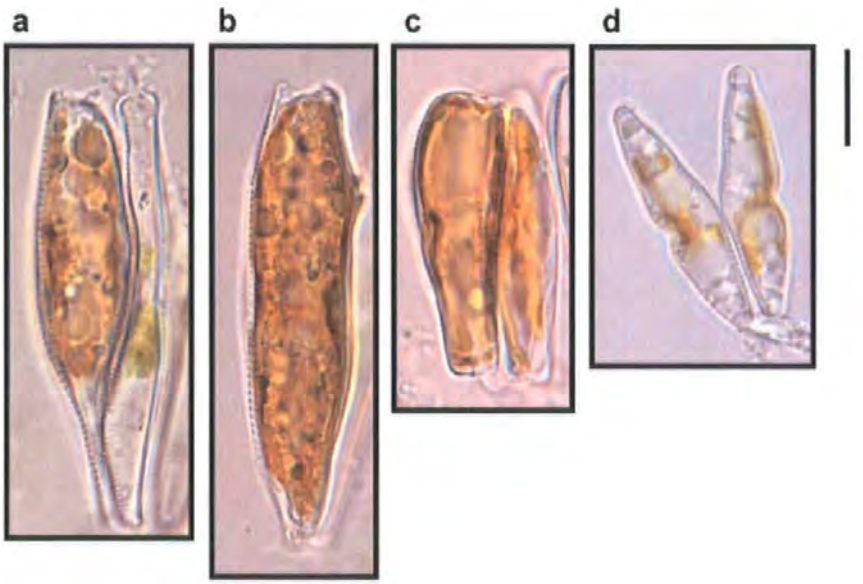


Figure 2.18: a-d

Examples of observational character: Teratologies (CODE: OTH2). Scale bar = 10μm

2.8 STATISTICAL ANALYSES

Raw data and descriptive statistics (mean, range, standard deviation and coefficient of variation) for each analysis are provided on the accompanying CD.

2.8.1 Analysis of Variance

The statistical analyses performed on the different sets of data in this study were carried out using a combination of Minitab v14.13 and XLSTAT® v. 2006.3 (Addinsoft©, 2006).

To test the null hypothesis that there was no significant difference in Mean Exponential Growth Rate (MEGR) between each treatment, per regime or per clone, either a two-way (= two factor, for the light & temperature regime) or one-way (= one factor, for all other regimes) ANOVA was carried out. Examination of observational and measurement data for morphological analysis, determined whether ANOVA, Kruskal-Wallis and pairwise comparison tests were appropriate for the analysis of stock cultures. Additional Kruskal-Wallis tests were performed to test for significant differences between morphological characters occurring on both the primary and secondary side of the diatom valve, i.e. central striae, or occurring on both sides of the valve face transapically, i.e. upper and lower raphe and, head and foot pole shape. The significance level in all cases was set to 95%.

Diagnostic checks were performed to verify that each data set held to ANOVA assumptions (the Anderson-Darling test for normality and Bartlett's or Levene's test for

equal variances on normal and non-normal data respectively) (Fry, 1993). Where non-normality and/or heterogeneous data were observed, it is usual to transform raw data to modify the scale upon which the data are expressed so that valid statistical assumptions are expressed. Where cell counts had already been transformed via Equation 1 into growth rates, further transformation was not appropriate. Any indication of non-normality and heterogeneous variance was therefore subject to an ANOVA equivalent, non-parametric test, either Kruskal-Wallis (KW) or Moods Median. **Figure 2.19** illustrates the steps taken in deciding which test to proceed with.

A full statistical analysis of each clone within each experimental regime was not possible for several reasons:

- not all clones grew at all assay levels,
- growth is recorded, but insufficient harvestable material survived the digestion process,
- poor quality slides/material: despite repeated washing an unidentified coating obscured diatom morphology on some slides,
- loss of data (see Preface)
- assays for each regime were conducted at different times, thus diatom size reduction occurred between each regime and would not allow sensible comparison of quantitative characters.

2.8.2 Pairwise Comparisons

Where the ANOVA indicated a significant difference between MEGRs, the null hypothesis was rejected and a comparison of between treatment MEGR performed to determine where the differences lay, using the Bonferroni method. Bonferroni was chosen over both the Tukey or Sidak methods due to small sample size and the presence of extreme

values that may otherwise produce false positives in terms of significance (Berk & Carey, 2000). It is a conservative method that is robust to violations of ANOVA assumptions. For all other regimes, the Dunnett method was used to test the difference between each treatment MEGR and control MEGR. The Bonferroni method was additionally used to test for significant differences with increasing temperature/light and pH, i.e. between neighbouring treatment groups, e.g. between pH 5.0 and 5.5 or copper concentration x2 and x4, but not between, e.g. pH 5.0 and 6.0. The significance level in all cases was set to 95%. For those clones in which the null hypothesis was accepted, no pairwise comparisons were made.

All data sets comprised of a number of occasional negative (when enough cells died to reduce overall numbers) and nil (when no growth took place) growth rates. Such data are often revealed as outliers or extreme values, and may be expressed as skewed histograms. These data were valid with a potential to yield important information, it was therefore considered inappropriate to eliminate them from the analysis as their removal would have artificially inflated the mean and reduced the variance but increased the probability of finding a significant difference where none existed.

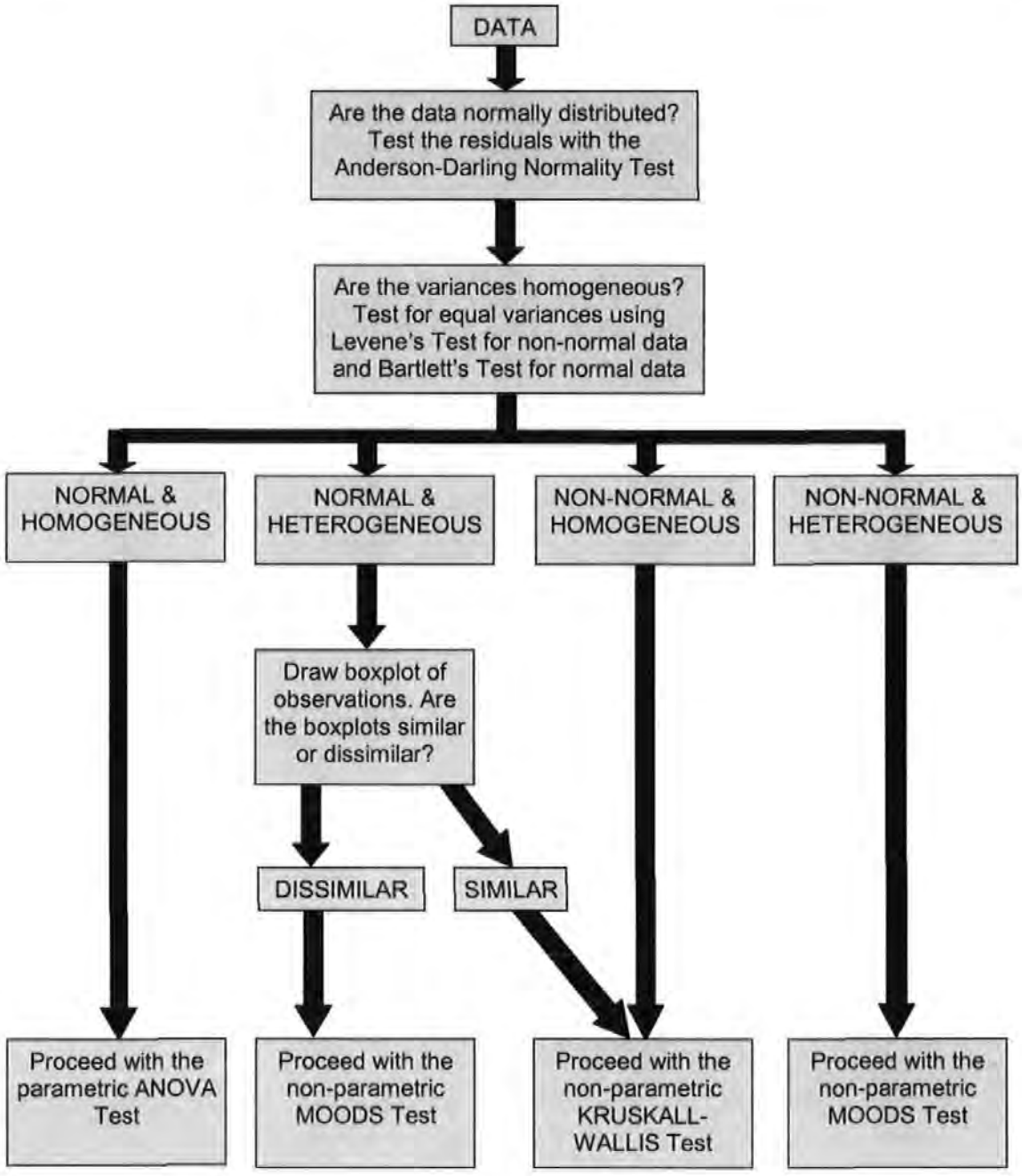


Figure 2.19

Flowchart of procedural steps in the analysis of mean exponential growth rates.

2.9 CLASSIFICATION TECHNIQUES

Data were binary-coded and the simple matching resemblance function/similarity coefficient used. This takes account of both positive 1, 1 and negative 0, 0 matches (Fry, 1993), and in which absence, presence, and matches (+/+ and -/-) or mismatches (+/- and -/+) all have equal weights, to produce a symmetrical similarity matrix from which the cluster method could produce its classification. No character weighting was used, as it would imply that one character was preferable to another.

The study used the Unweighted Pair-Group cluster Method, with Arithmetic averages (UPGMA cluster analysis), as it is a good compromise between the extremes exhibited by single and complete-linkage, and because it is reported to work best on most data sets (Fry, 1993; Sneath & Sokal, 1973). Cluster results are presented as UPGMA dendrograms. Where data was missing from a dataset, it was omitted from the final raw data matrix.

All cluster analyses were performed using XLSTAT® v. 2006.3 (Addinsoft©, 2006) to produce a similarity matrix on which a hierarchical classification could be produced and presented as a truncated dendrogram. XLSTAT® allows for automatic truncation of the full dendrogram, determined according to the structure of the levels in an accompanying histogram of node levels.

3 ECOPHYSIOLOGY

3.1 INTRODUCTION

This study uses clones collected from ten sites from which single *G. parvulum* cells were isolated and subsequently grown as uniclonal cultures. The results of experiments on *G. parvulum* clones are investigated and discussed. The chapter aims to provide an ecophysiological framework from which inferences about *G. parvulum's* ecological and taxonomic status can be discussed and applied to diatom indices of water quality. It will identify those ecophysiological factors that influence *G. parvulum* growth rate.

3.2 SAMPLE SITE CHARACTERISTICS

Of the many sites visited, ten provided specimens of *G. parvulum* for subsequent culture and experimentation. Their geographical location and site details are provided in **Table 3.1.** and full water chemistry for all ten sites is shown in **Table 3.2.** Nearly all water quality variables measured were within regulatory limits (= World Health Organisation (WHO), European Union Drinking Water Directive 80/778/EEC and The Water Supply (Water Quality) Regulations 2000 UK) with the exception of chlorine (all samples except for Llyn Idwal), iron (samples for Kings Mere, Ham Gate Pond and Llyn Idwal), ammonium (samples for Barnwood Pond and Kings Mere) and nickel (sample for Llyn Idwal).

Table 3.1

Site name, reference, collection date, sample date, sample description and substratum type.

Site Name	Site Ref.	Landranger Grid Ref.	Collection Date	Sample Sediment	Sample Type
Richmond Park Pond	35/01	TQ200737	12/09/2001	Macrophyte	Epiphytic
Scion Pond, Putney	39/01	TQ228734	25/09/2001	Sandy Sediment	Episammic
Kings Mere Pond, Putney	40/01	TQ232732	25/09/2001	Fine Gravel	Episammic
Ham Gate Pond, Richmond	41/01	TQ188717	25/09/2001	Sandy Sediment	Episammic
Abbey Lakes River, Nr Wigan	56/01	SD535041	28/09/2001	Fine Gravel	Episammic
Lyn Idwal Lake, N. Wales	59/01	SH646597	28/09/2001	Rock	Epilithic
River Kennet, Wiltshire	01/02	SU152688	01/02/2002	Mud	Epipelic
Pen-y-Bryn, N. Wales	14/02	SJ164422	16/04/2002	Rock	Epilithic
Llyn Ogwen, N. Wales	16/02	SH661603	16/04/2002	Sandy Sediment	Episammic
Parys Mountain Pond, Anglesey	17/02	SH437900	17/04/2002	Fine Sediment	Episammic

Table 3.2

Concentrations of anions and cations in filtered water samples. **NB:** All values are in mg/l (ppm). **m** = missing. **nf** = not found as below detection limits. <0.01 indicates below the detection limit.

	SITE NAME and REFERENCE									
	Barnwood Pond	Scion Pond	Kings Mere Pond	Ham Gate Pond	Abbey Lakes River	Lyn Idwal Lake	River Kennet	Pen-y-Bryn	Llyn Ogwen	Parys Mountain Pond
Ion Conc.	35/01	39/01	40/01	41/01	56/01	59/01	01/02	14/02	16/02	17/02
Total P	0.3	nf	nf	nf	nf	nf	0.3	<0.01	<0.01	<0.01
PO_4^{3-}	2.1	nf	nf	nf	nf	nf	<0.01	<0.01	<0.01	<0.01
Total S	18.0	10.0	16.0	19.0	15.0	4.3	m	m	m	m
SO_4^{2-}	47.5	24.7	39.0	52.0	39.5	7.4	19.6	6.0	2.0	9.7
NH_4^+	0.9	nf	3.3	nf	nf	nf	0.2	<0.1	<0.1	<0.1
NO_3^-	7.9	0.4	0.6	0.8	7.9	0.5	26.6	5.3	0.4	0.2
Li	0.0	0.0	0.0	0.0	0.0	nf	<0.01	<0.01	<0.01	<0.01
Na	26.0	28.5	16.5	24.5	14.5	2.6	11.8	5.0	3.2	12.8
K	7.0	6.8	5.9	5.1	4.8	0.1	4.7	4.1	0.2	1.7
Mg	6.2	4.4	3.9	10.8	8.0	0.5	1.6	3.4	0.6	2.5
Ca	57.0	25.5	13.5	36.0	39.0	2.0	100.0	9.3	1.5	6.7
Zn	0.2	0.1	0.3	nf	0.0	0.2	0.1	0.0	<0.01	0.0
Ni	nf	nf	nf	nf	nf	0.2	<0.01	<0.01	<0.01	<0.01
Mn	0.0	0.2	0.4	0.2	0.1	0.1	<0.00	<0.01	<0.01	0.0
Fe	nf	0.2	1.5	0.3	0.1	1.1	<0.01	<0.01	<0.01	<0.01
Cd	nf	nf	nf	nf	nf	nf	<0.01	<0.01	<0.01	<0.01
Co	nf	nf	nf	nf	nf	nf	<0.01	<0.01	<0.01	<0.01
Si	nf	nf	nf	nf	nf	nf	3.3	0.7	0.1	<0.01
Pb	nf	nf	nf	nf	nf	nf	<0.01	<0.01	<0.01	<0.01
Cr	nf	nf	nf	nf	nf	0.2	<0.01	<0.01	<0.01	<0.01
Cu	nf	nf	nf	nf	nf	nf	<0.01	<0.01	<0.01	<0.01
Al	nf	nf	nf	nf	nf	nf	0.1	0.0	0.0	0.0
Sr	0.2	0.1	0.1	0.2	0.1	0.0	0.2	0.0	0.0	0.0
Ba	0.1	0.1	0.2	0.0	0.0	0.0	0.1	0.0	0.0	0.1
F^-	0.1	0.2	0.3	0.1	0.1	nf	0.2	0.0	<0.01	0.0
Cl^-	30.0	38.0	18.0	28.0	19.0	2.6	13.8	24.6	6.1	20.6

Pen-y-Bryn, Lake Ogwen and Parys Mountain were the most nutrient poor, whilst Barnwood Pond was the most nutrient rich. Barnwood Pond, Kings Mere, Ham Gate Pond and Abbey Lakes were particularly high in sulphates (39 to 52mg/l), whilst Barnwood, Scion and Ham Gate Ponds were high in sodium (24.5 to 28.5 mg/l). The River Kennet sample was particularly high in nitrates (26.6mg/l) and showed a high concentration of calcium (100mg/l). All sites except Llyn Idwal and Lake Ogwen had high levels of chlorine (13.8 to 38mg/l). Conversely, aluminium, barium, cadmium, cobalt, chromium, copper, fluorine, lithium, manganese, lead, silicon, strontium and zinc concentrations were all very low.

3.3 ASSAY RESULTS

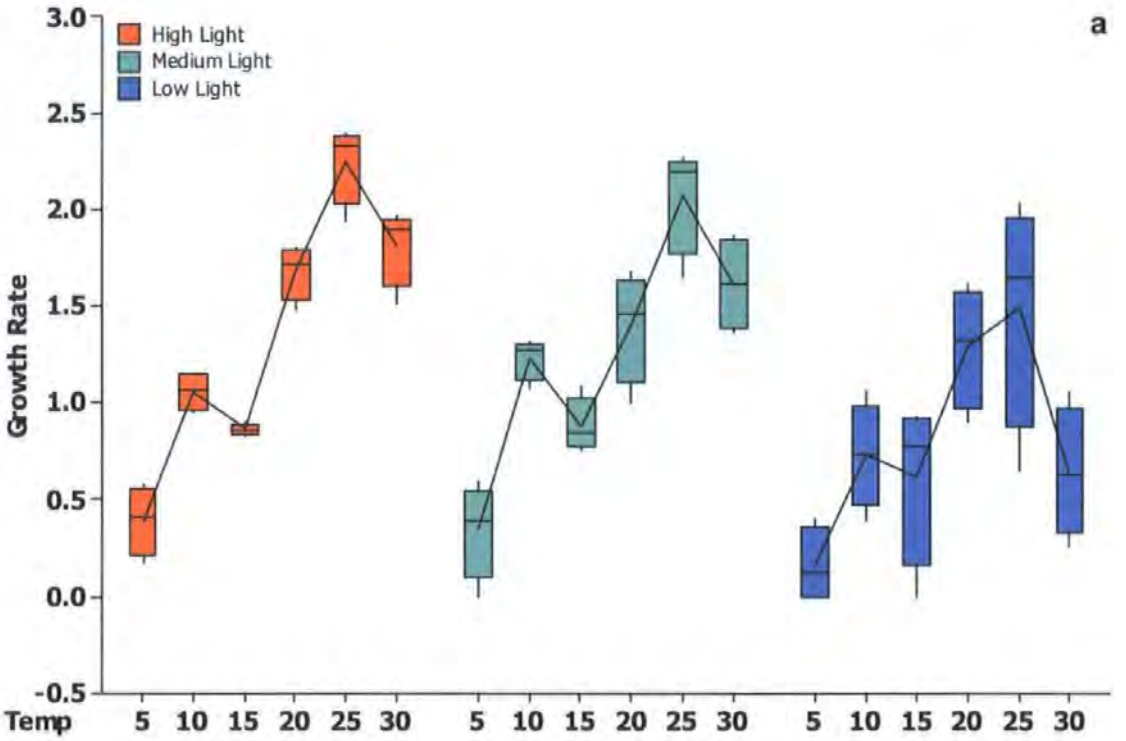
Results of tests for normality, equal variances and subsequent ANOVA, Kruskal-Wallis or Mood's tests for each experimental regime and multiple pairwise comparisons of treatment MEGRs, are available on the enclosed CD. **Figures 3.2 to 3.22** show the MEGRs for each clone for each treatment as boxplots.

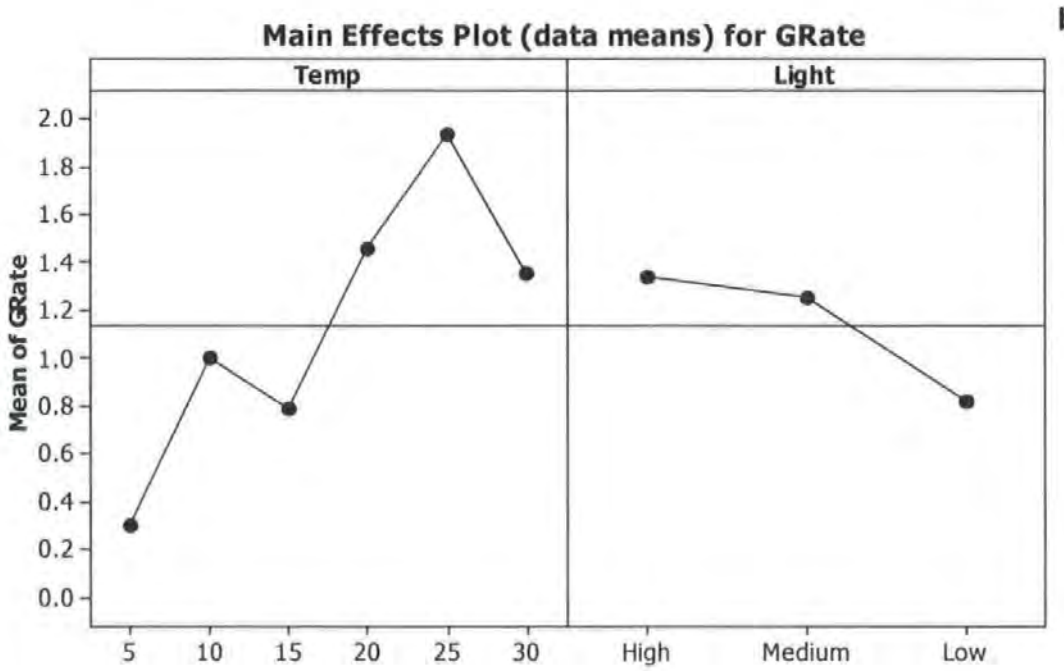
3.3.1 Light and Temperature Regime

Figures 3.1 to 3.13. In addition to the boxplots, a main effects plot for the data means, comparing the relative strengths of light and temperature on growth rate is also shown. Most clones were able to grow across the full light and temperature ranges assayed in this study with the exception of clones from Kings Mere and Ham Gate Ponds (G_1 and G_2 clones). The former failed to grow at 10°C and 30°C at all light levels, and the latter failed to grow at 5°C high and low light. For the former, this suggests a narrow limit to light exposure, particularly at lower and higher temperatures. For the latter the temperature

may have been too low for the Ham Gate Pond clones. As expected, most clones generally exhibited an increase in MEGR with increasing light levels from low to medium and medium to high with the exception of clones from Scion Pond, Kings Mere (G_1) and Pen-y-Bryn. Most clones also exhibited a general increase in MEGR with increasing temperature from 5°C to 25°C, but falling sharply from 25 to 30°C. However, there were some marked drops in MEGR against the general trend. Some of these decreases can be explained by failed inoculation, e.g. the Kings Mere (G_1 and G_2) clones failed to grow at 10°C and 30°C high, medium and low light and 5°C high light, probably for the same reasons as the Kings Mere and Ham Gate Pond clones above. Alternatively, cells could have been damaged at the inoculation stage and thus failed to replicate, particularly as they were able to grow at temperatures higher and lower than this.

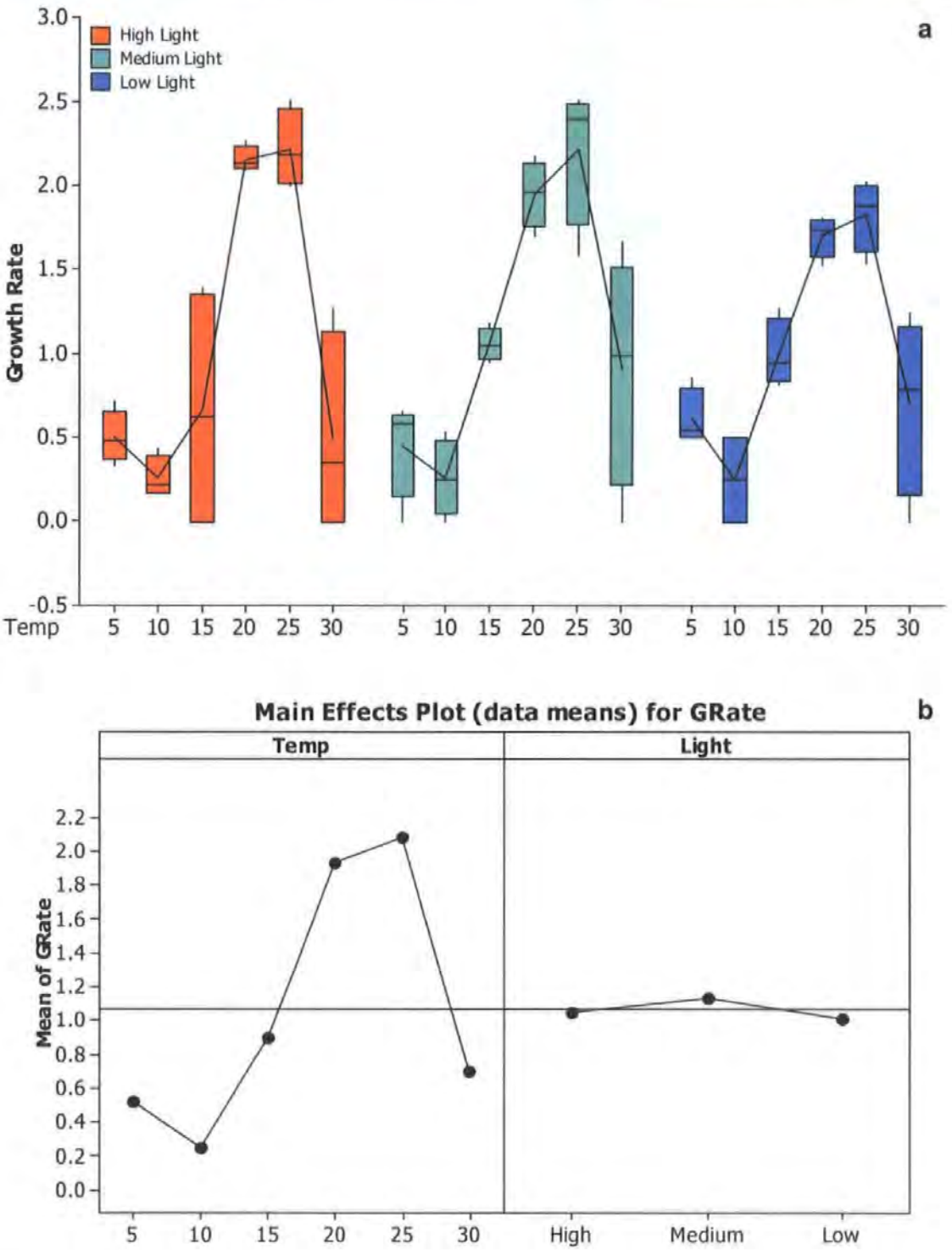
Multiple pairwise comparisons of treatment MEGRs, indicate growth rate was significantly affected by increasing light levels but only from low to medium light in 6 clones (Barn Wood Pond, Kings Mere (G_1 clone), Abbey Lakes River, Llyn Idwal (G_2 clone), Lake Ogwen and Parys Mountain). There were no significant results for any clone at medium to high light, and remaining clones showed no significant difference in MEGR either from low to medium or medium to high light. Pairwise comparisons also indicated that growth rate was significantly affected by temperature in seven clones (all clones except Kings Mere and Llyn Idwal: G_2 clones).





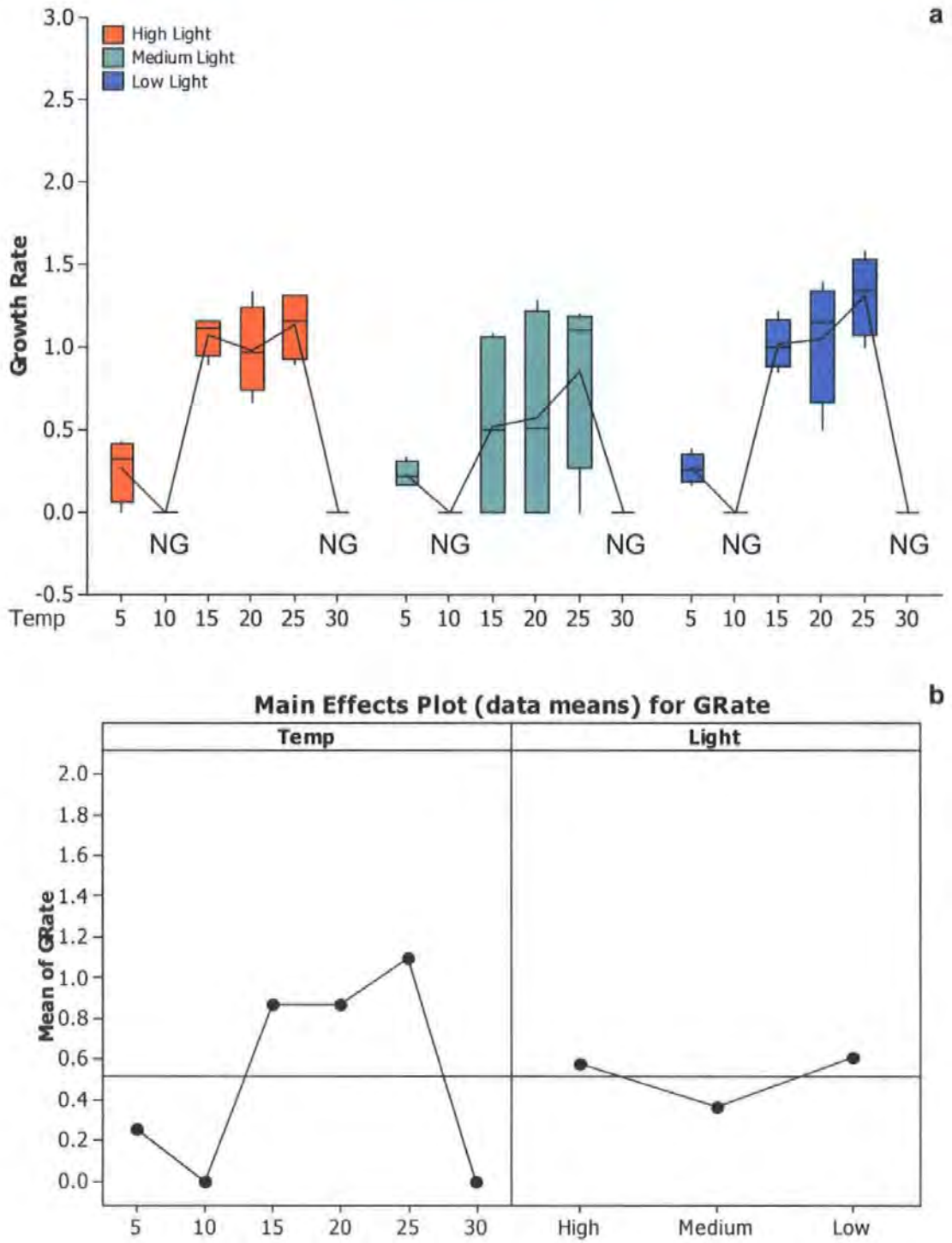
Figures 3.1: a-b

Barnwood Pond Clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means, comparing the relative strength of effect of light and temperature on growth rate.



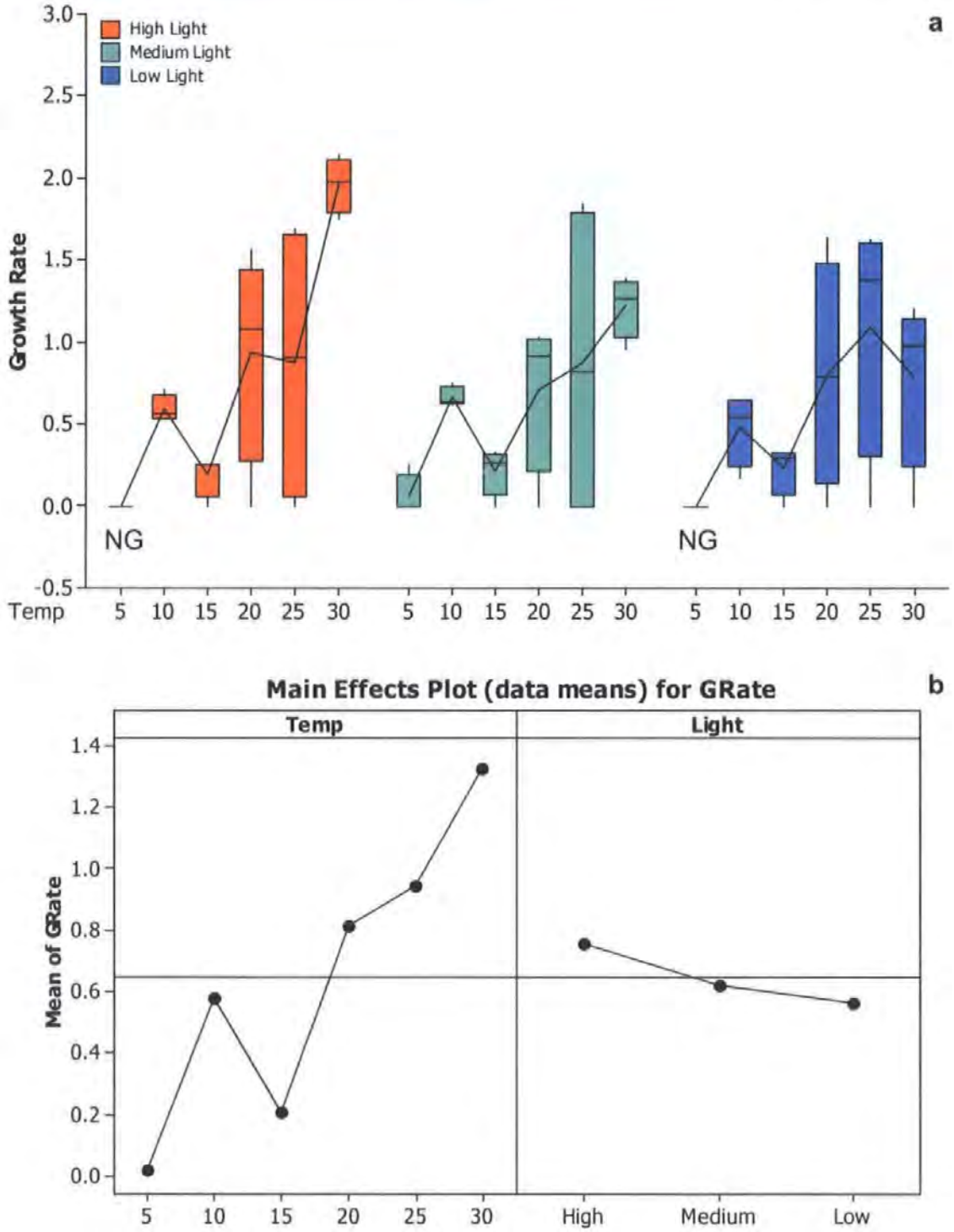
Figures 3.2: a-b

Scion Pond clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.



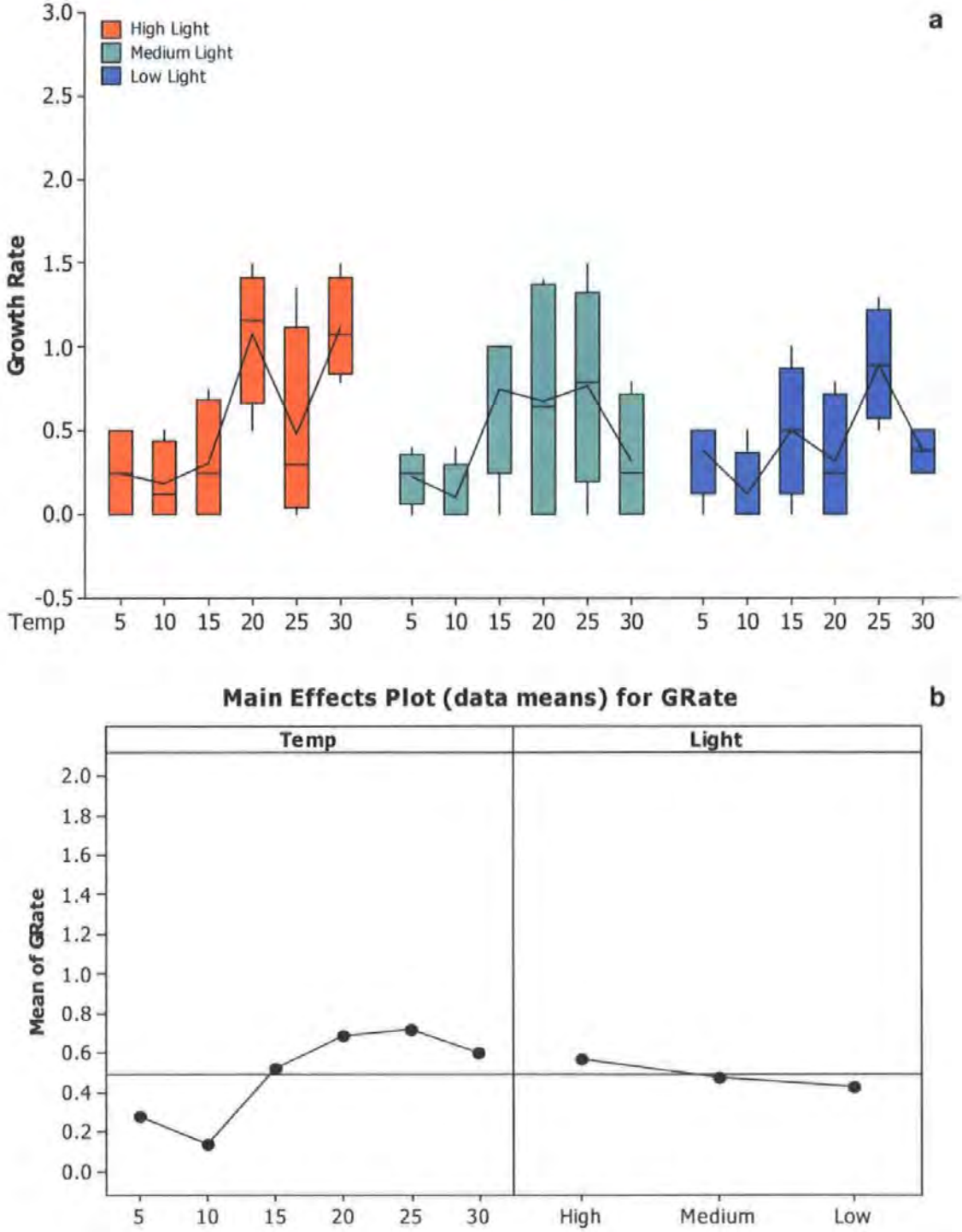
Figures 3.3: a-b

Kings Mere, G_1 clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate. **NG** = No Growth.



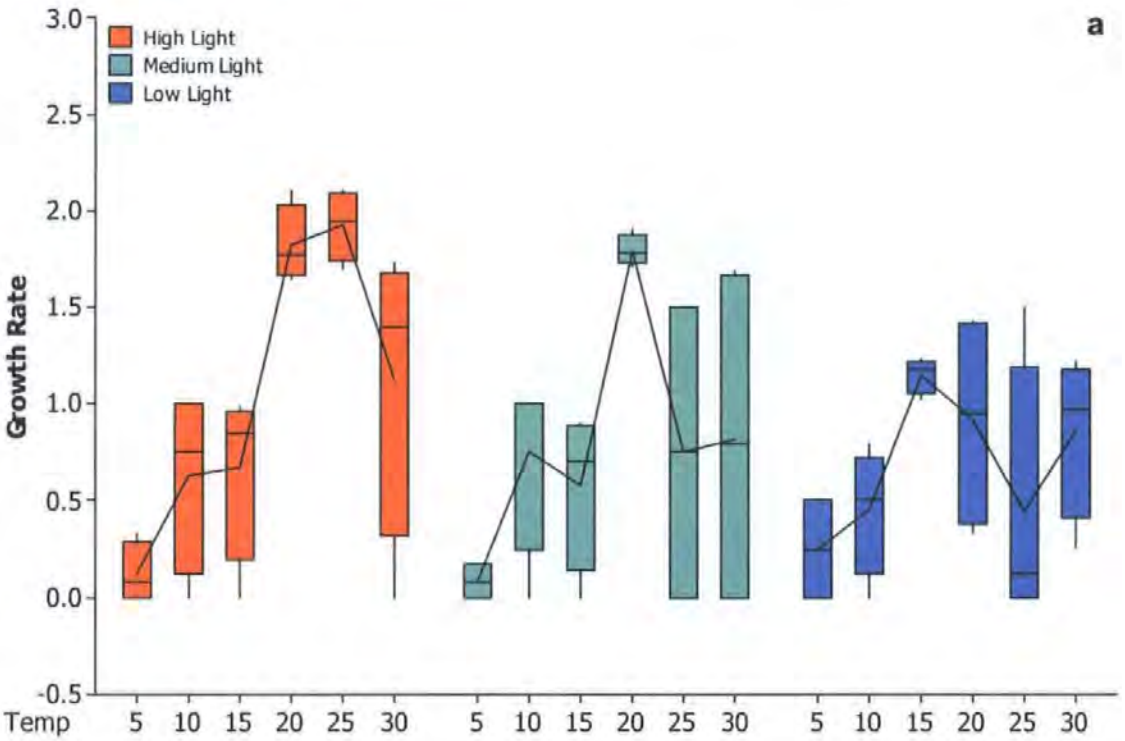
Figures 3.4: a-b

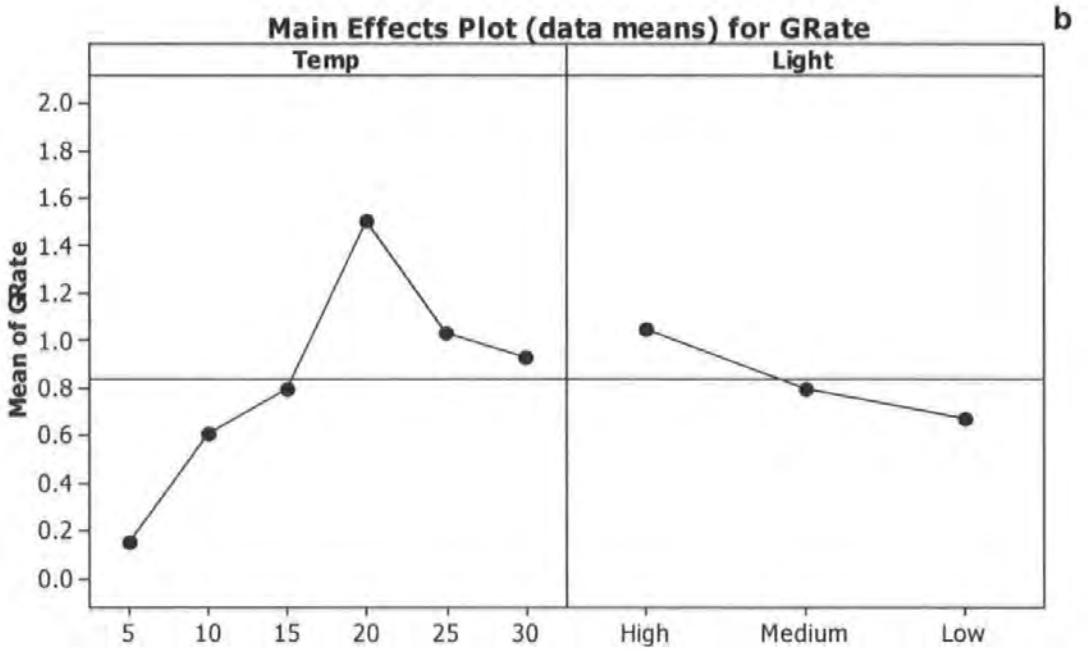
Kings Mere, G_2 clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate. **NG** = No Growth.



Figures 3.5: a-b

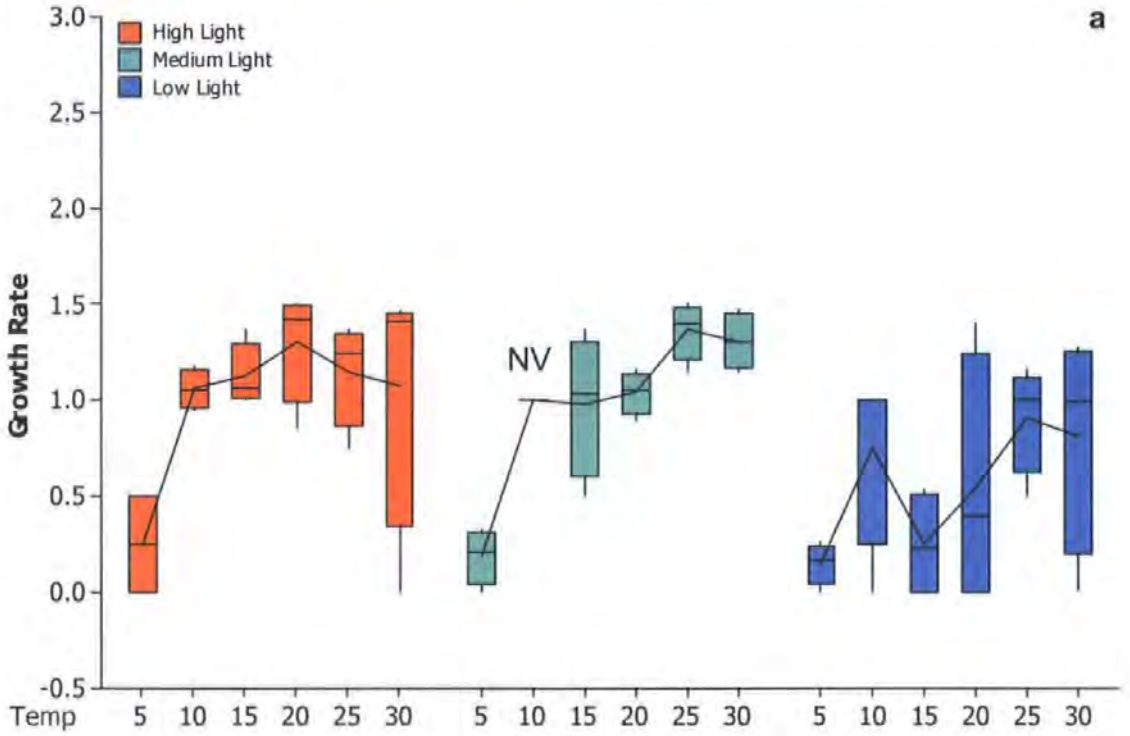
Ham Gate Pond, G_1 clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.

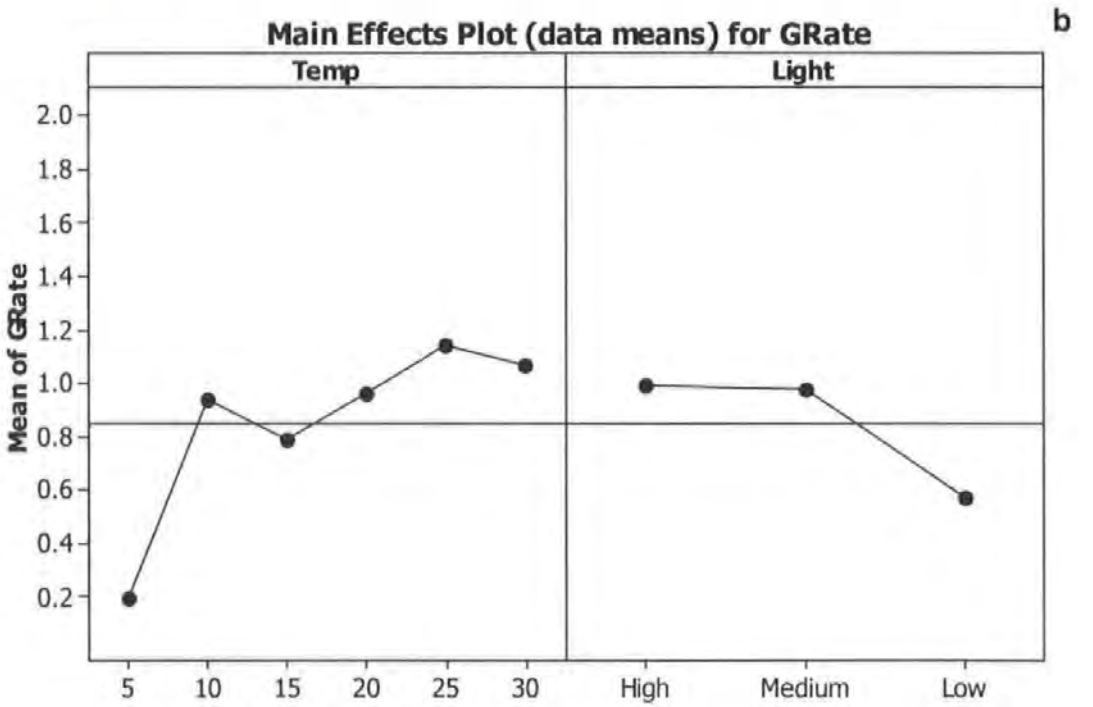




Figures 3.6: a-b

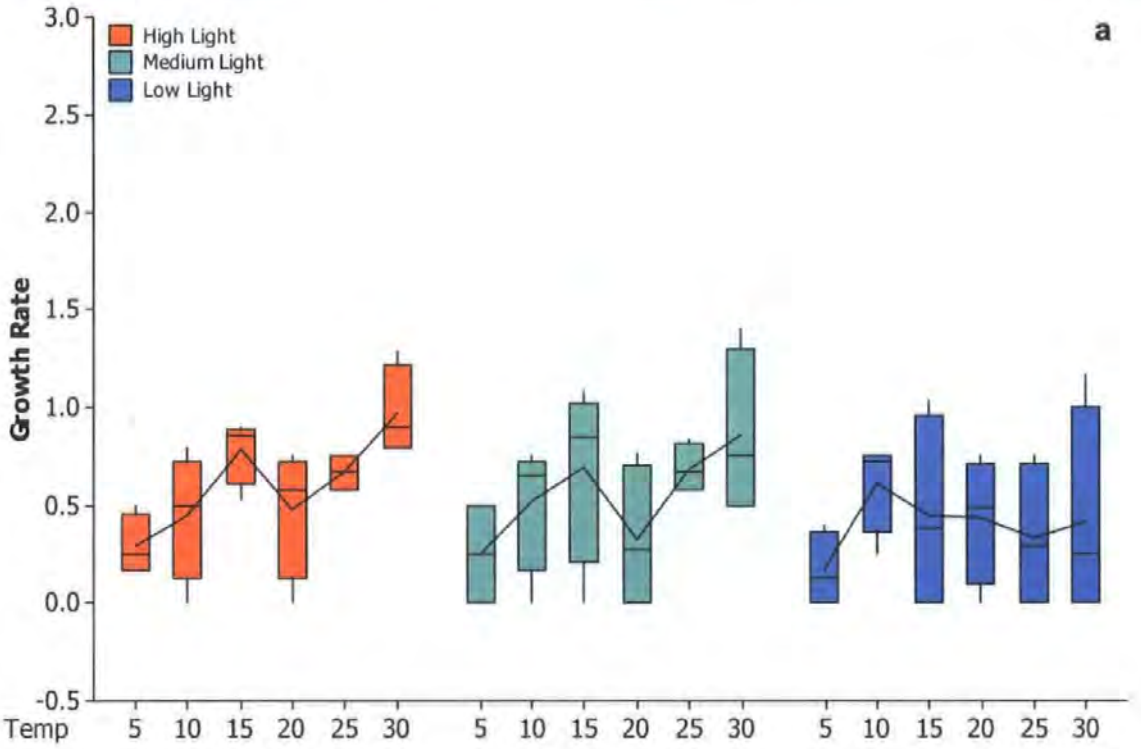
Ham Gate Pond, G_2 clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.

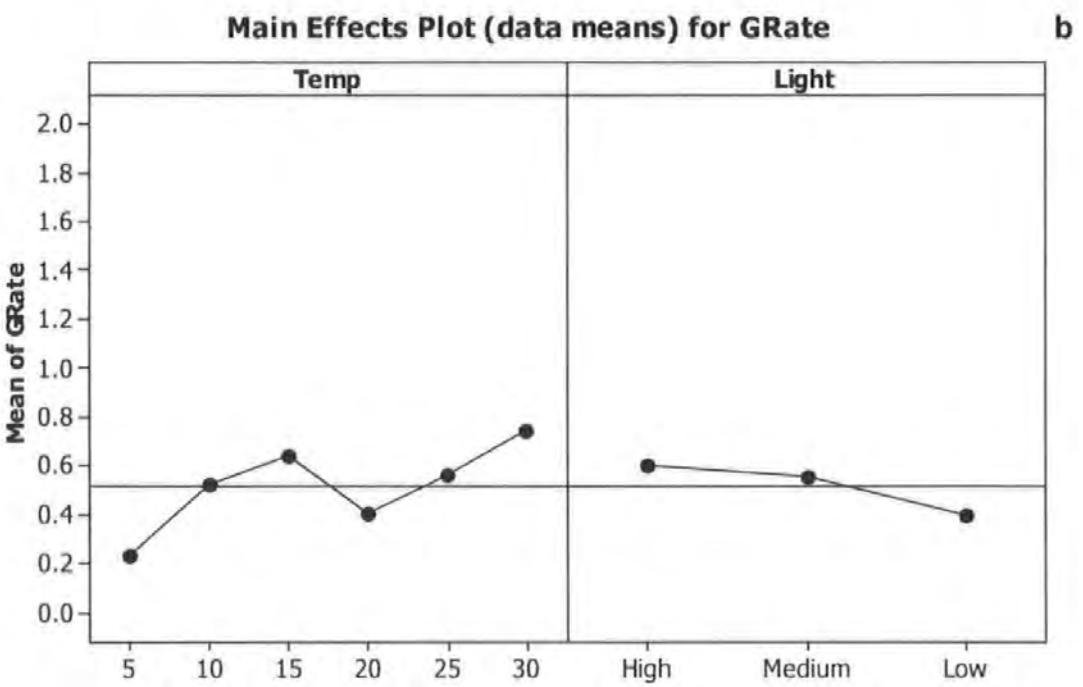




Figures 3.7: a-b

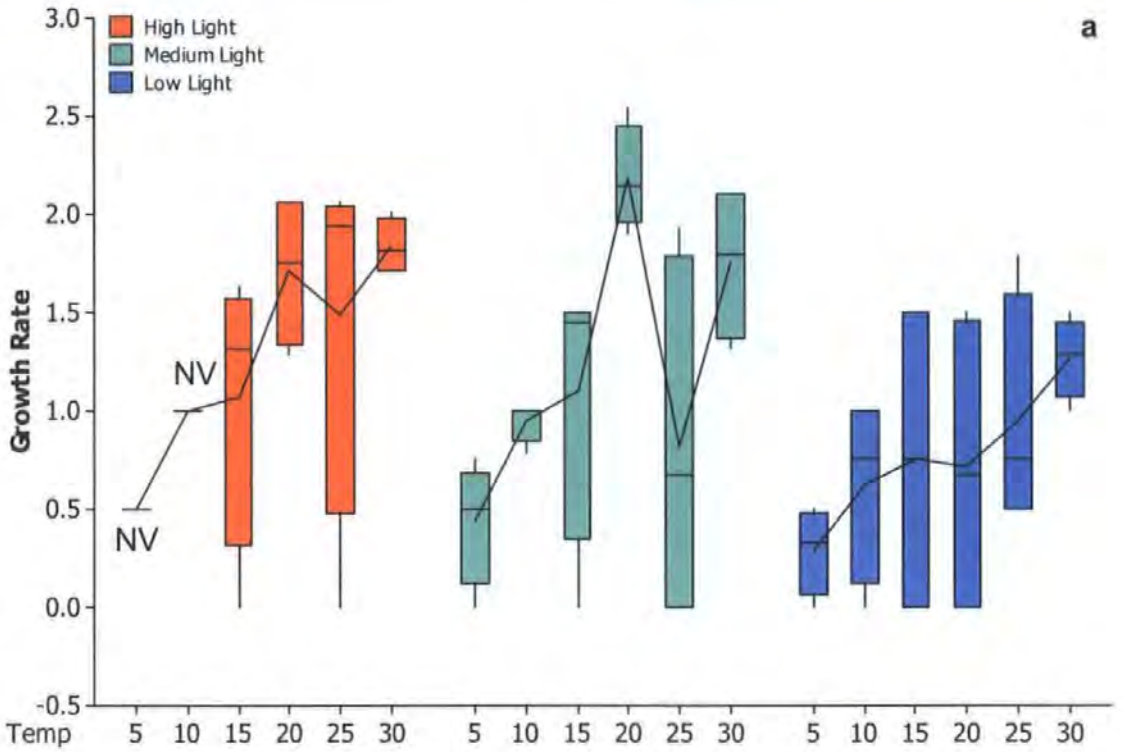
Abbey Lakes River clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate. **NV** = No variance.

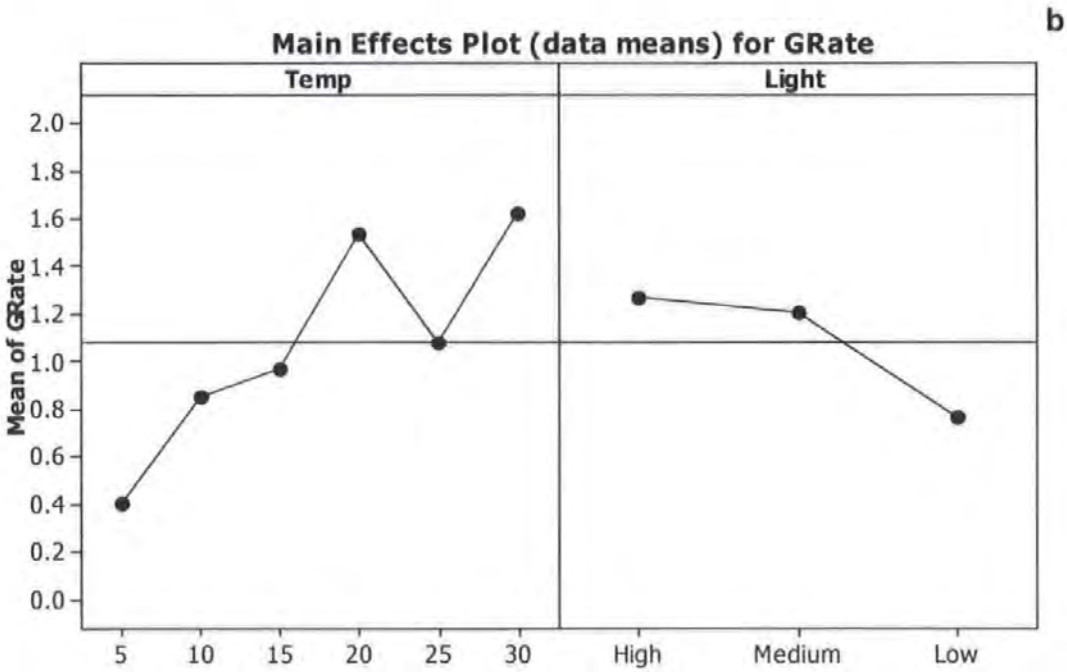




Figures 3.8: a-b

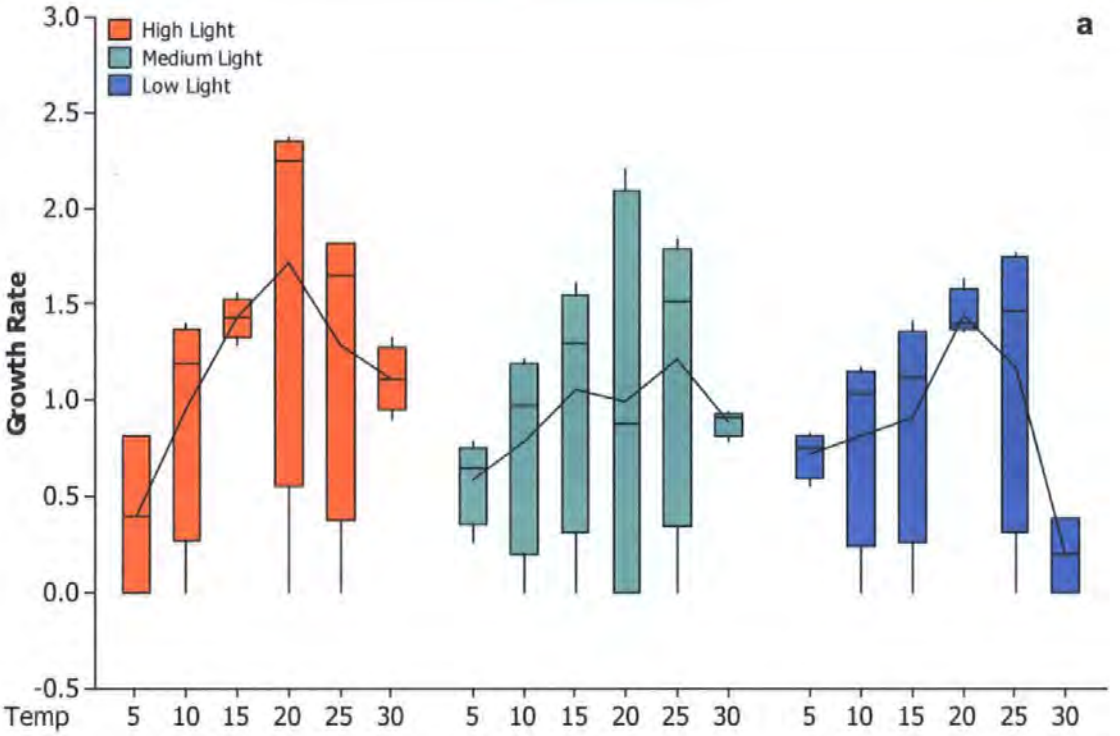
Llyn Idwal, G_1 clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.

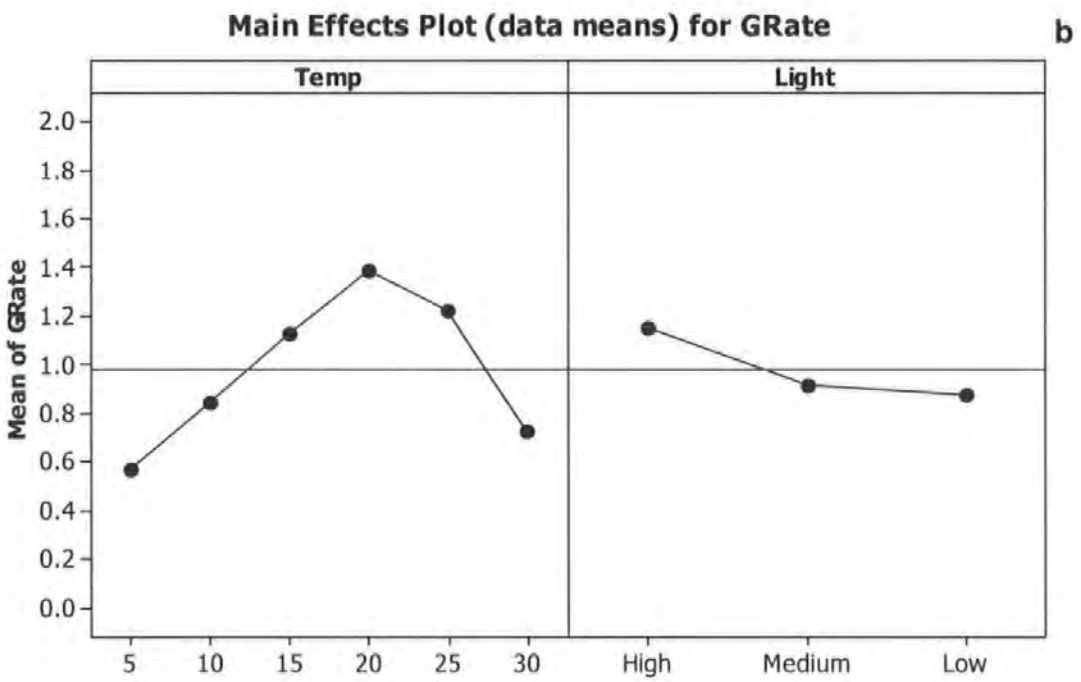




Figures 3.9: a-b

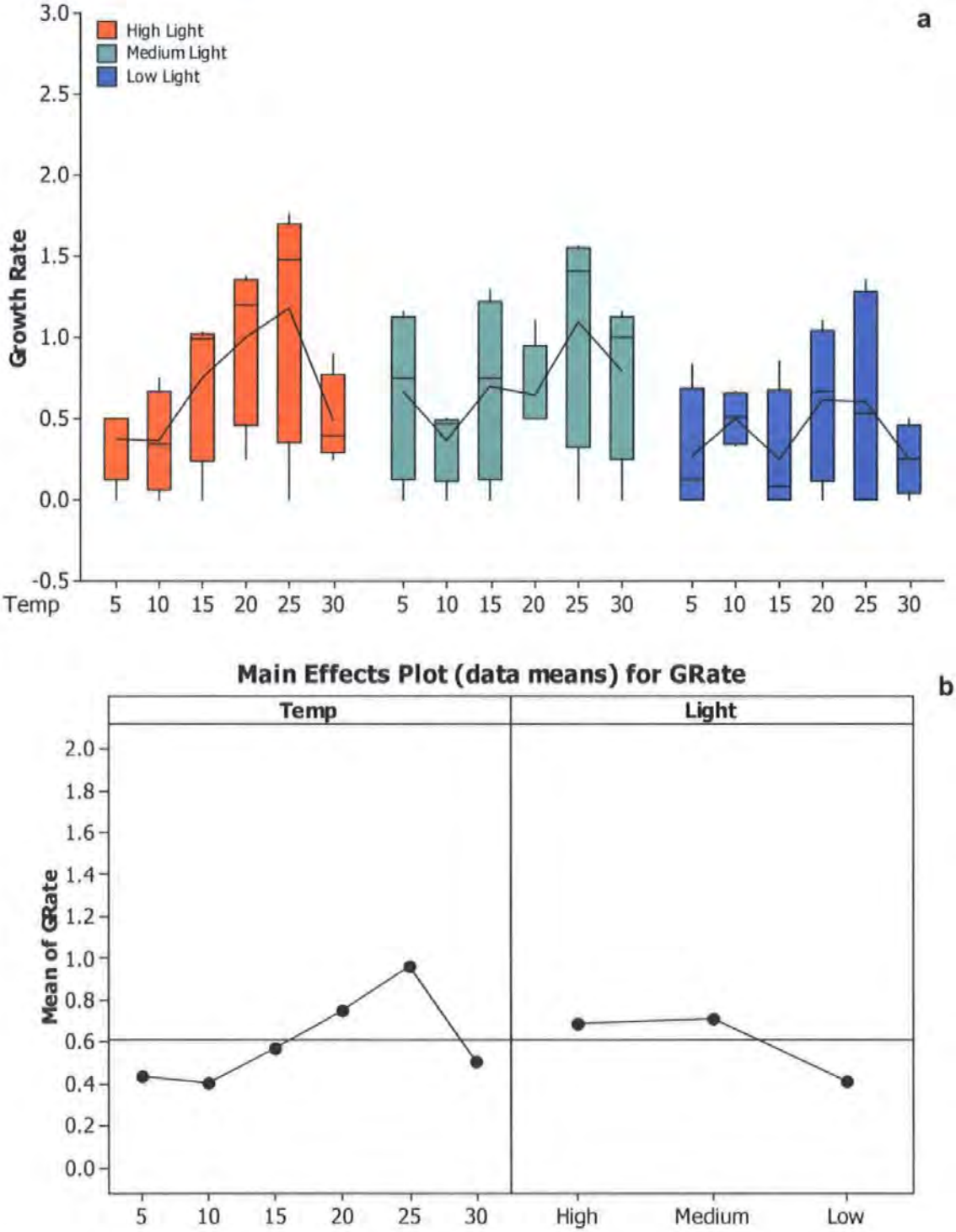
Llyn Idwal, G_2 clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate. **NV** = No variance.





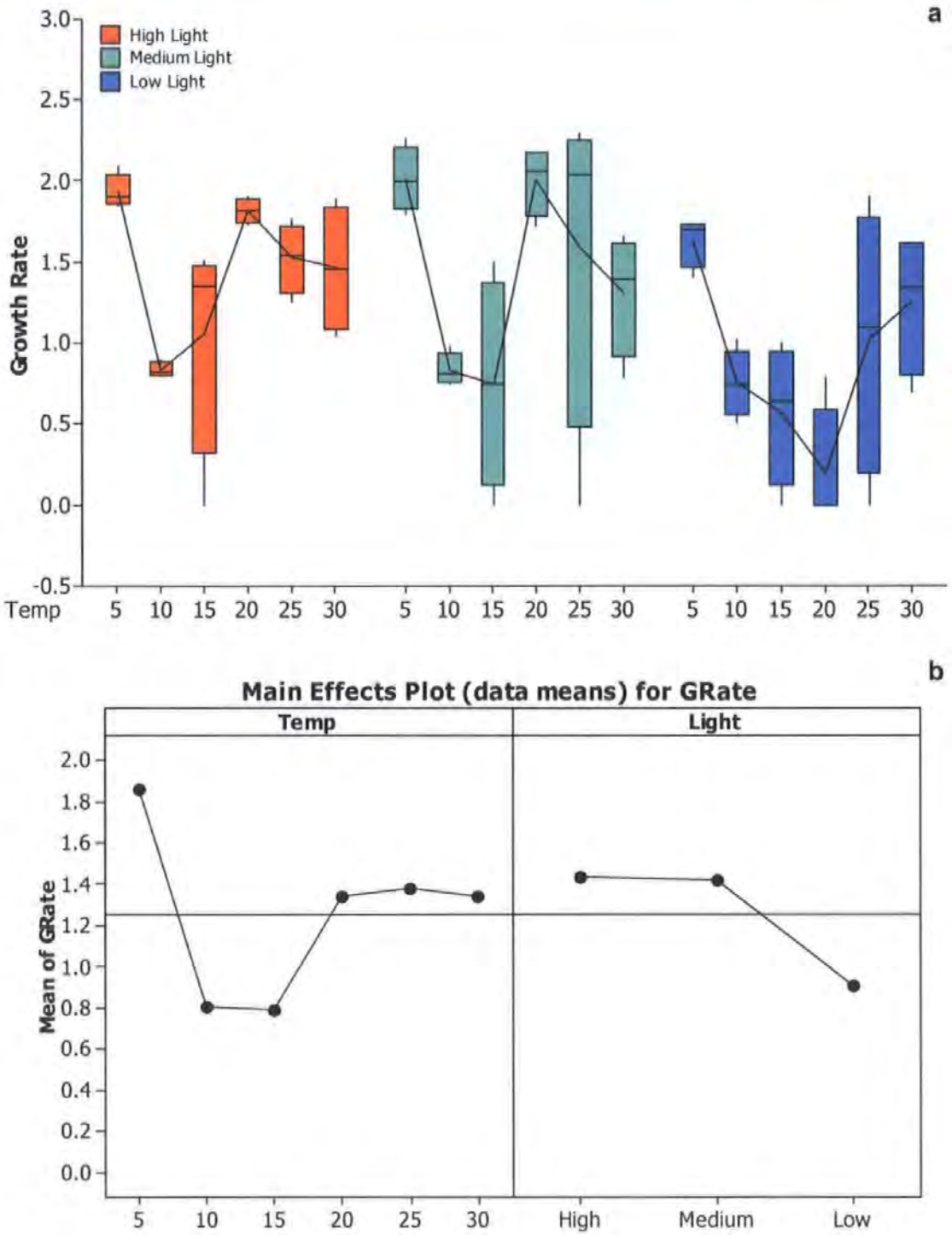
Figures 3.10: a-b

River Kennet clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.



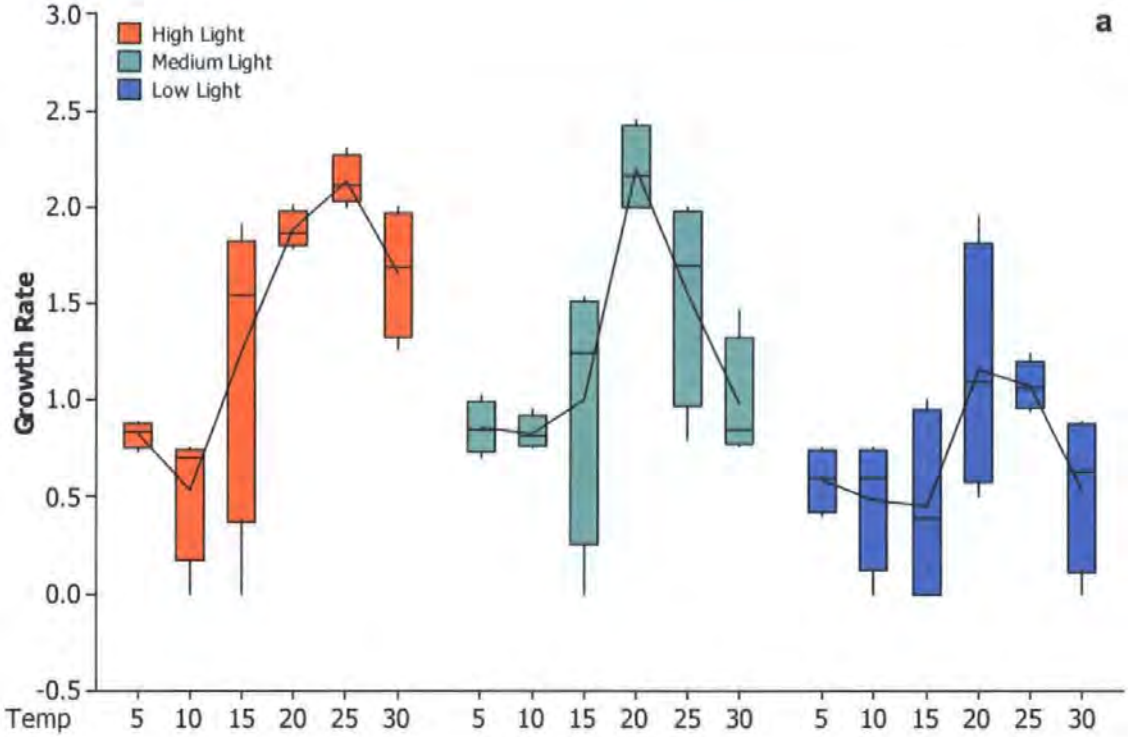
Figures 3.11: a-b

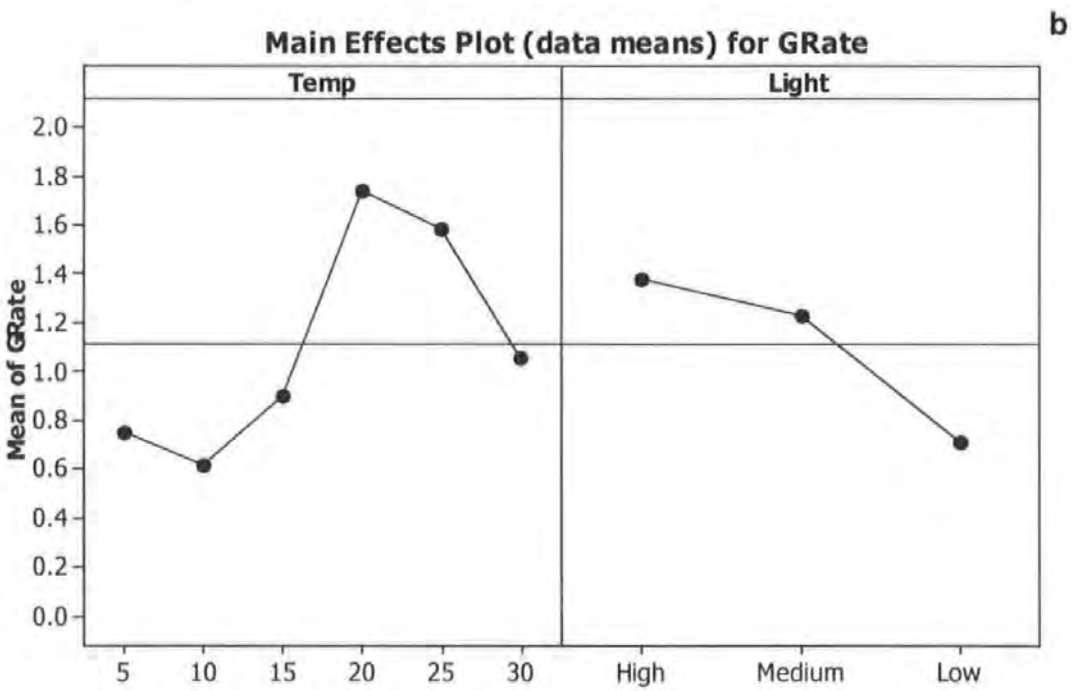
Pen-y-Bryn clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.



Figures 3.12: a-b

Lake Ogwen clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.





Figures 3.13: a-b

Parys Mountain, G_1 clone **(a)** Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit increase per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **(b)** Main effects plot for data means comparing the relative strength of effect of light and temperature on growth rate.

3.3.2 pH Regime

Figures 3.14 to 3.23. Most clones were able to grow between pH 4.0 to 10.5 and some clones were also able to grow at pH 3.5 and 11.0 (Scion Pond, Llyn Idwal: G_2, River Kennet and Pen-y-Bryn clones). Others did not grow at pH 3.5, but did grow at pH 11.0 (Kings Mere: G_2, Ham Gate Pond: G_2, Abbey Lakes River and Lake Ogwen clones). Clones from Scion Pond, Abbey Lakes River and River Kennet failed to grow at pH 5.0, 4.0 and 8.0 respectively, suggesting inoculation failure for the Scion Pond and River Kennet clones but a requirement for higher pH in the Abbey Lakes clone. Only the G_2 clone from Kings Mere showed a significant difference with increasing pH level and only between pH 7.0 and 7.5.

Overall, it was difficult to infer a consistent pattern of MEGR with increasing pH. Some clones (Scion Pond, Ham Gate Pond: G_2 clone and Llyn Idwal: G_2 clone) appeared to show no particular preference, the growth rates rising and falling randomly across the pH range assayed, though these clones had a clear peak MEGR at pH 7.0, 7.0 and 4.5 respectively. Generally however, clones show an increased MEGR with increasing pH from between pH 3.5 and 4.5 to between pH 5.5 and 9.0, declining thereafter. Therefore, all clones assayed appear to have a circumneutral optimum but with a wide tolerance range. The clone from Abbey Lakes River has a clear optimum of pH 8.5 and the clones from the River Kennet an optimum of pH 9.5.

Figure 3.14

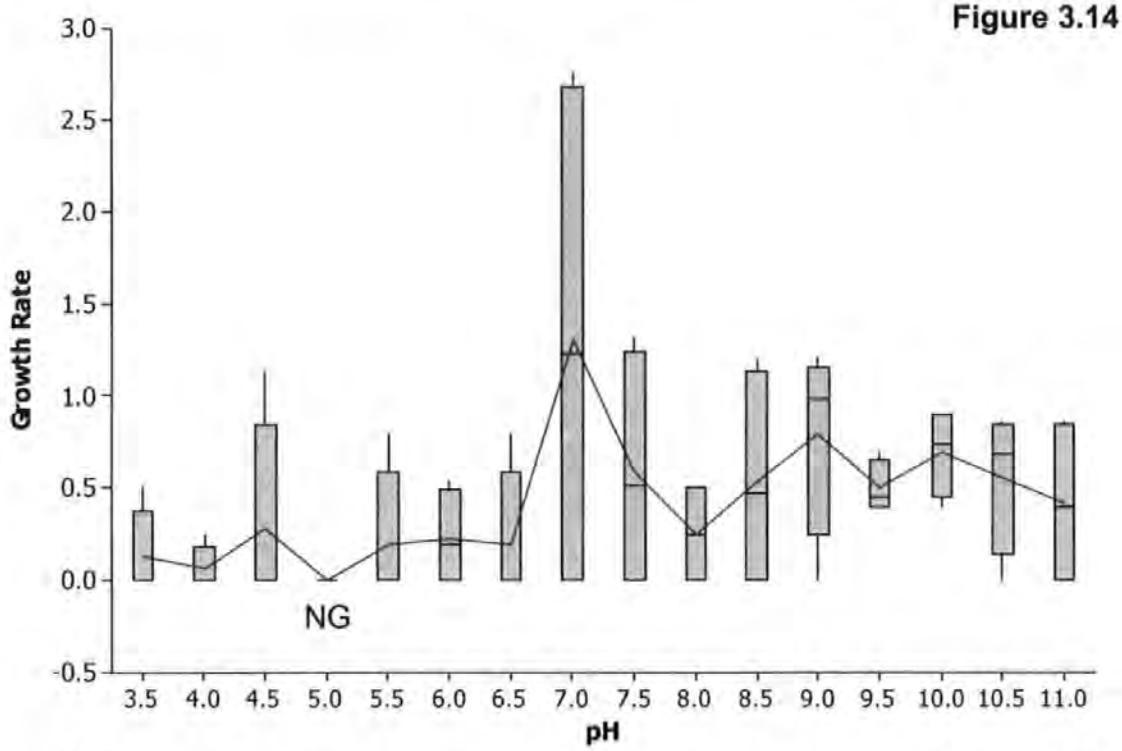
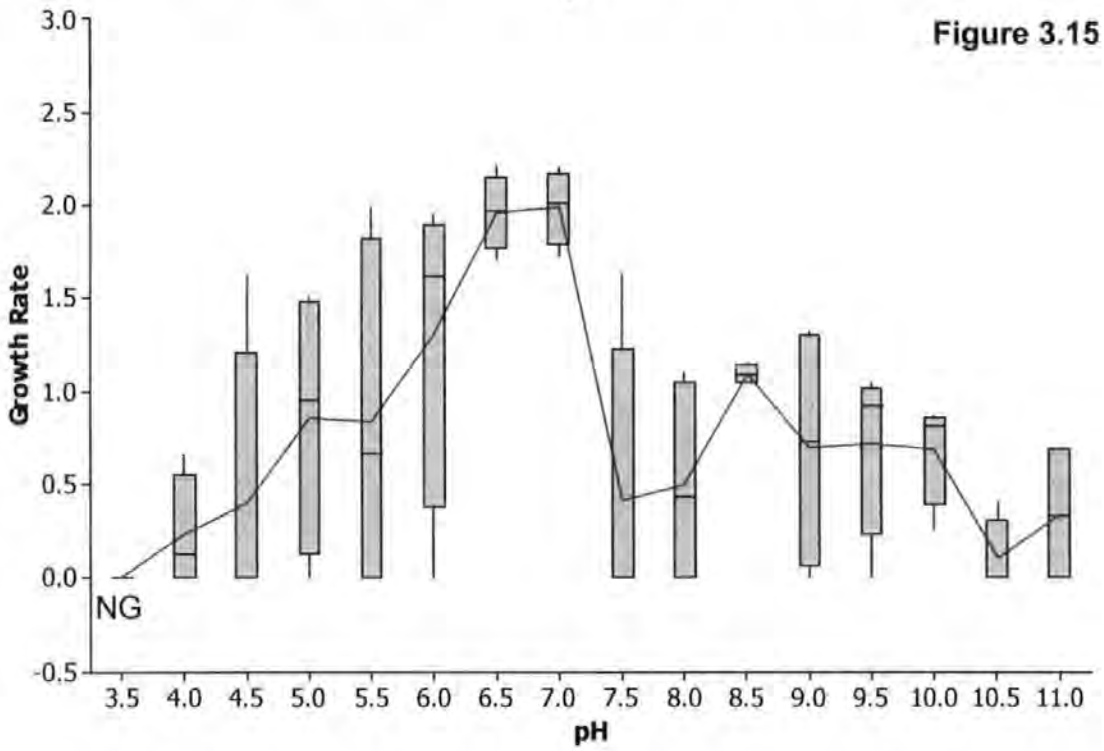


Figure 3.15



Figures 3.14 and 3.15

pH Regime, clones from Scion Pond and Kings Mere (G_2) respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.

Figure 3.16

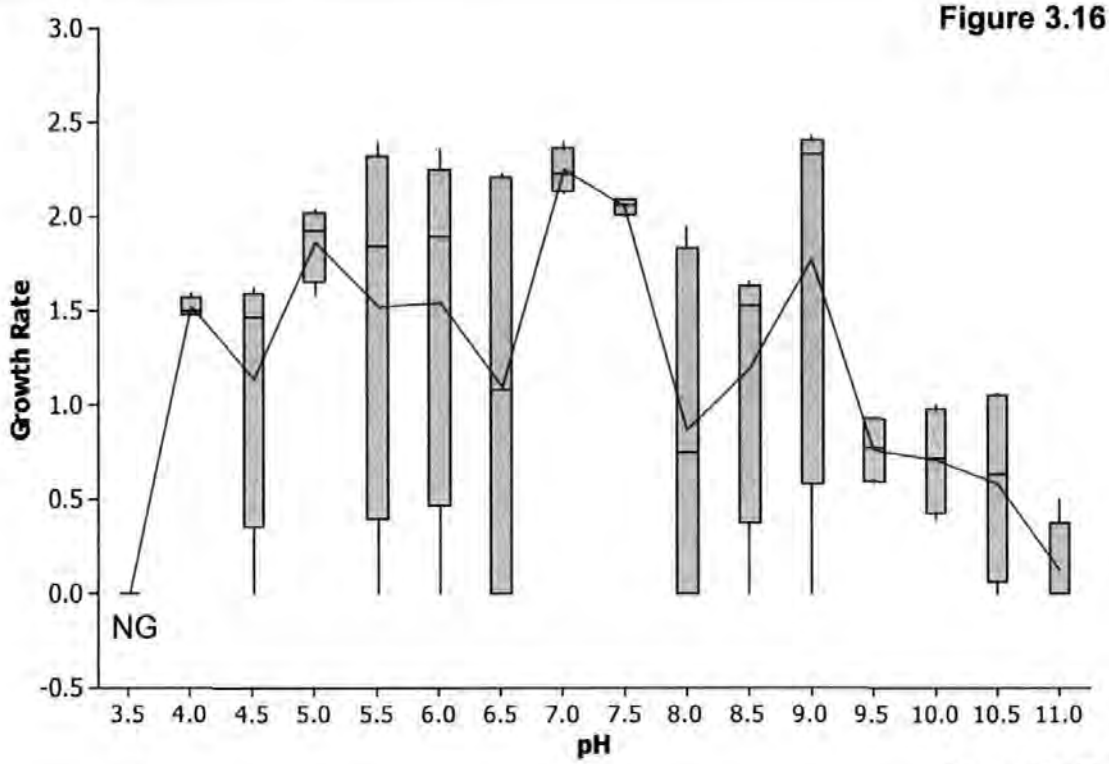
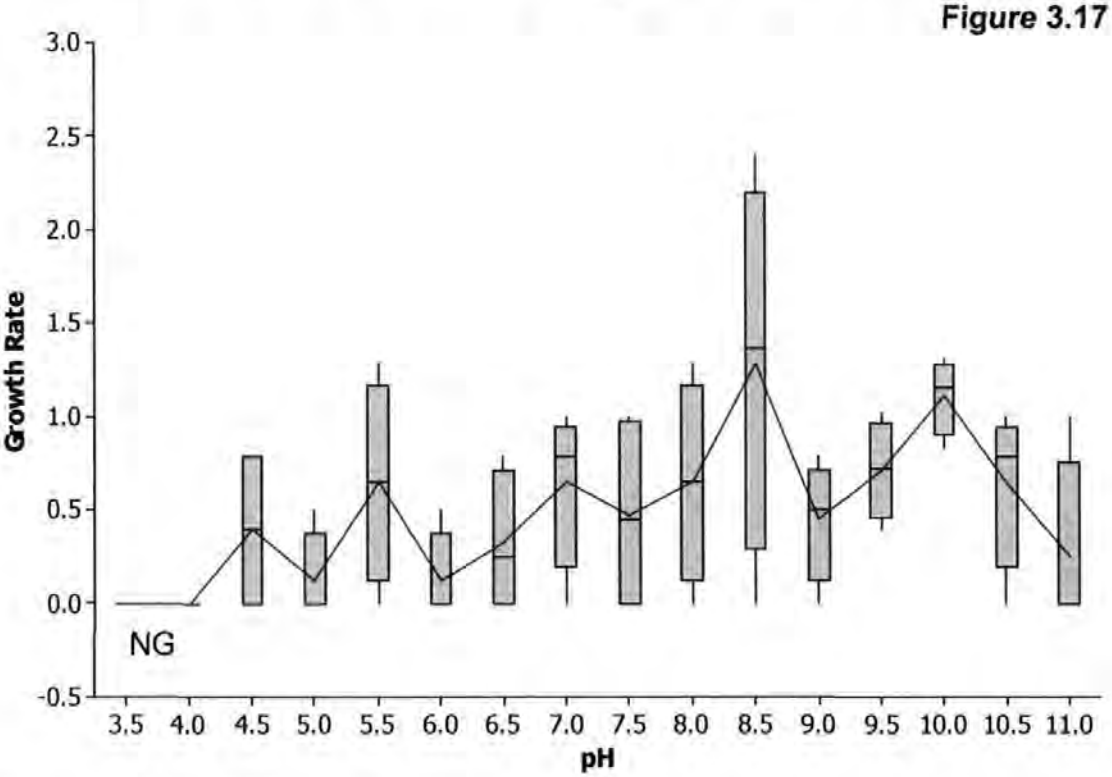


Figure 3.17



Figures 3.16 and 3.17

pH Regime, clones from Ham Gate Pond (G_2) and Abbey Lakes River respectively Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.

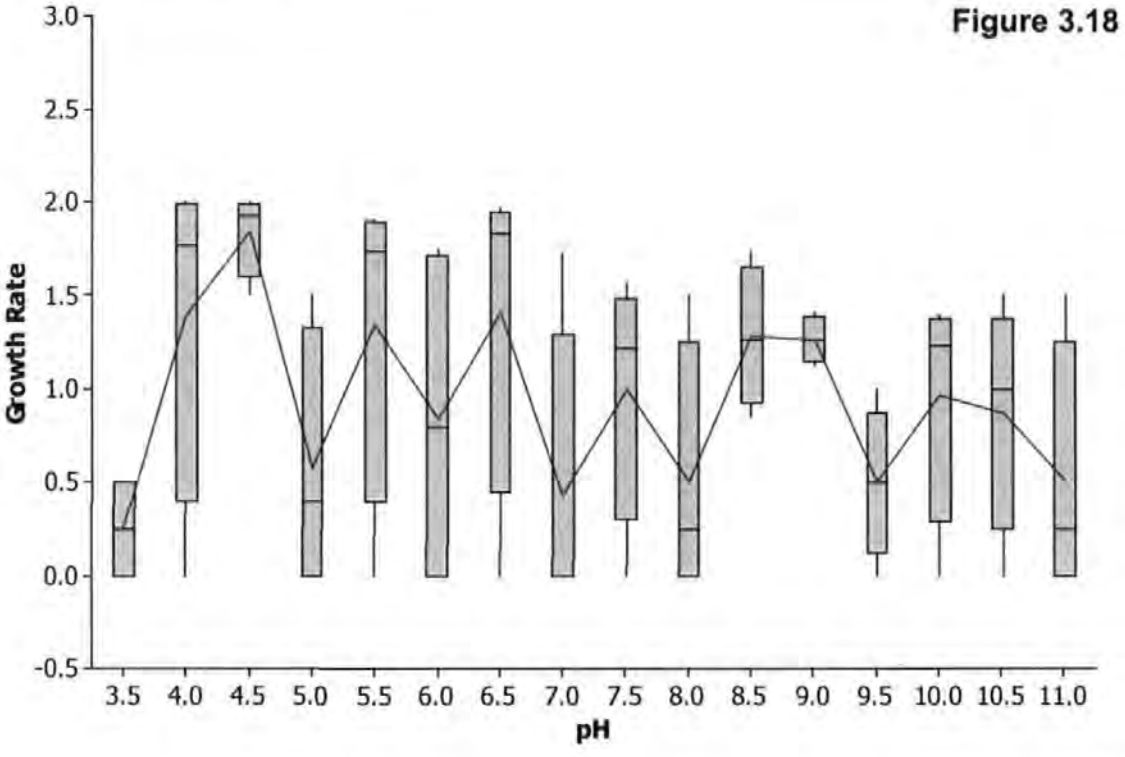


Figure 3.18

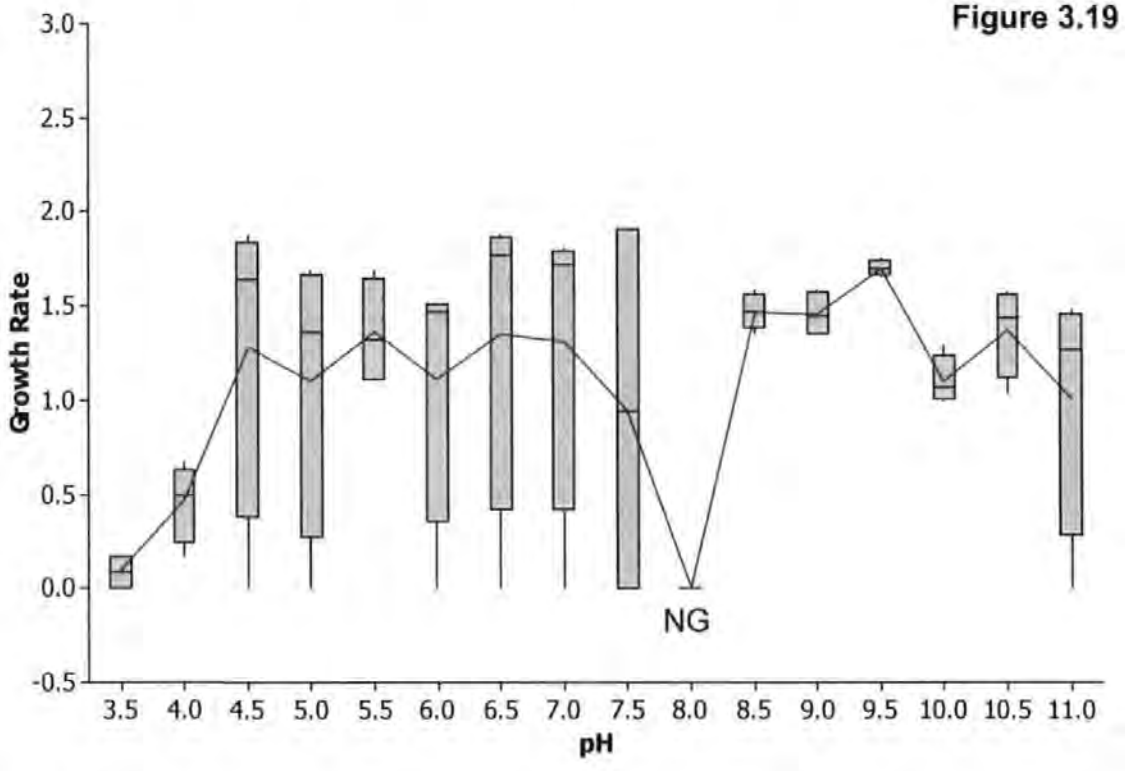


Figure 3.19

Figures 3.18 and 3.19

pH Regime, clones from Llyn Idwal (G_2) and River Kennet respectively Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.

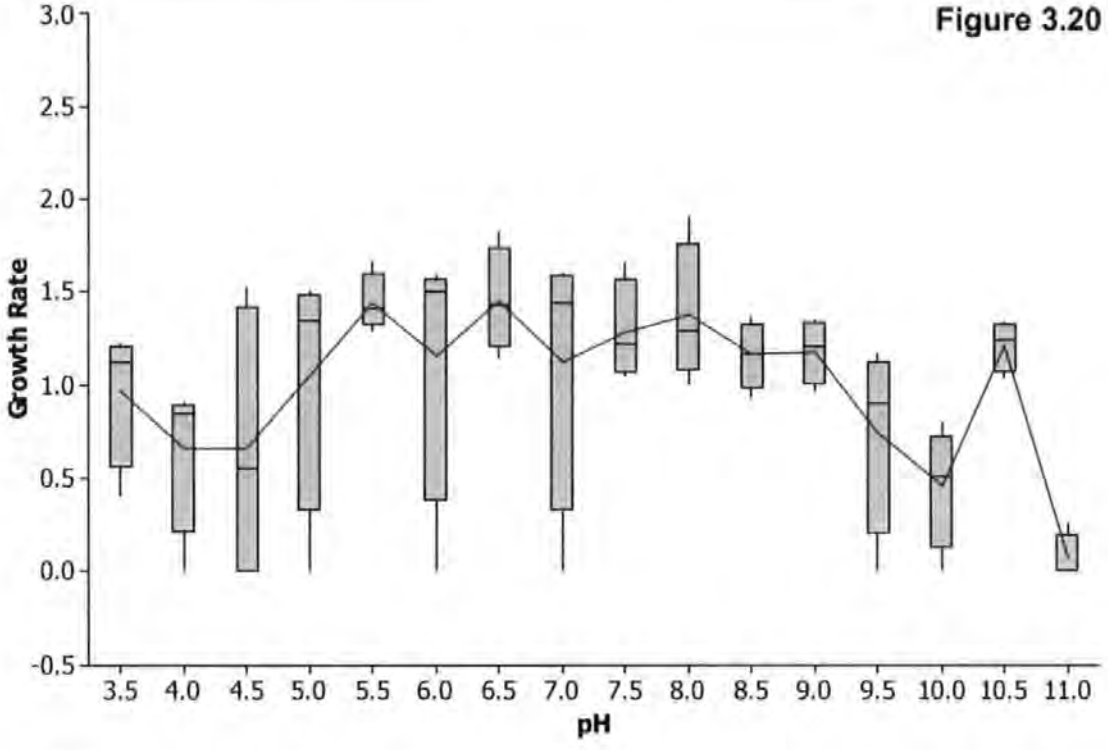


Figure 3.20

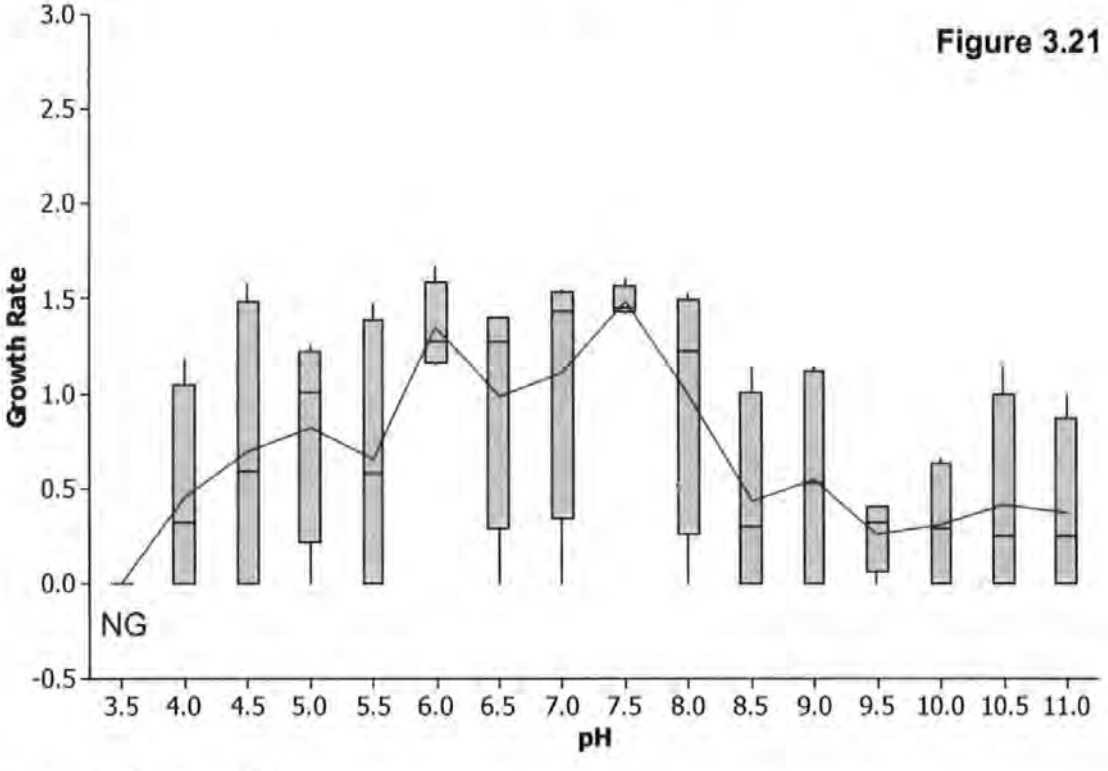


Figure 3.21

Figures 3.20 and 3.21

pH Regime, clones from Pen-y-Bryn and Lake Ogwen respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.

Figure 3.22

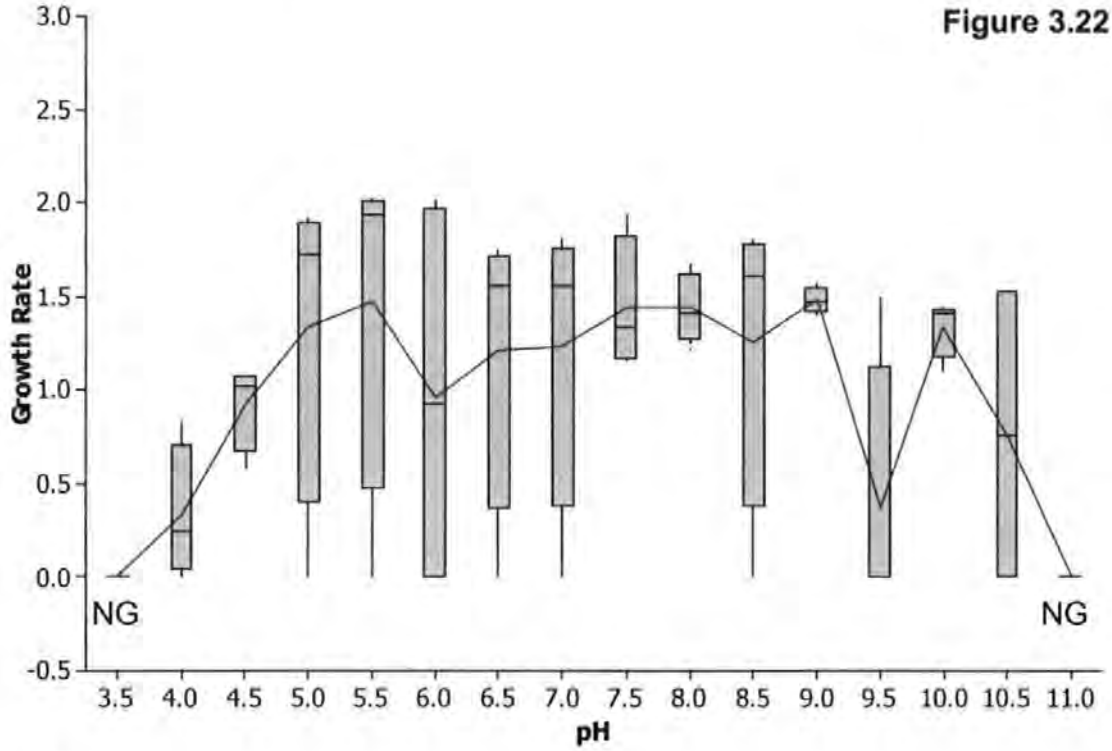
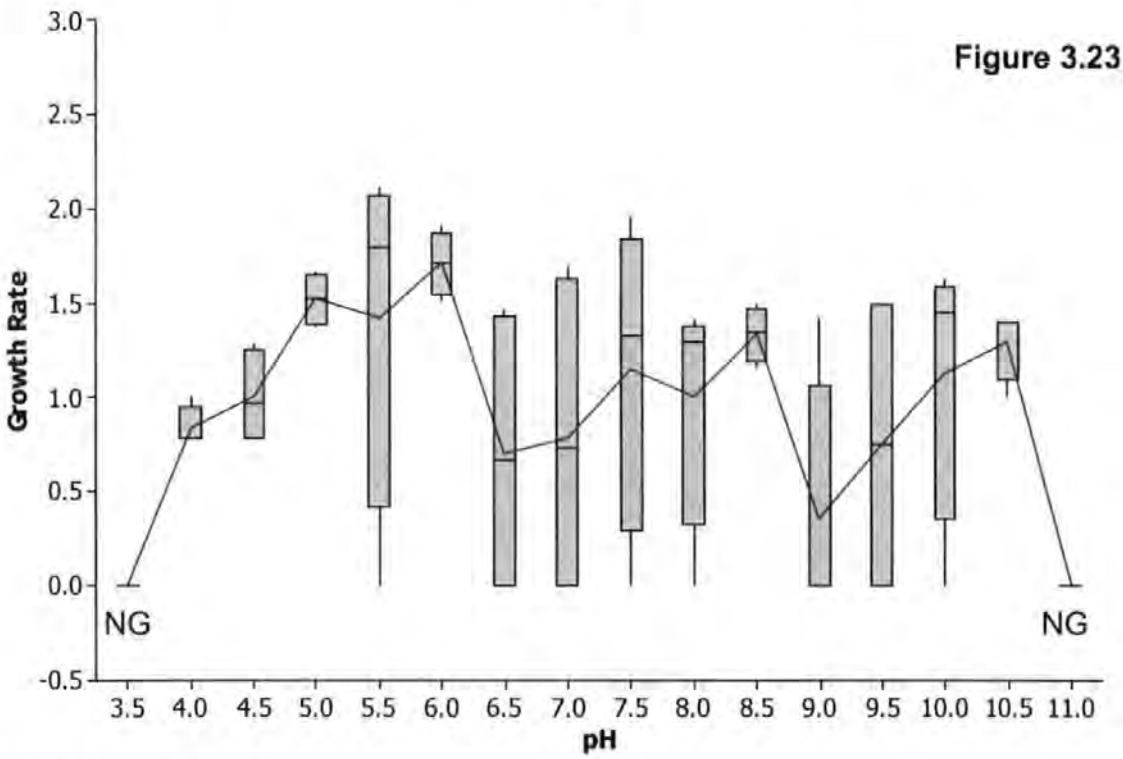


Figure 3.23



Figures 3.22 and 3.23

pH Regime, clones from Parys Mountain (G_1 and G_2 respectively) Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.

3.3.3 Artificial Sewage Regime

Figures 3.24 to 3.32. All clones were able to grow at all strengths of sewage. Clones from Scion Pond, Ham Gate Pond (G_2), Pen-y-Bryn, Lake Ogwen and Parys Mountain (G_1 and G_2), however showed no significant differences between treatments. Boxplots indicate decreasing MEGR with decreasing sewage strength in the Scion Pond clone, but the opposite in the Pen-y-Bryn clone. Each clone had clear optima (excluding the control) as follows:

- 1/5 strength for clones from Scion Pond, Ham Gate Pond (G_2) and the River Kennet;
- 1/10 strength for clones from Llyn Idwal (G_2), Pen-y-Bryn and Parys Mountain (G_1);
- 1/2 strength for clones from Ham Gate Pond (G_2) and Lake Ogwen and
- 1/25 strength for G_2 clones from Parys Mountain.

Overall, it was difficult to interpret a firm pattern in MEGR with decreasing sewage strength, though generally, clones show a decreased MEGR with decreasing sewage strength from Full to 1/25th strength sewage. Nearly all clones exhibited their highest MEGR in the control group of Nil sewage (standard MBL medium) with the exception of the G_2 clone from Ham Gate Pond, in which the highest MEGR was at 1/2 strength, and the G_2 clone from Llyn Idwal in which, 1/10 strength sewage and the control group, were almost the same. The results suggest all clones assayed appear to be broadly tolerant of eutrophic environments however, they show a preference for non-eutrophic environments.

Figure 3.24

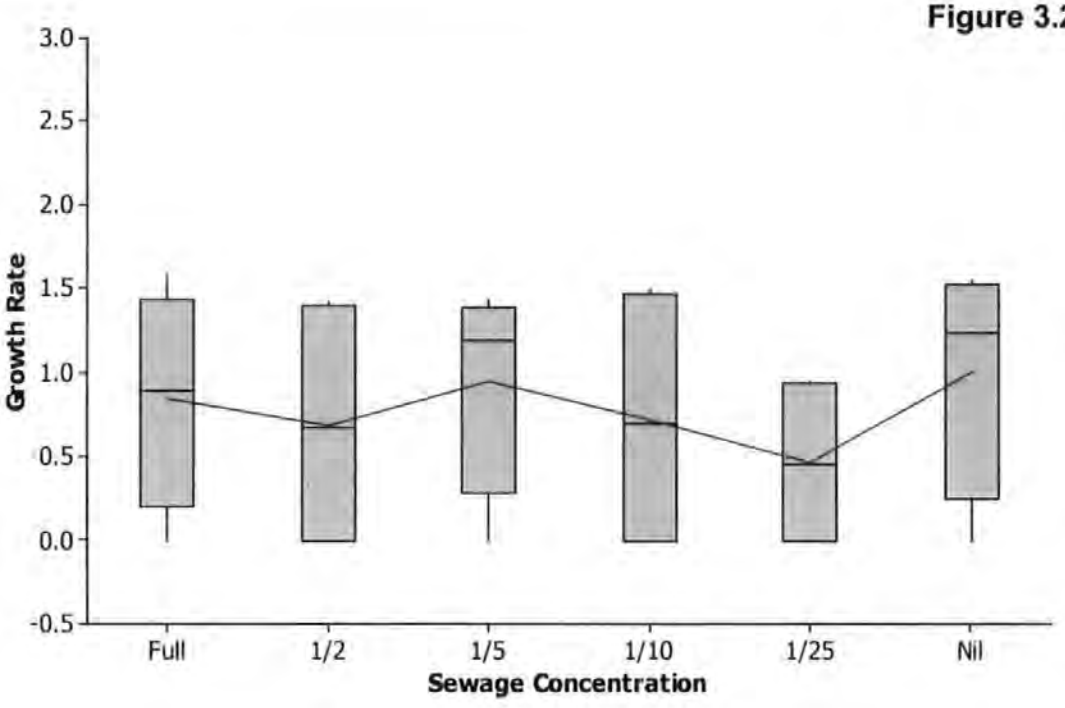
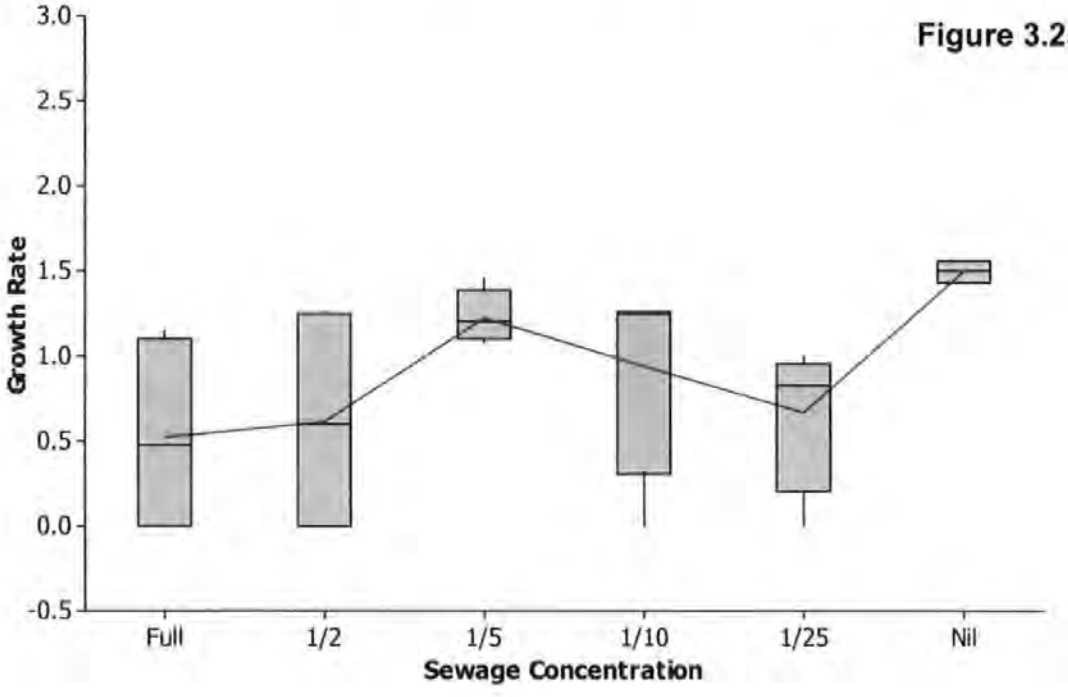


Figure 3.25



Figures 3.24 and 3.25

Sewage Regime, Scion Pond and Kings Mere G_1 clones respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.

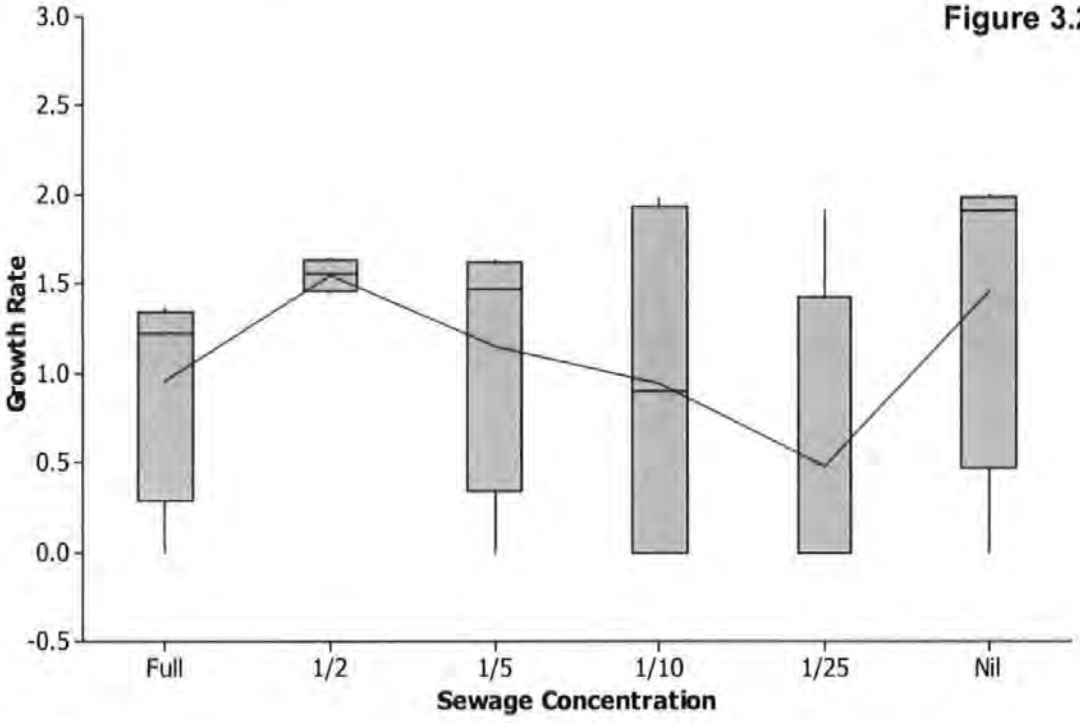


Figure 3.26

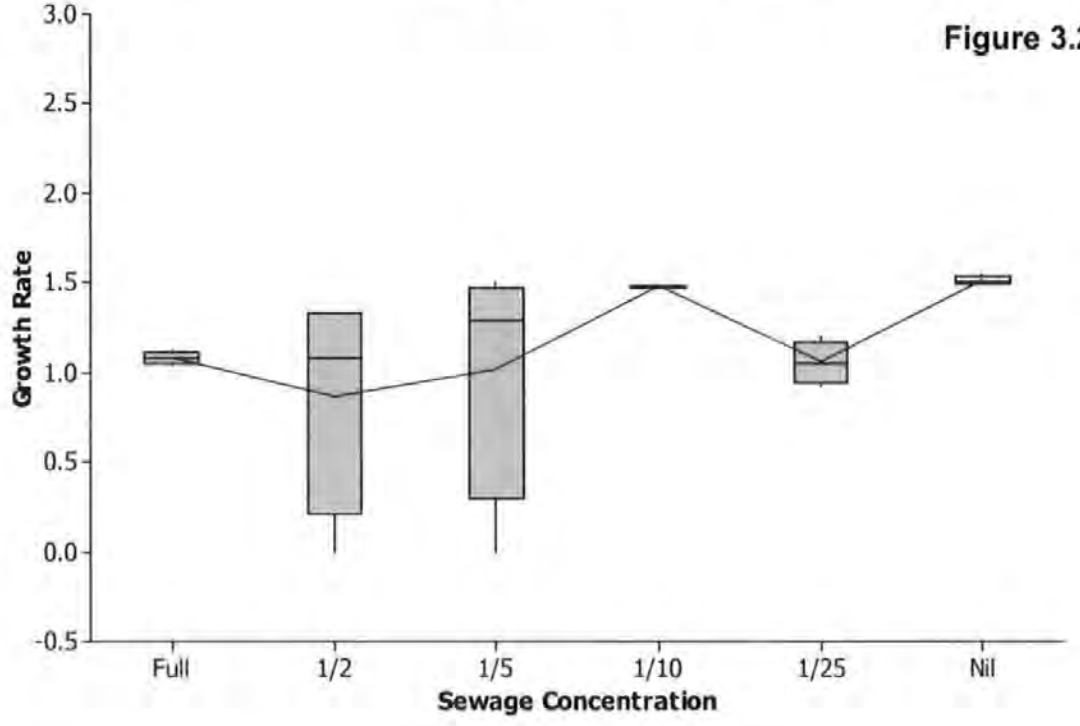


Figure 3.27

Figures 3.26 and 3.27

Sewage Regime, clones Ham Gate Pond G_2 clone and Llyn Idwal G_2 clone respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.

Figure 3.28

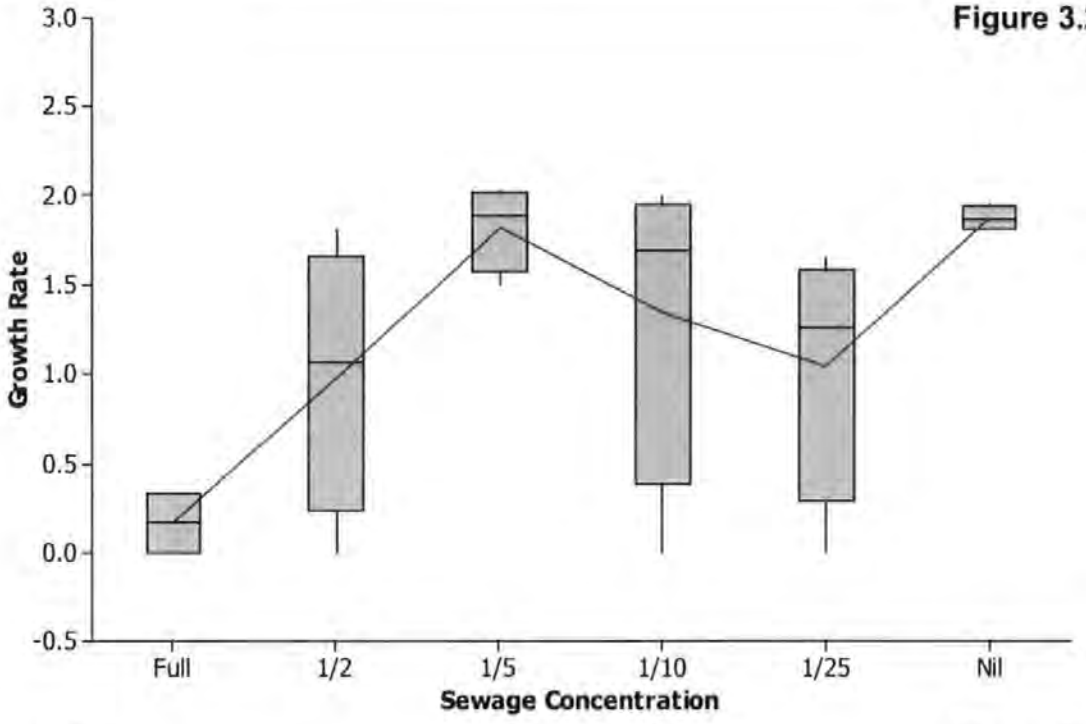
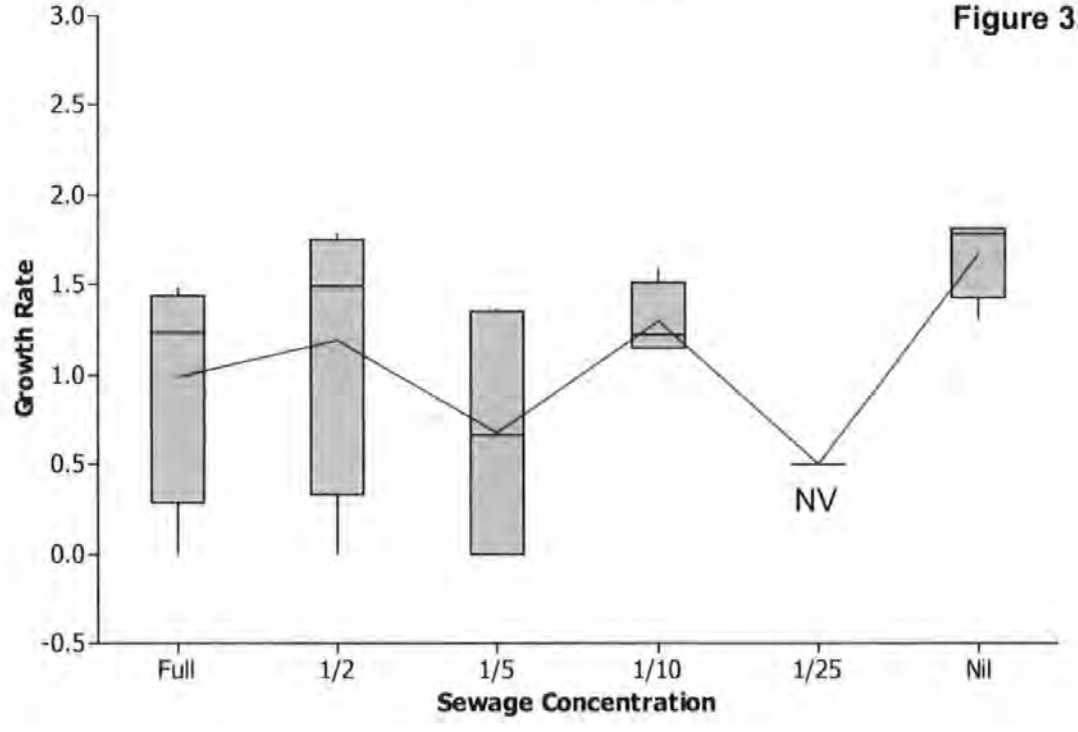


Figure 3.29



Figures 3.28 and 3.29

Sewage Regime, River Kennet and Pen-y-Bryn clones respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NV** = No variance.

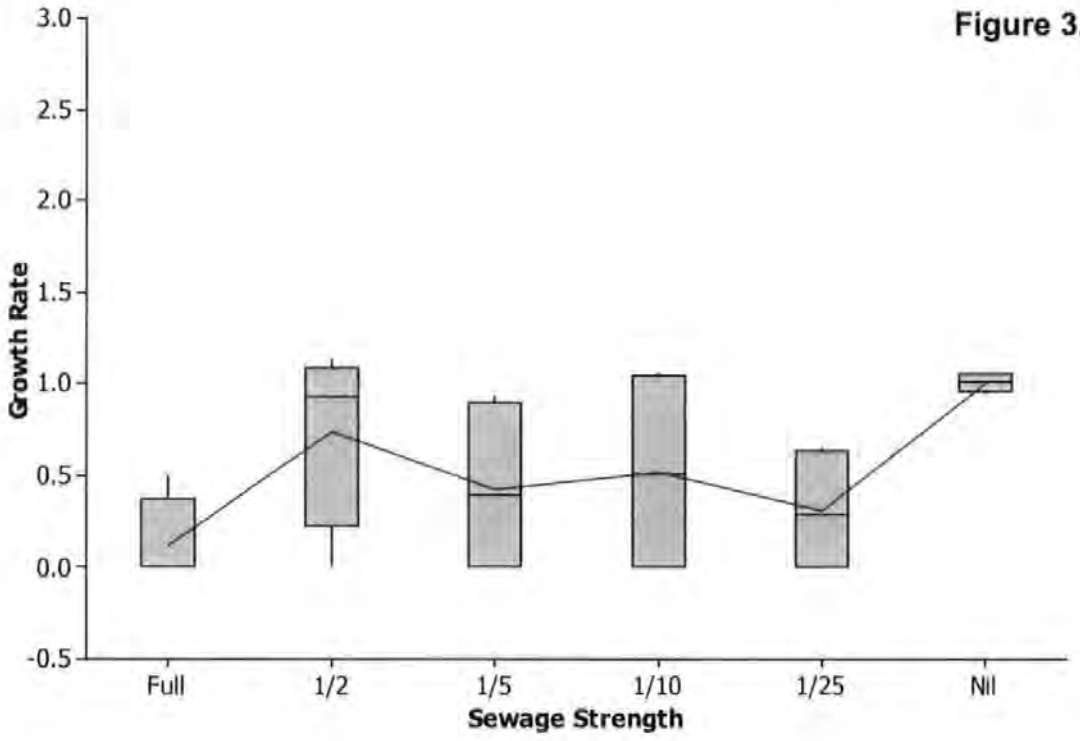


Figure 3.30

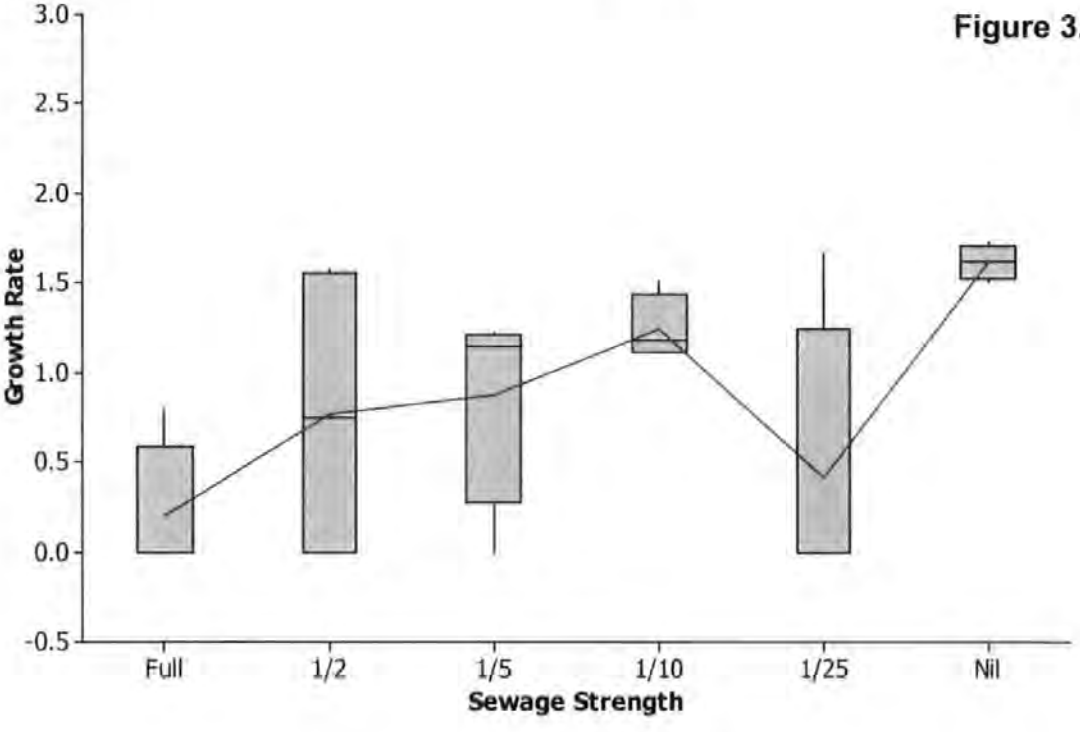


Figure 3.31

Figures 3.30 and 3.31

Sewage Regime, Llyn Ogwen and Parys Mountain G_1 clone respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.

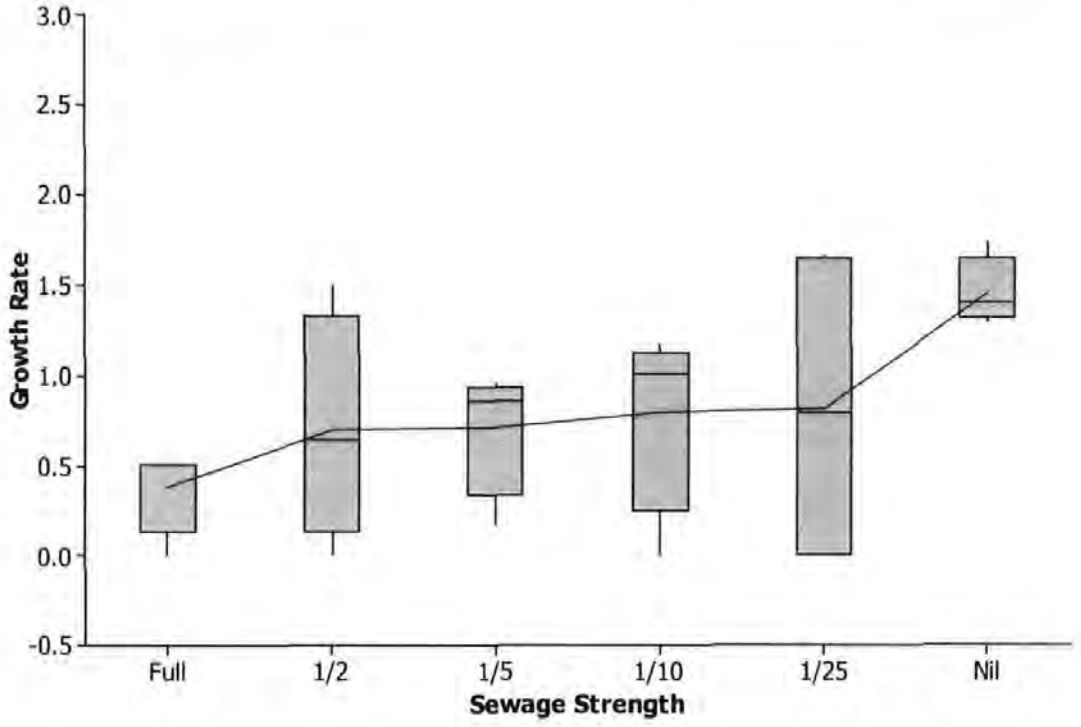


Figure 3.32

Sewage Regime, Parys Mountain G_2 clone. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.

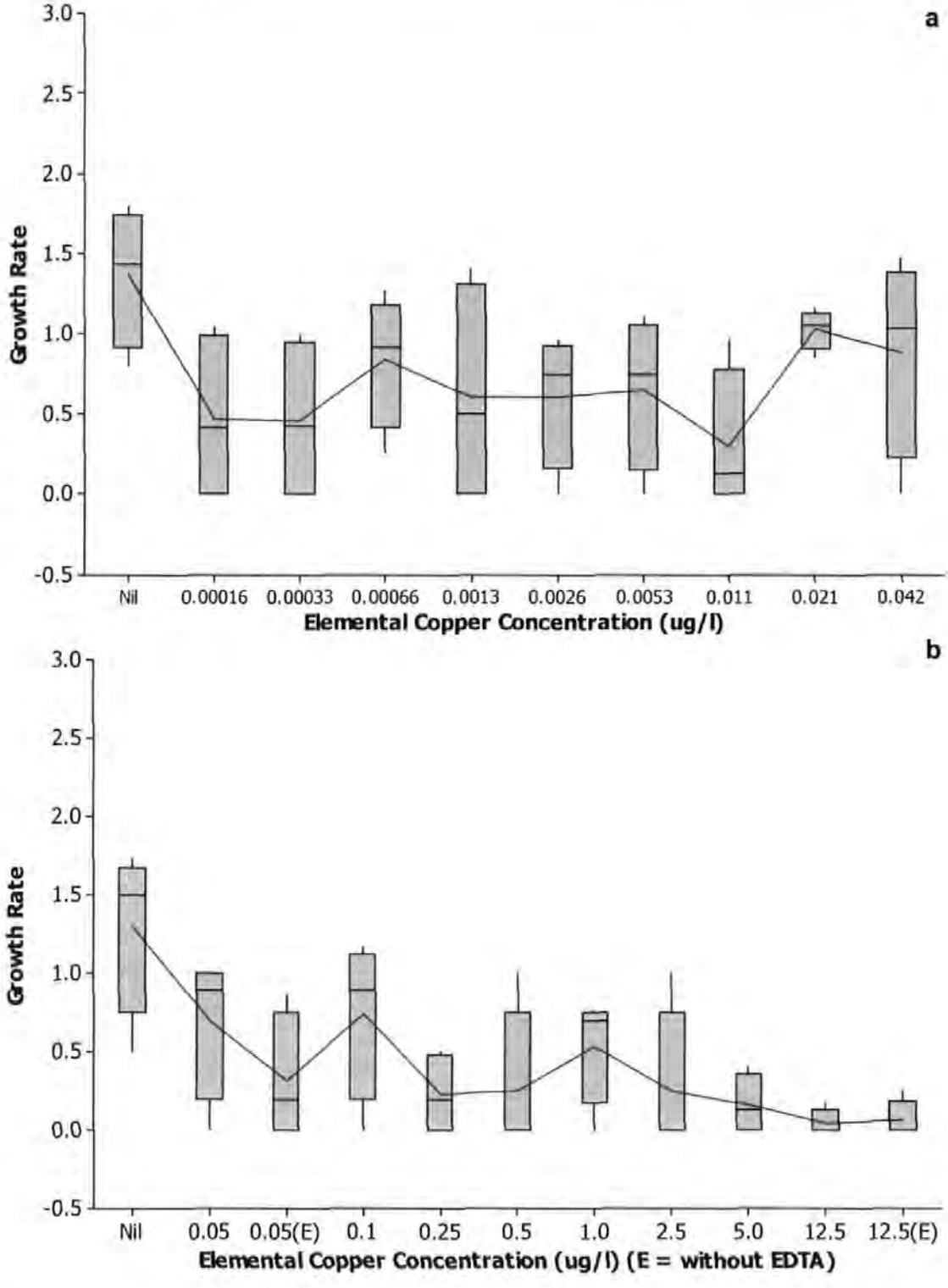
3.3.4 Copper Regime

Figures 3.34 to 3.42. Only the Scion Pond clone was able to grow across the full range of copper concentrations in both the initial and extended runs. In the initial run, only the Lake Ogwen clone was not able to grow across the full copper concentration range, failing to grow at 0.00066µg/l and 0.042µg/l. The failure at 0.00066µg/l is probably due to inoculation failure. In the extended run however, several clones were unable to grow at higher concentrations. No clone was able to grow at copper concentrations of 12.5µg/l with or without the addition of EDTA. Additionally, clones from Kings Mere, Ham Gate Pond, Llyn Idwal and Parys Mountain (all G_2 clones) failed to grow in the extended run at copper concentrations of 0.10, 0.25, 1.0 and 0.25µg/l respectively, probably due to inoculation failure rather than copper concentration.

Nearly all clones exhibited a higher MEGR in media containing EDTA compared to that without EDTA (0.05 and 12.5µg/l) with the exception of clones from Llyn Idwal (G_2) and the River Kennet, which both showed a slight increase in MEGR in the absence of EDTA. However, none of these differences was significant at the 95% probability level.

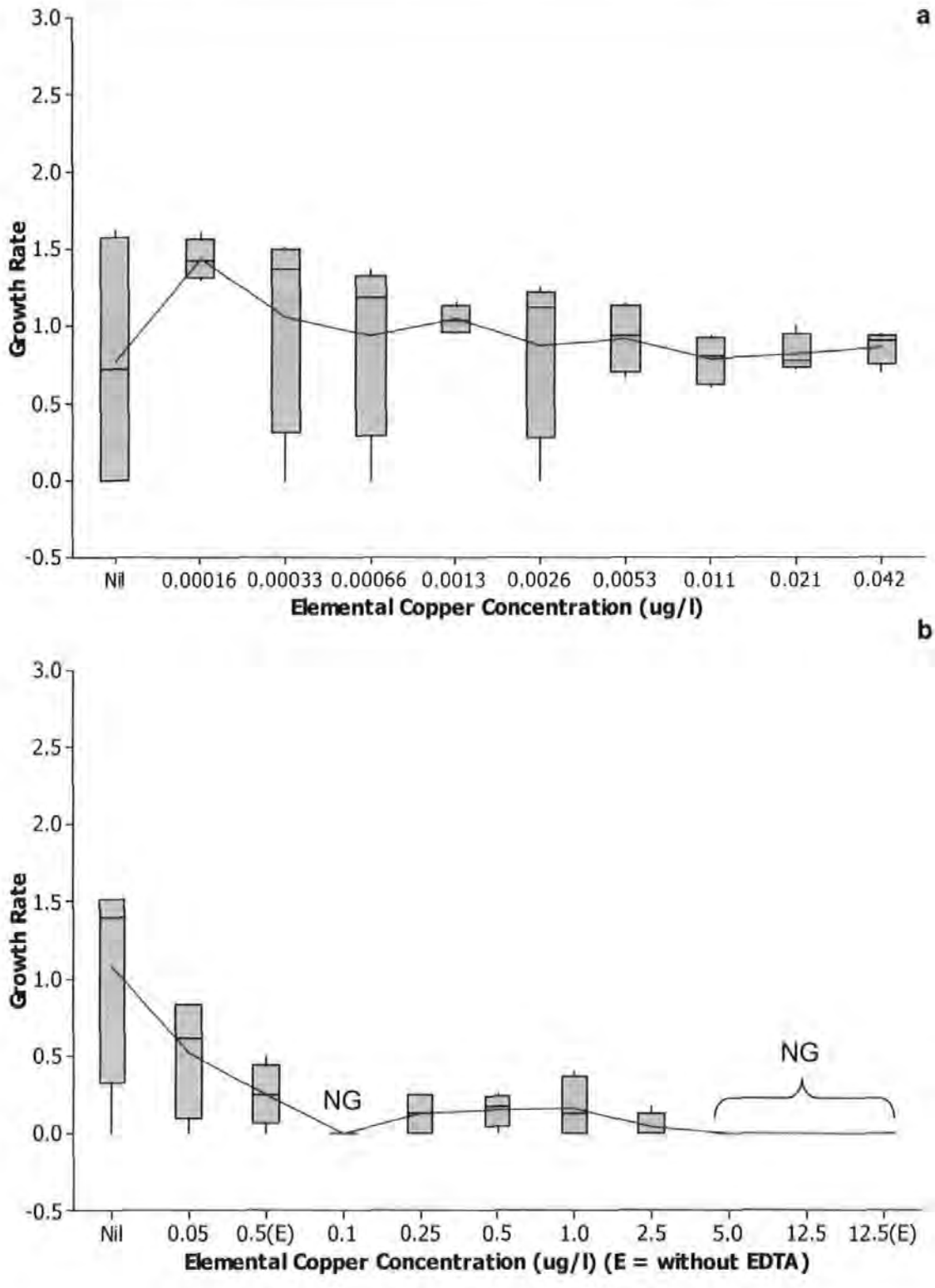
Subsequent pairwise comparisons of treatment groups with the control group (Nil Copper) in the extended run, indicated [often highly] significant differences, with the exception of the Ham Gate Pond (G_2) clone, in which none of the pairwise comparisons in the initial run were significant. Generally, all clones exhibited a decrease in MEGR with increasing copper concentration. This was particularly marked in the extended run where three clones (Ham Gate Pond (G_2), River Kennet and Parys Mountain (G_2)) exhibited a

significant difference with increasing copper concentration from 0.05 to 0.10µg/l. Interestingly, approximately half the clones assayed exhibited higher MEGRs in the absence of copper than in its presence, i.e. clones from Scion Pond and lake Ogwen for both the initial and extended runs, and G_2 clones from Kings Mere, Ham Gate Pond, Llyn Idwal and Parys Mountain in the extended run. The results suggest that overall, all clones assayed are broadly tolerant of high copper environments with the exception of the clone from Lake Ogwen and the G_2 clone from Parys Mountain, which appear more sensitive.



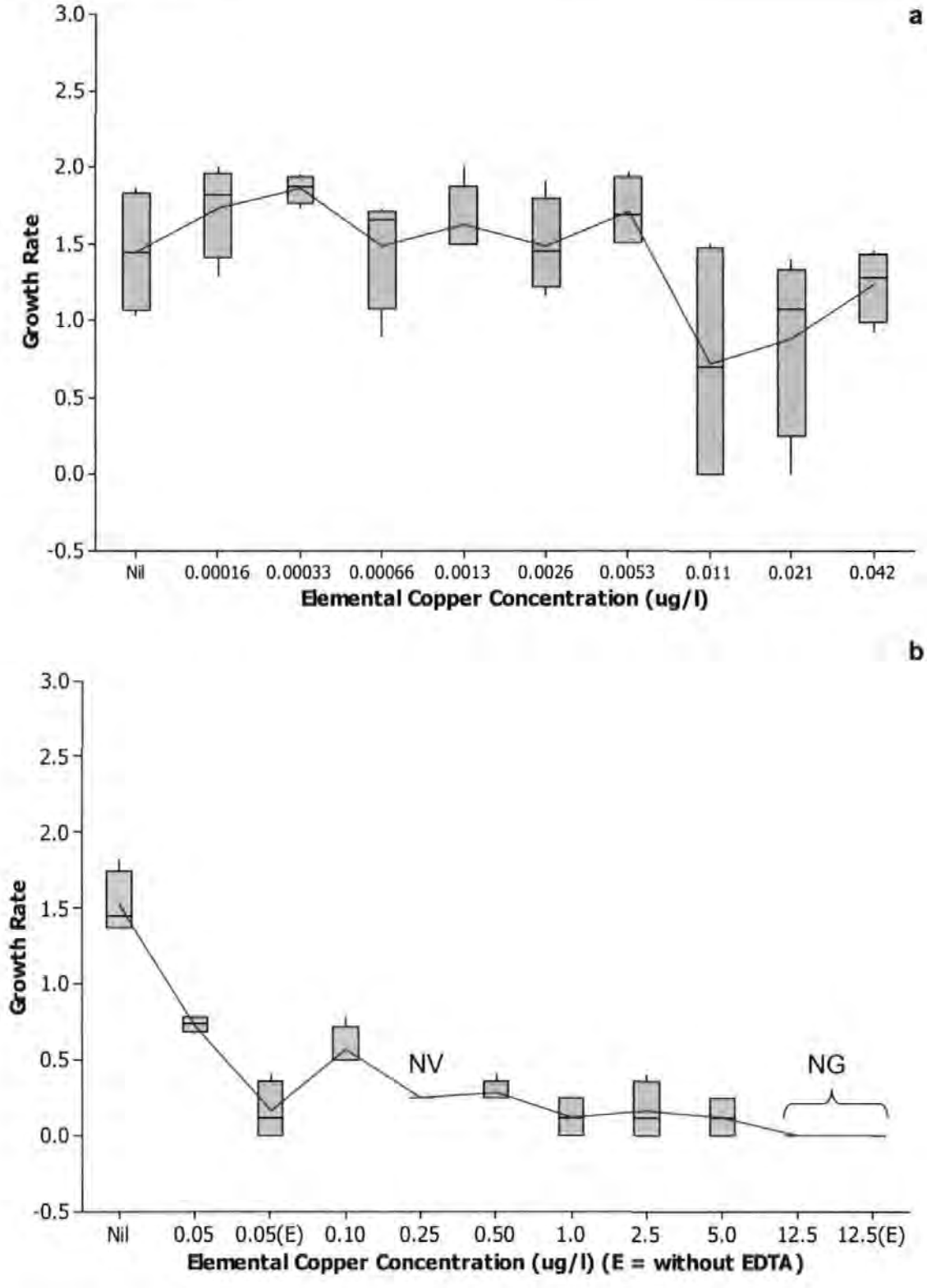
Figures 3.33: a-b

Copper Regime, Scion Pond clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.



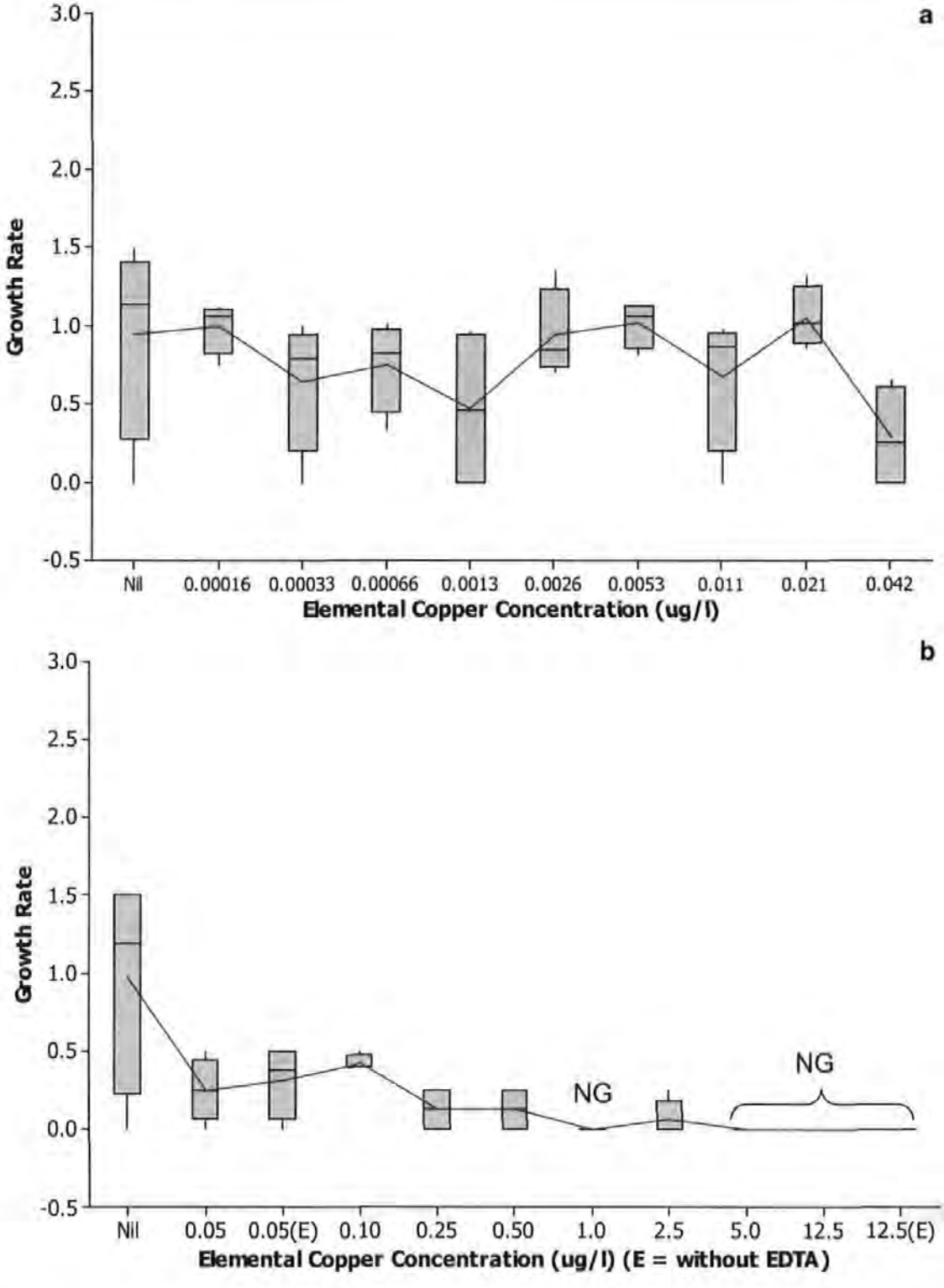
Figures 3.34: a-b

Copper Regime, Kings Mere G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



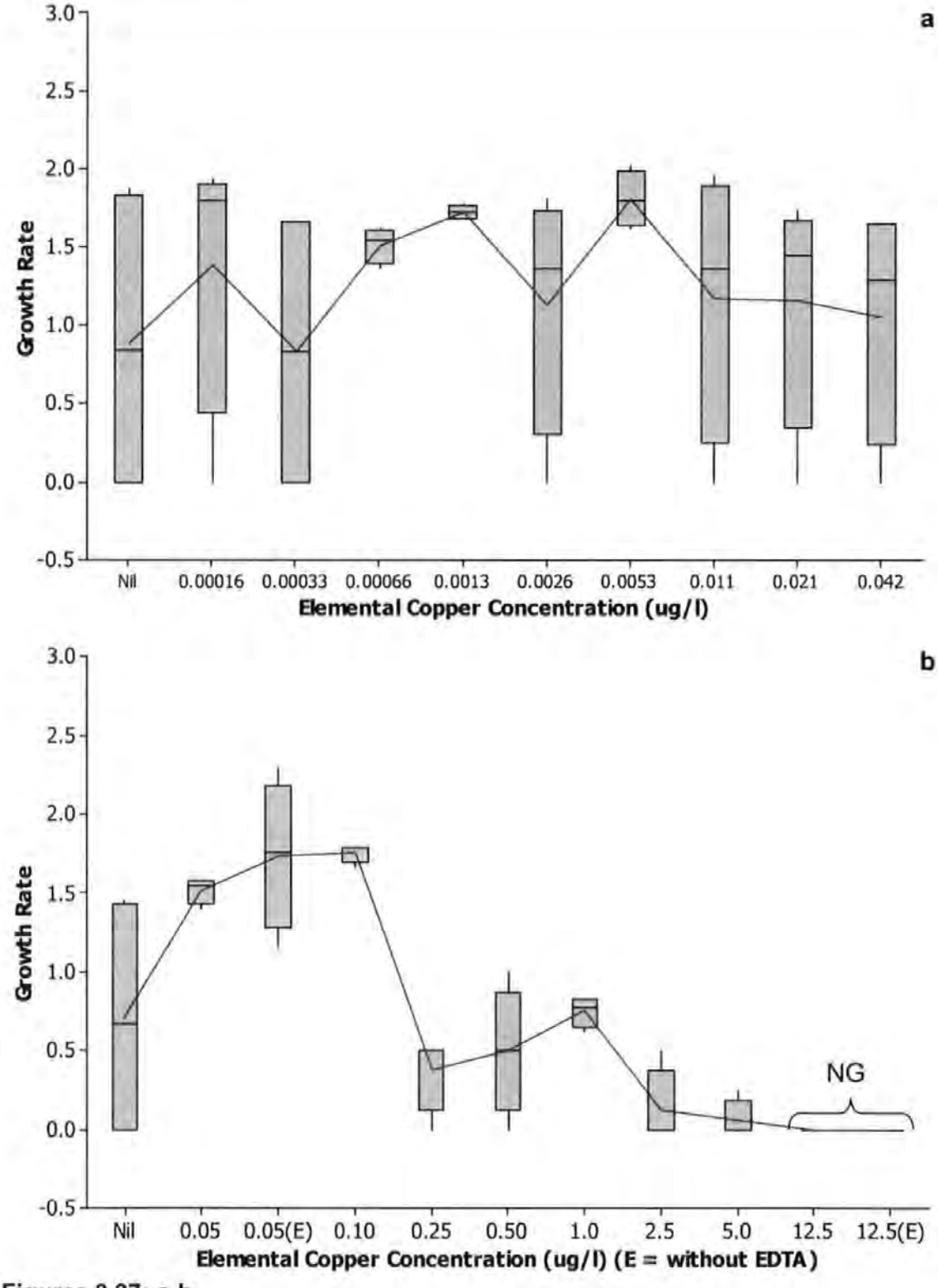
Figures 3.35: a-b

Copper Regime, Ham Gate Pond G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth. **NV** = No Growth.



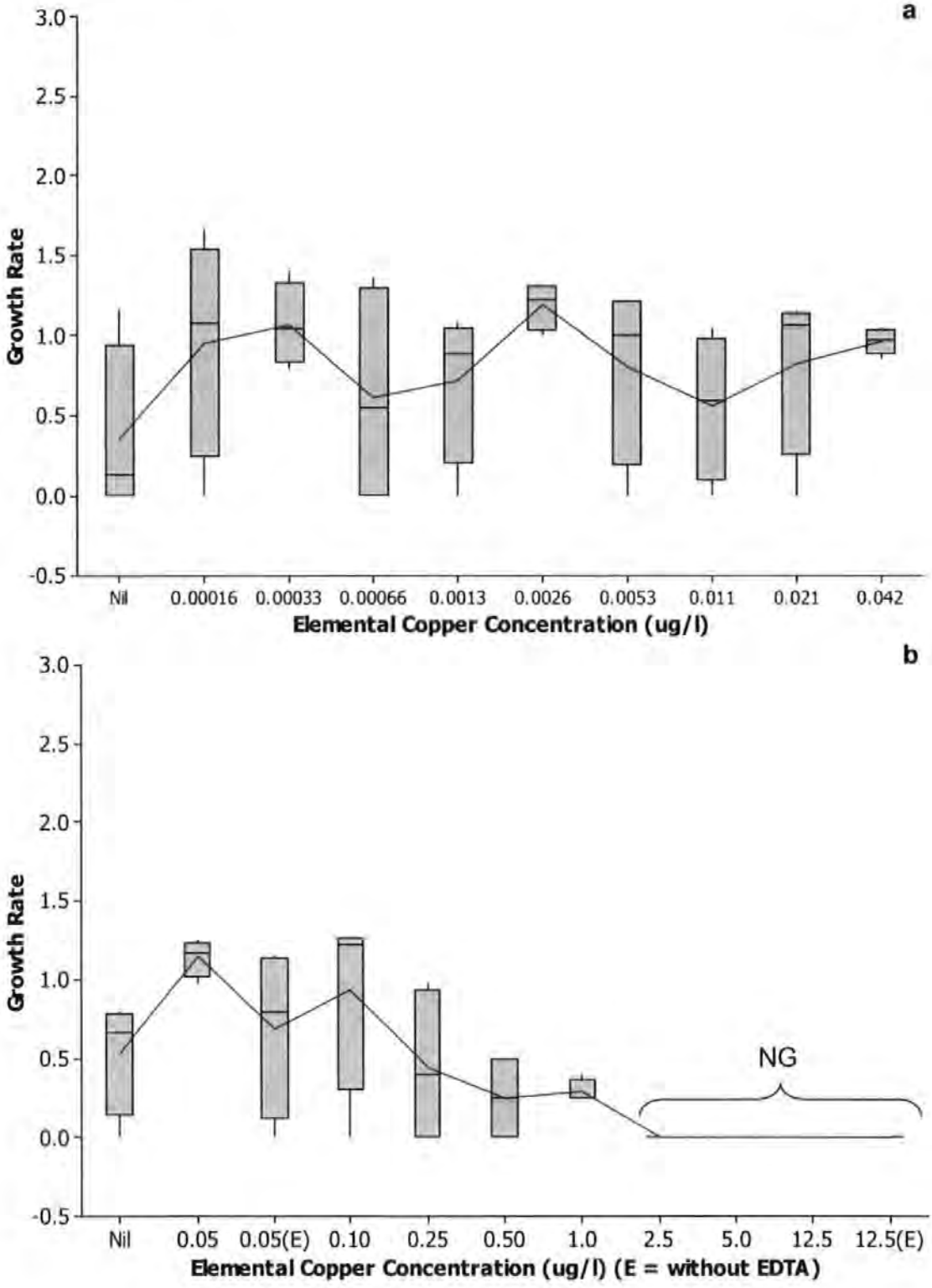
Figures 3.36: a-b

Copper Regime, Llyn Idwal G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth



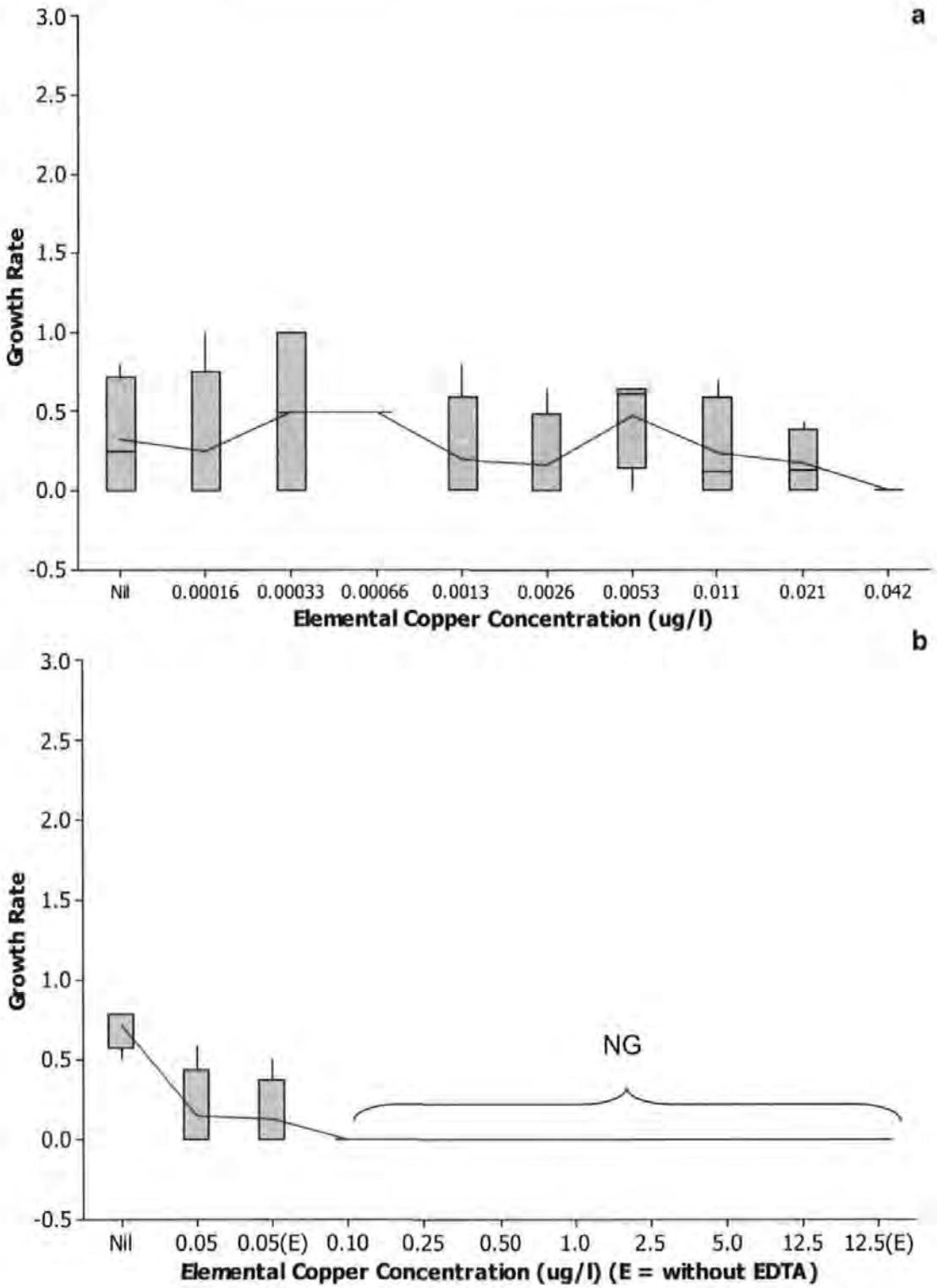
Figures 3.37: a-b

Copper Regime, River Kennet clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



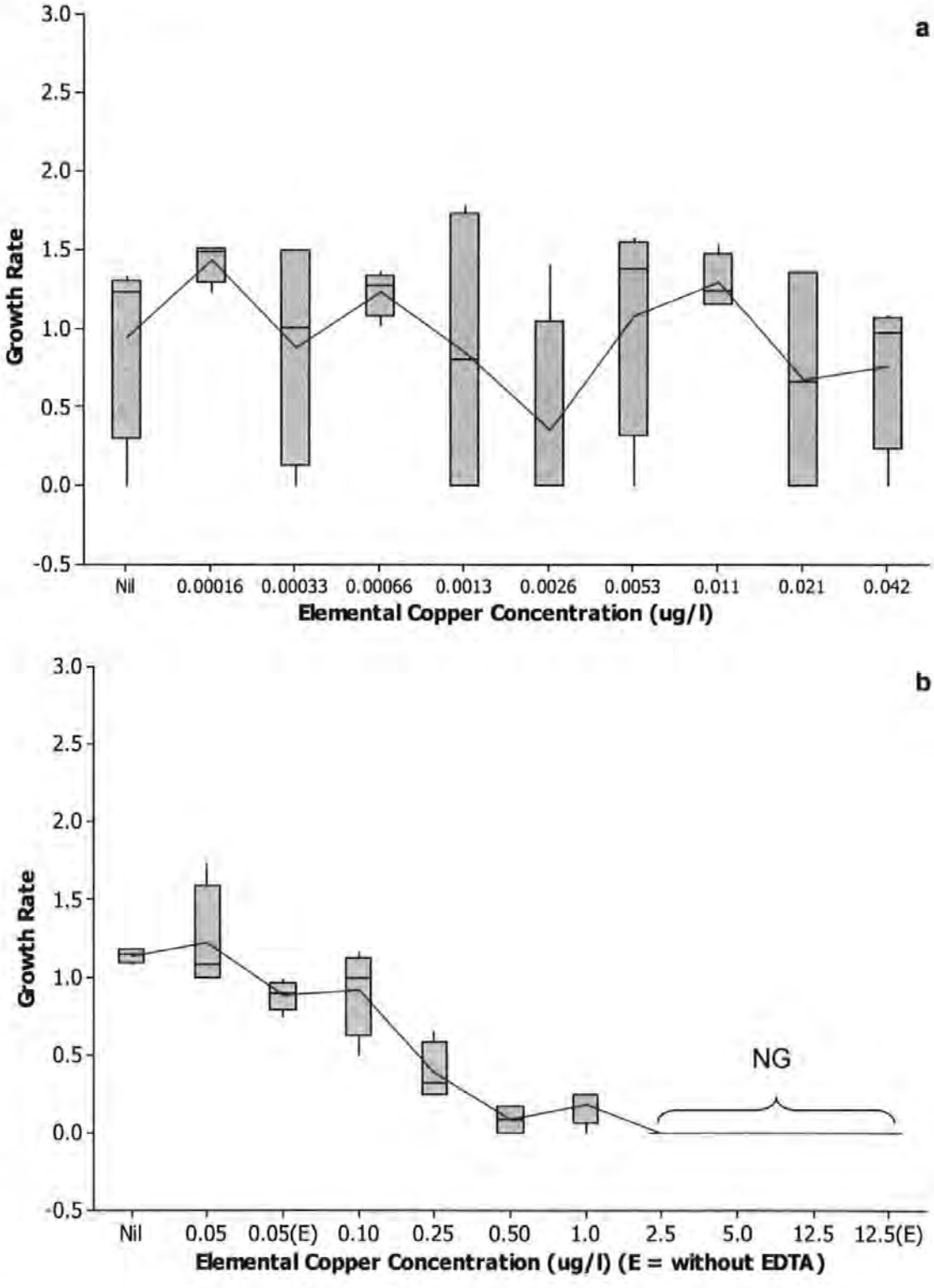
Figures 3.38: a-b

Copper Regime, Pen-y-Bryn clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



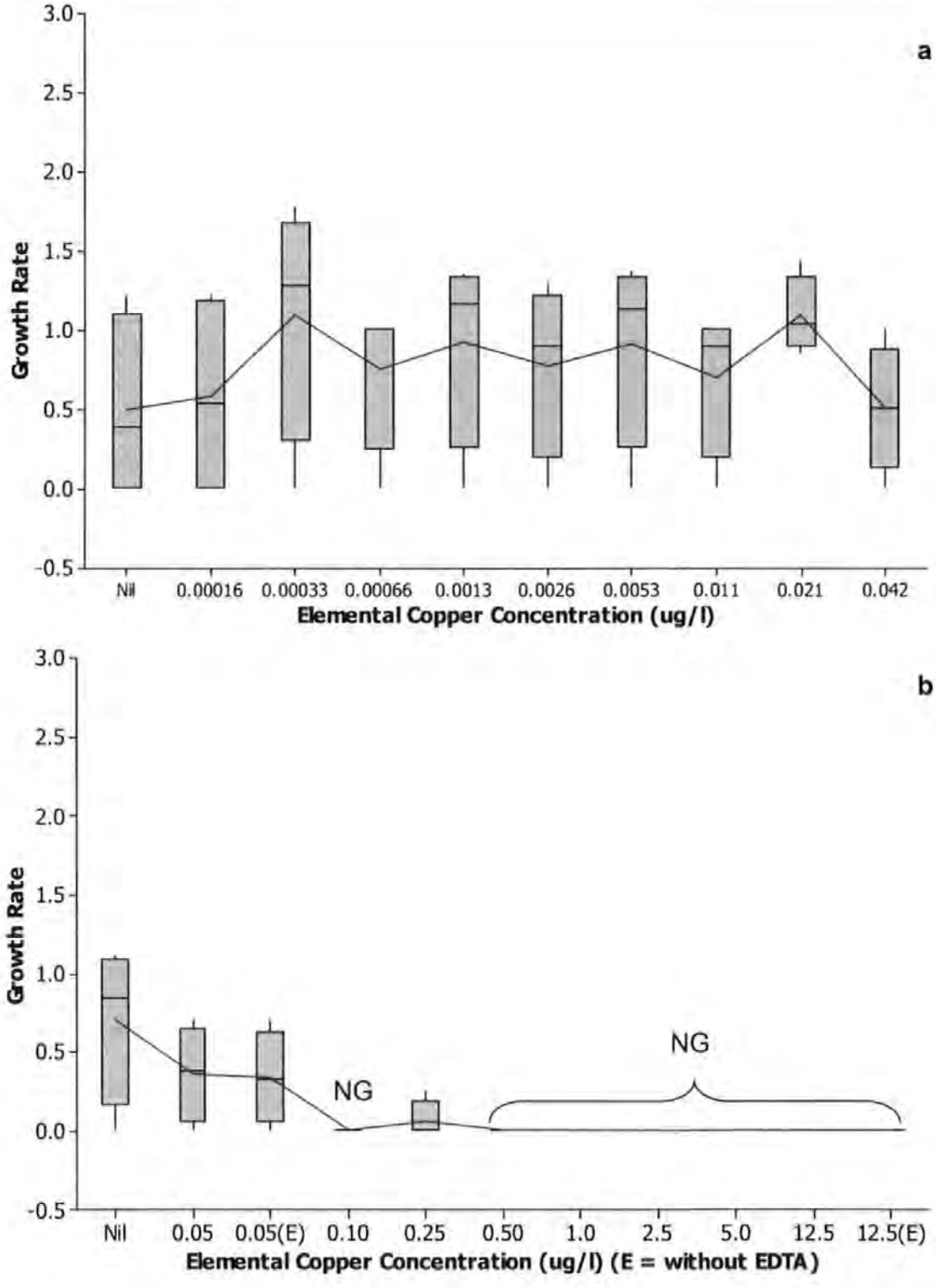
Figures 3.39: a-b

Copper Regime, Lake Ogwen clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth. **NV** = No variance.



Figures 3.40: a-b

Copper Regime, Parys Mountain G_1 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



Figures 3.41: a-b

Copper Regime, Parys Mountain G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.

3.3.5 Zinc Regime

Figures 3.43 to 3.51. No single clone was able to grow across the full range of zinc concentrations, though several grew at all initial run concentrations, i.e. clones from Scion Pond, G_2 clones from Ham Gate Pond and Kings Mere, G_1 and G_2 clones from Parys Mountain and clones from Pen-y-Bryn. The Llyn Idwal clone (G_2) failed to grow at a zinc concentration of 0.042 µg/l in the initial run and clones from the River Kennet and Lake Ogwen failed to grow at a zinc concentration of 0.011 µg/l, though this was probably due to inoculation failure as it grew well at 0.0053 and 0.021 µg/l copper concentration in the initial run.

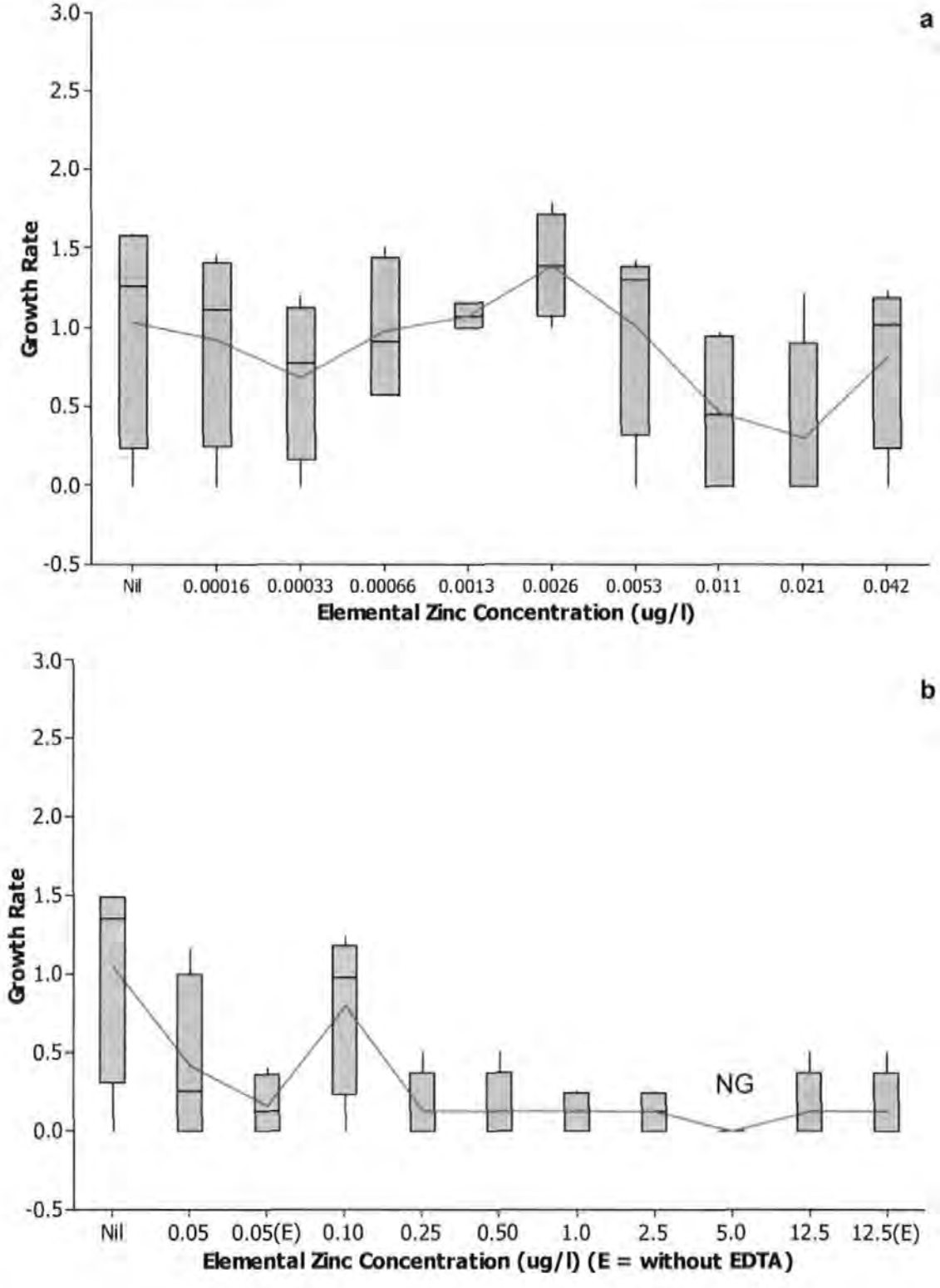
In the extended run, clones from Kings Mere (G_2), River Kennet, Pen-y-Bryn and Parys Mountain (G_1) ceased to grow at zinc concentrations of 2.5, 1.0, 2.5, and 0.5µg/l respectively. Additionally, clones from Scion Pond, Kings Mere (G_2), Ham Gate Pond (G_2), Llyn Idwal (G_2) and Lake Ogwen did not grow at 5.0, 0.25, 5.0, 2.5 and 0.10µg/l zinc concentrations respectively. Again, these later failures are probably due to inoculation failure as the clones were able to grow at higher concentrations.

Nearly all clones exhibited a higher MEGR in the extended run at the zinc concentration of 0.05µg/l with EDTA, compared to that without EDTA, with the exception of the Lake Ogwen clone in which the MEGRs were the same for both the EDTA and non-EDTA replicates. The same was true at zinc concentration of 12.5µg/l, in which clones Ham Gate Pond (G_2), Llyn Idwal (G_2), Lake Ogwen and Parys Mountain (G_2), in the presence of EDTA, exhibited a higher growth rate than in its absence. Clones from River Kennet,

Pen-y-Bryn and Parys Mountain (G_1) did not exhibit any growth at zinc concentrations 12.5µg/l or without EDTA. Only the Kings Mere (G_2) clone had a higher growth rate at a zinc concentration of 12.5µg/l without EDTA added. None of these differences was significant at the 95% probability level.

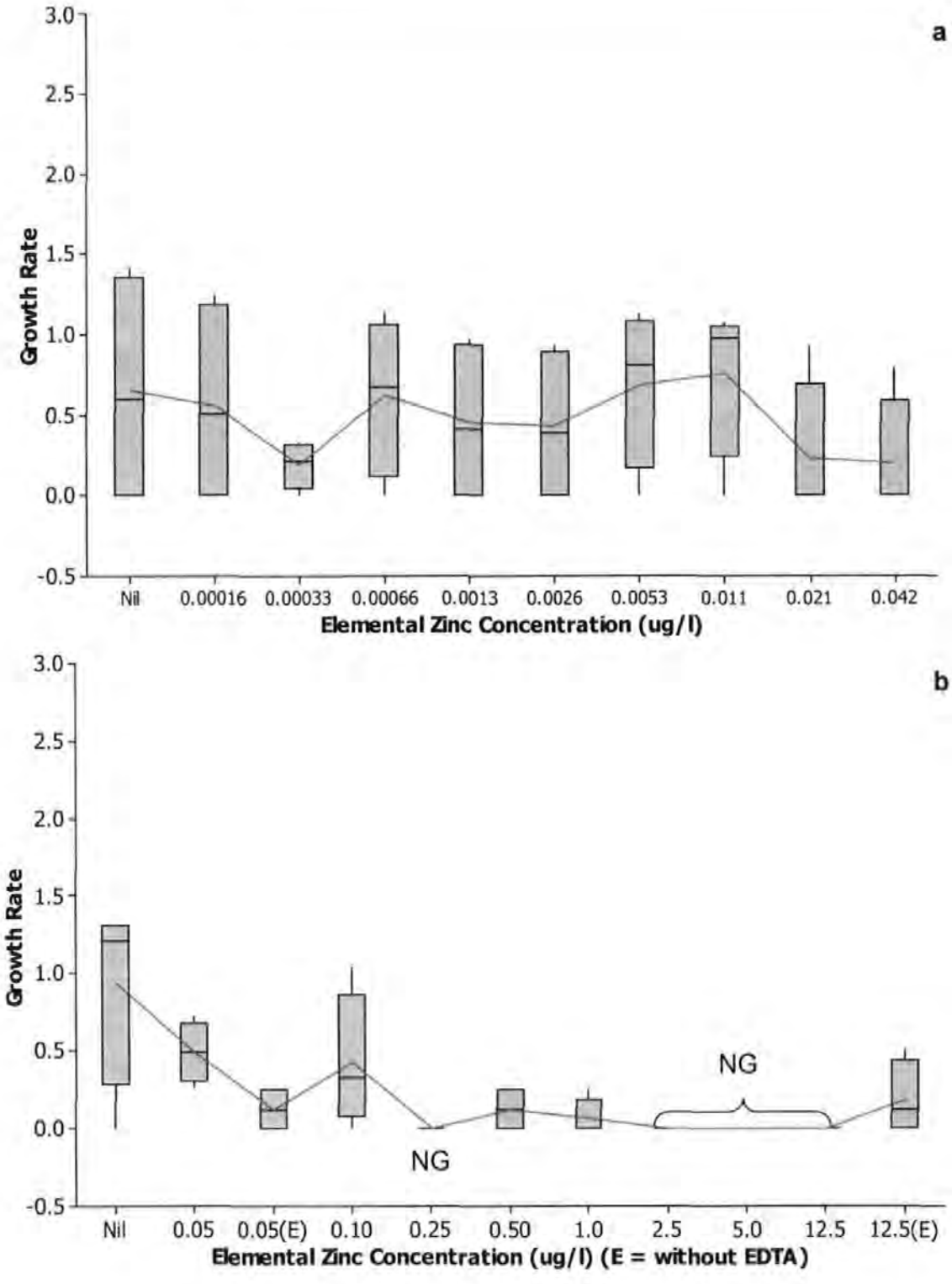
Pairwise comparison of treatment MEGR and control MEGR for the Ham Gate Pond, G_2 clone on the initial run showed significant differences between the control group and zinc concentrations of 0.00033, 0.0053, 0.011 and 0.042µg/l. In the extended run, pairwise comparisons indicated [often highly] significant differences (see enclosed CD). Therefore, all clones assayed appear to be broadly tolerant of high Zn environments with the exception of clones from the River Kennet, Pen-y-Bryn and the G_1 clone from Parys Mountain.

Generally, all clones exhibited a decrease in MEGR with increasing zinc concentration. However, only the Ham Gate Pond clone (G_2) exhibited a significant difference with increasing zinc concentration and only in the extended run with zinc concentrations between 0.0053 and 0.011µg/l. In both runs, nearly all clones exhibited higher overall MEGRs in the absence of zinc than in its presence (at any concentration), with the exception of clones from Scion Pond, Pen-y-Bryn and the G_2 clones from Parys Mountain (initial run) and the Pen-y-Bryn clone (extended run).



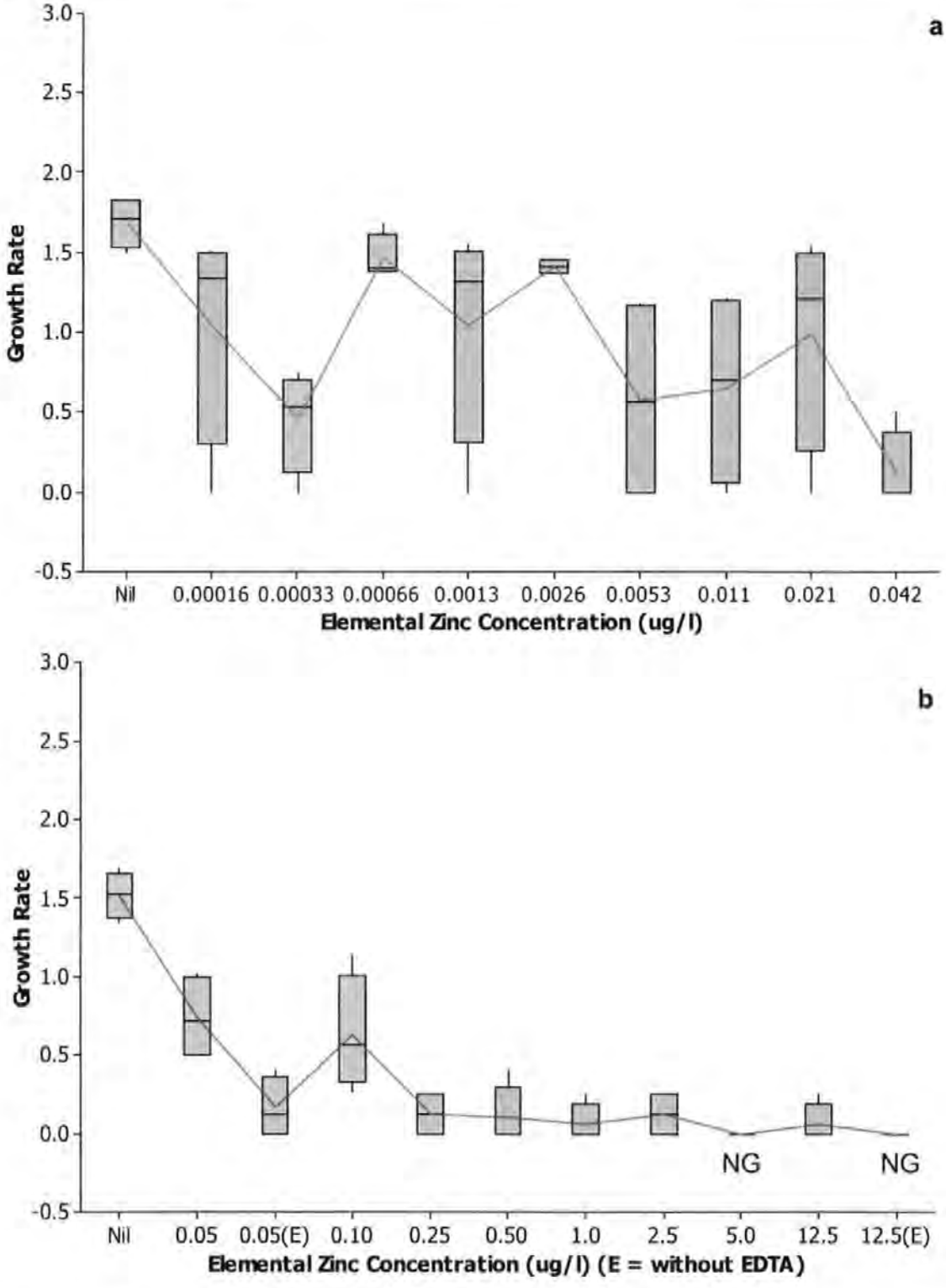
Figures 3.42: a-b

Zinc Regime, Scion Pond clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



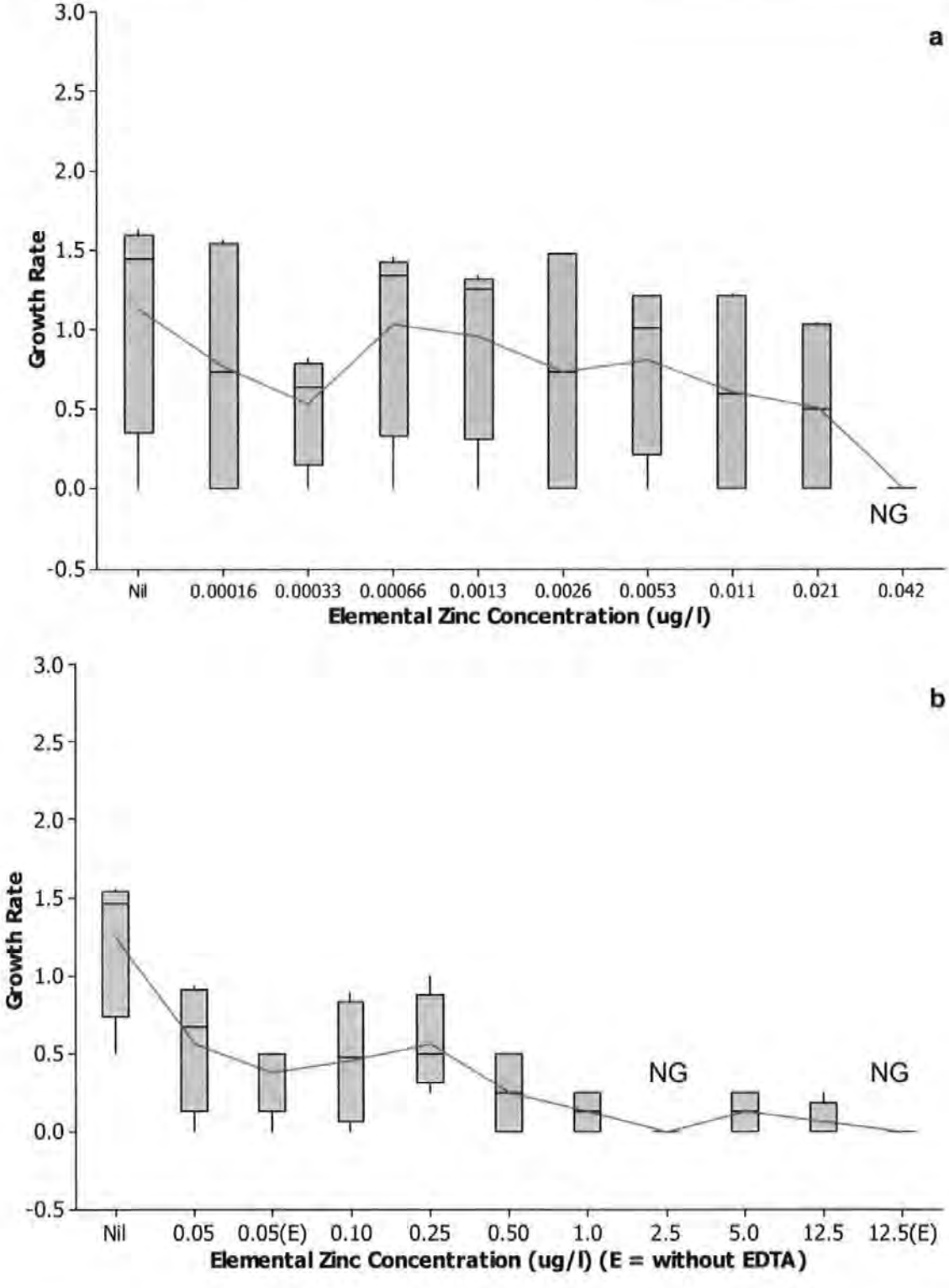
Figures 3.43: a-b

Zinc Regime, Kings Mere G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



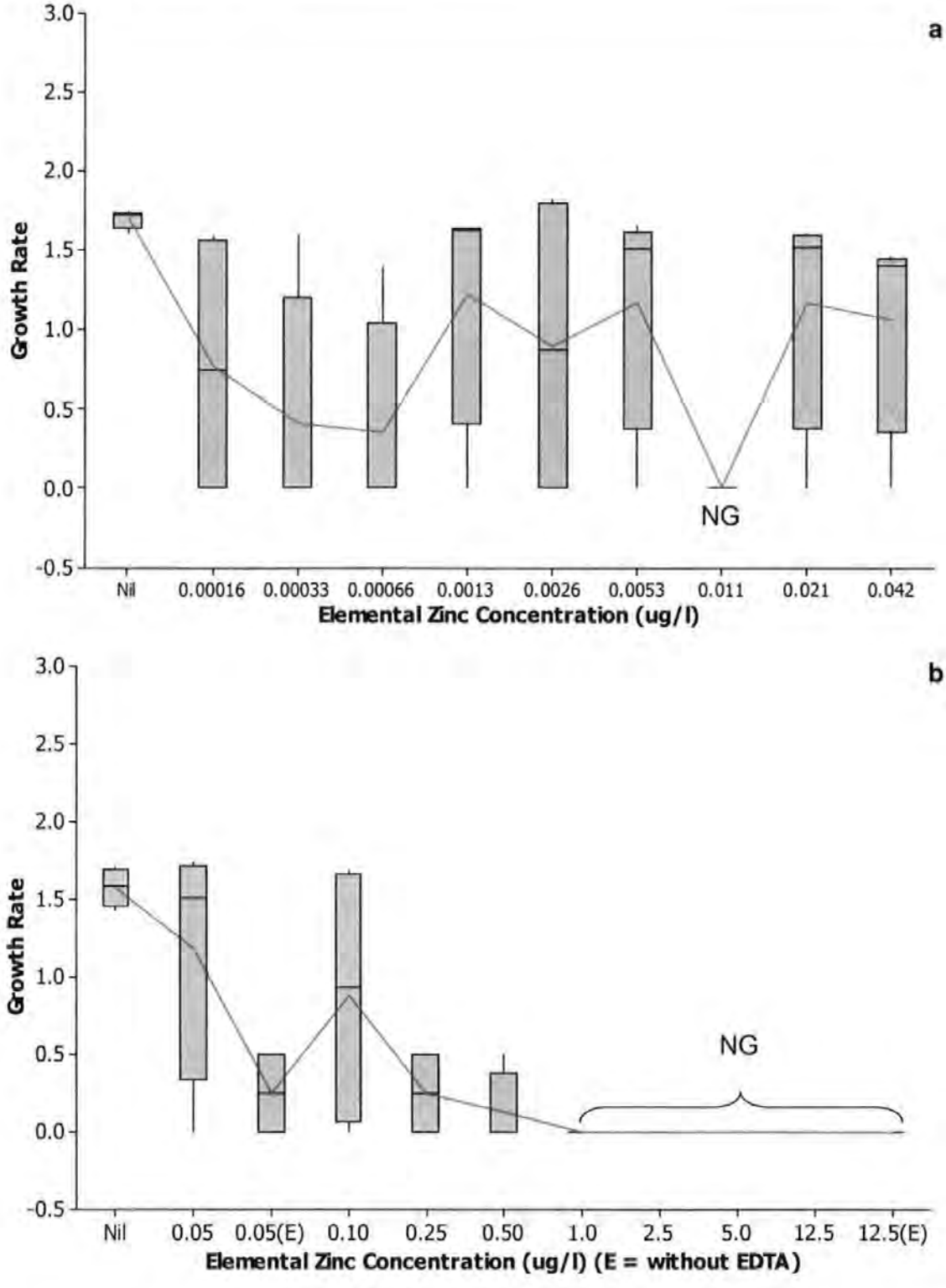
Figures 3.44: a-b

Zinc Regime, Ham Gate Pond G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



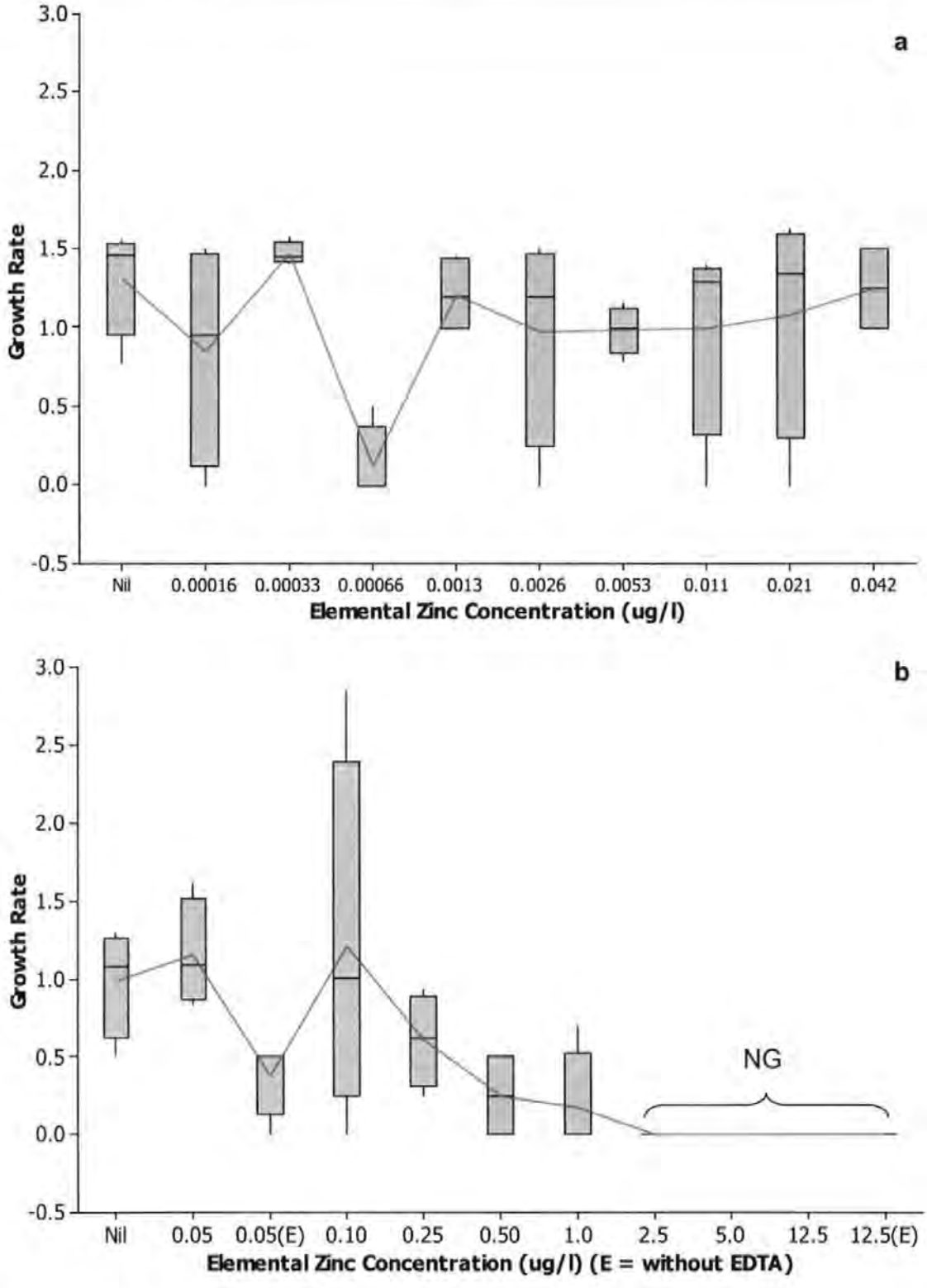
Figures 3.45: a-b

Zinc Regime, Llyn Idwal G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



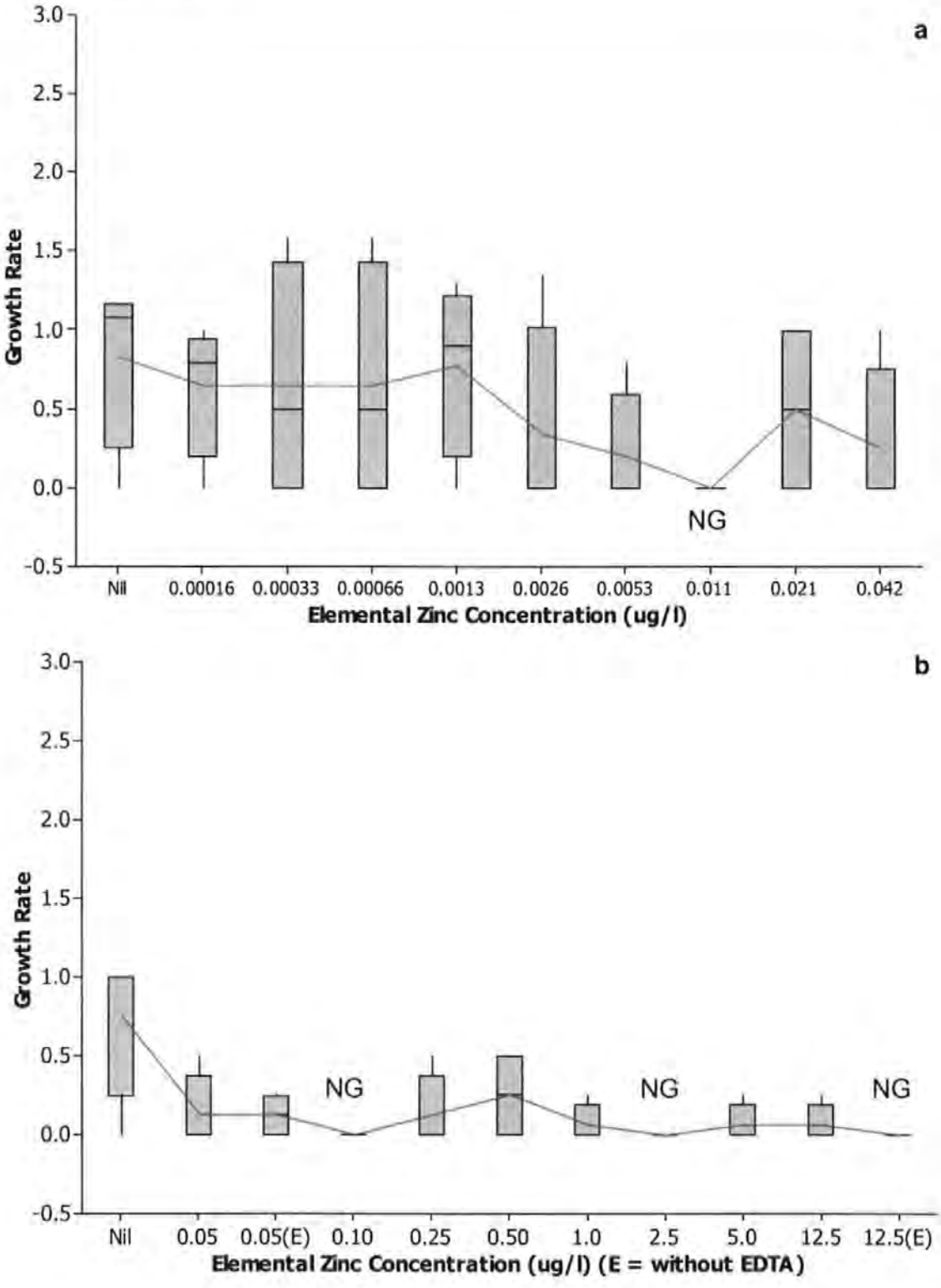
Figures 3.46: a-b

Zinc Regime, River Kennet clone. **a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



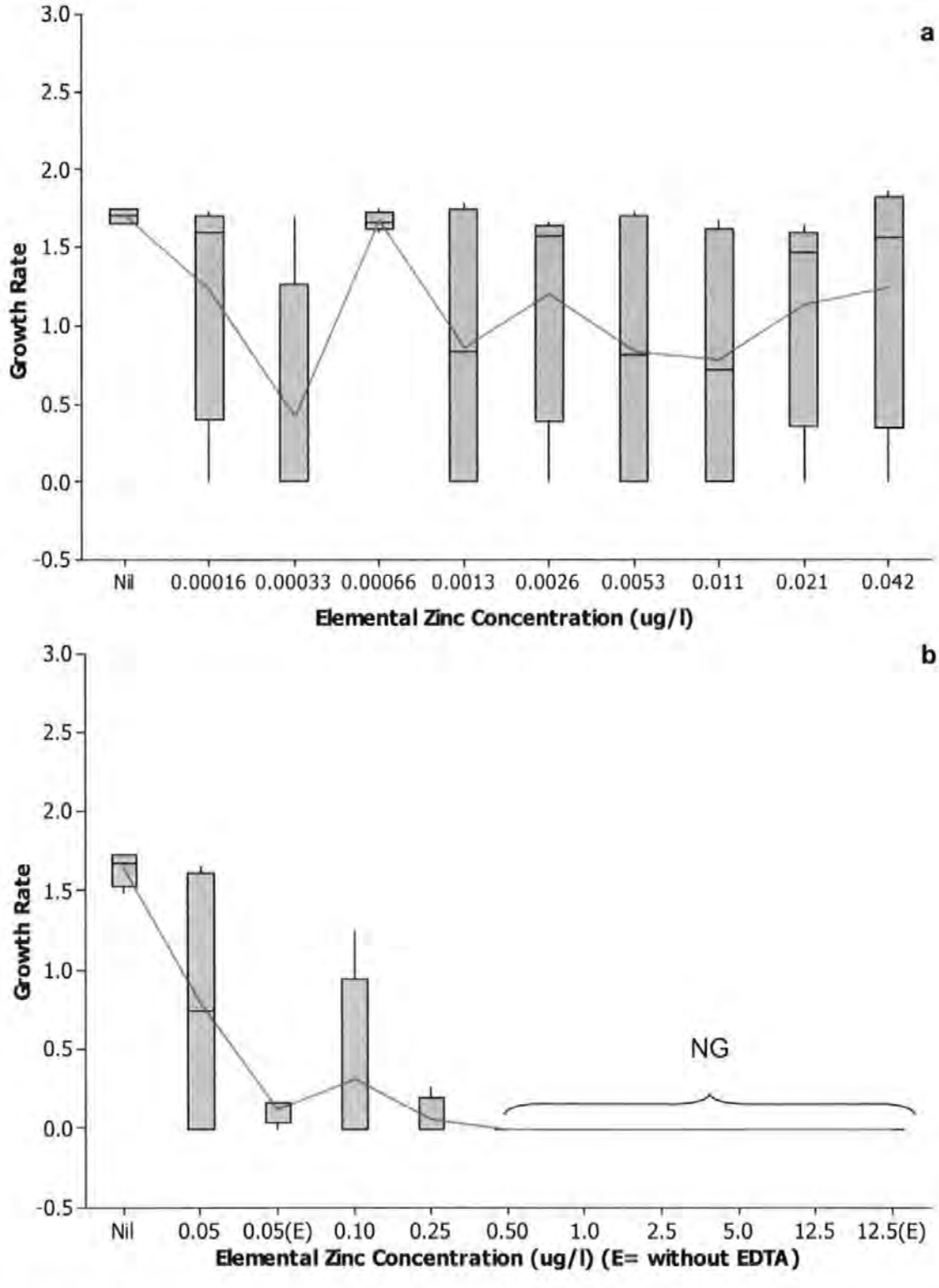
Figures 3.47: a-b

Zinc Regime, Pen-y-Bryn clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



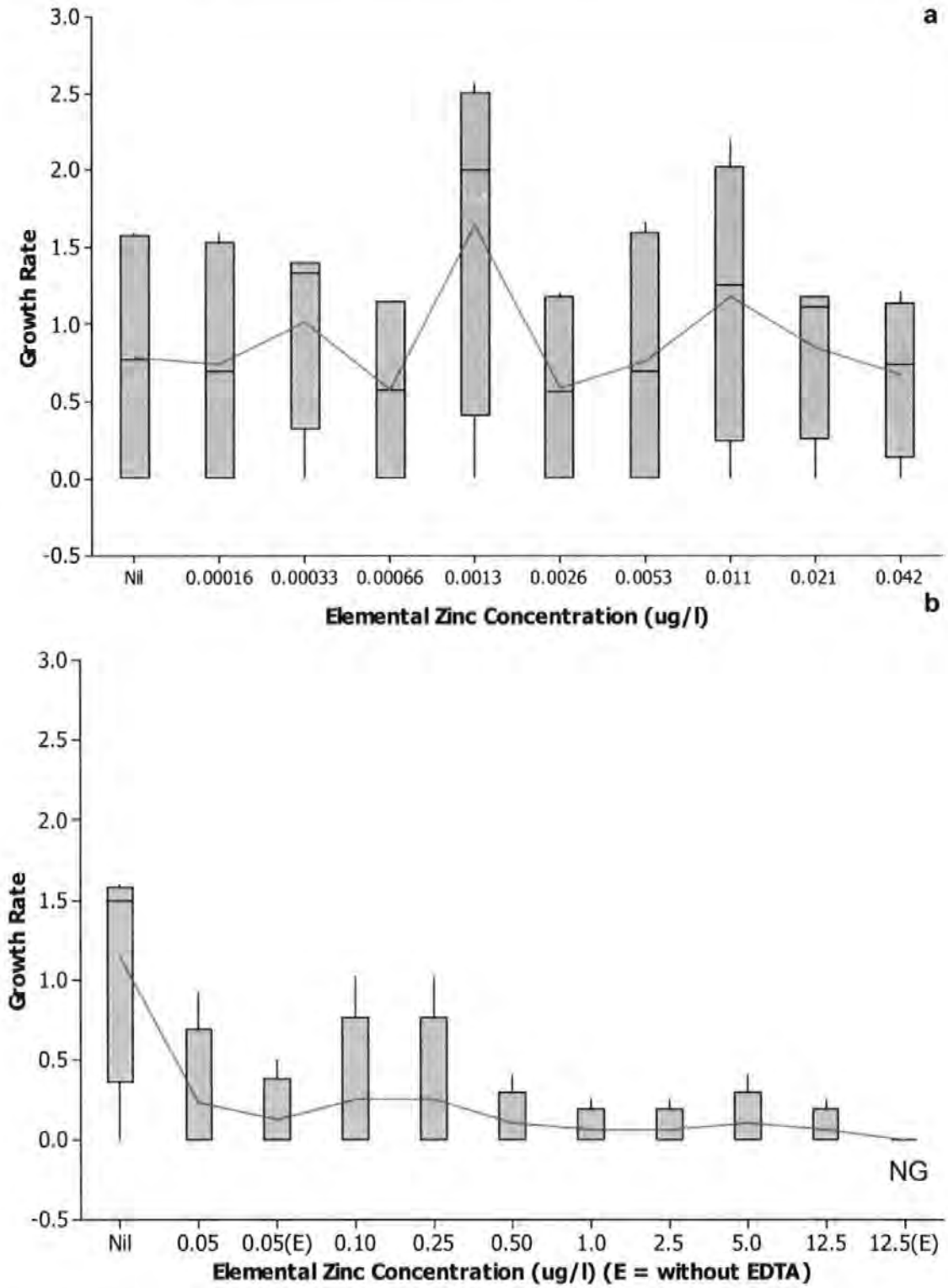
Figures 3.48: a-b

Zinc Regime, Lake Ogwen clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



Figures 3.49: a-b

Zinc Regime, Parys Mountain G_1 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.



Figures 3.50: a-b

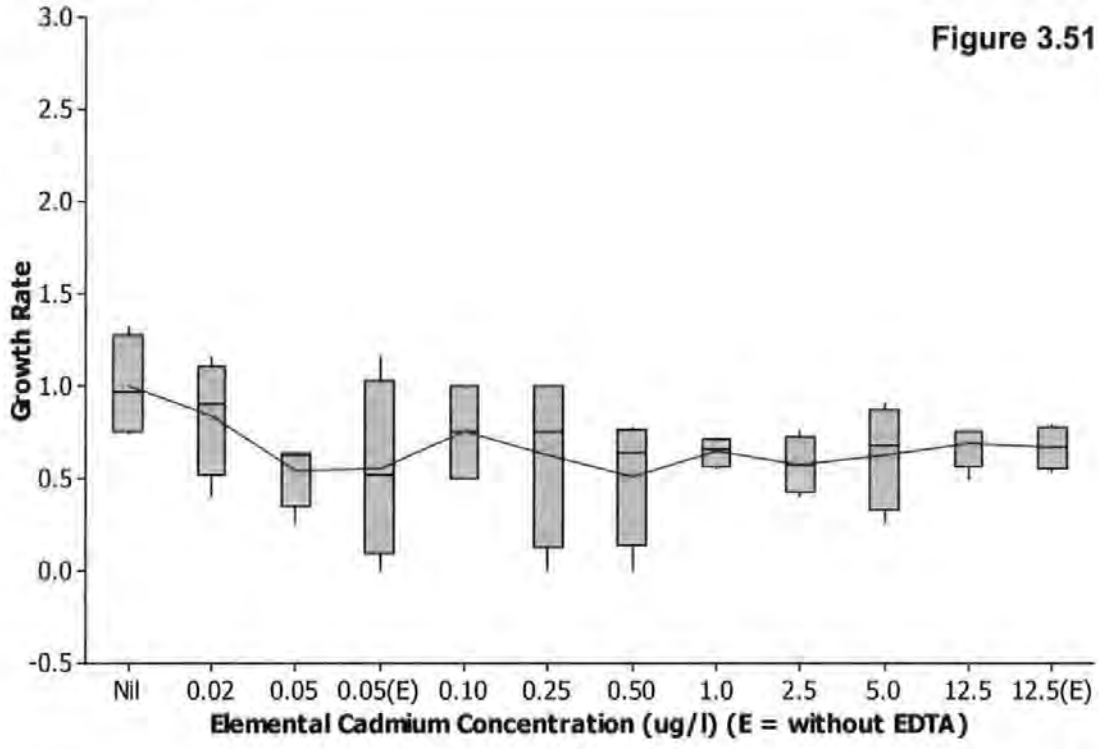
Zinc Regime, Parys Mountain G_2 clone. **(a)** initial run **(b)** extended run. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group. **NG** = No Growth.

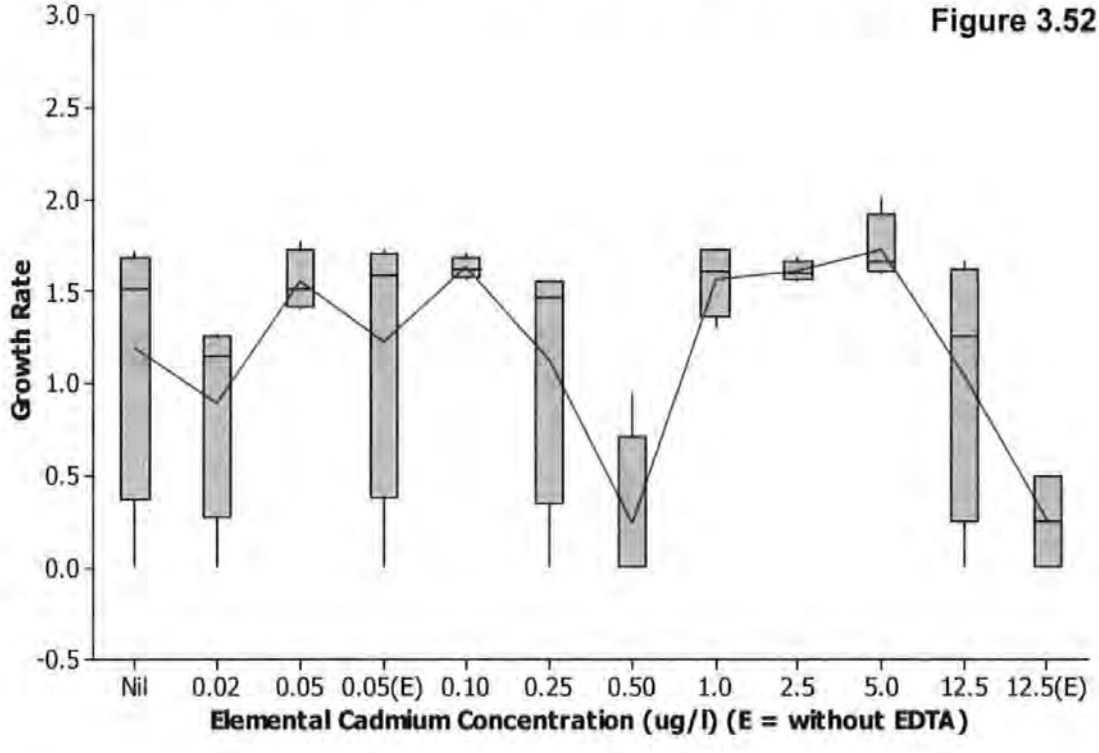
3.3.6 Cadmium Regime

Figures 3.52 to 3.58. All clones were able to grow across the full range of cadmium concentrations. It was difficult to interpret a clear pattern in MEGR with increasing cadmium concentration for any clone, as MEGRs rose and fell randomly. Nearly all clones however, exhibited their lowest MEGR at cadmium concentrations of 12.5µg/l with or without EDTA, with the exception of clones from Scion Pond and River Kennet in which the lowest MEGR was at a cadmium concentration of 0.5 and 0.02µg/l respectively.

Nearly all clones exhibited a higher MEGR in media containing EDTA compared to that without, with the exception of clones from Scion Pond and Pen-y-Bryn, which showed an increased MEGR in the absence of EDTA at cadmium concentrations of 0.05 and 12.5µg/l. None of these differences was significant at the 95% probability level.

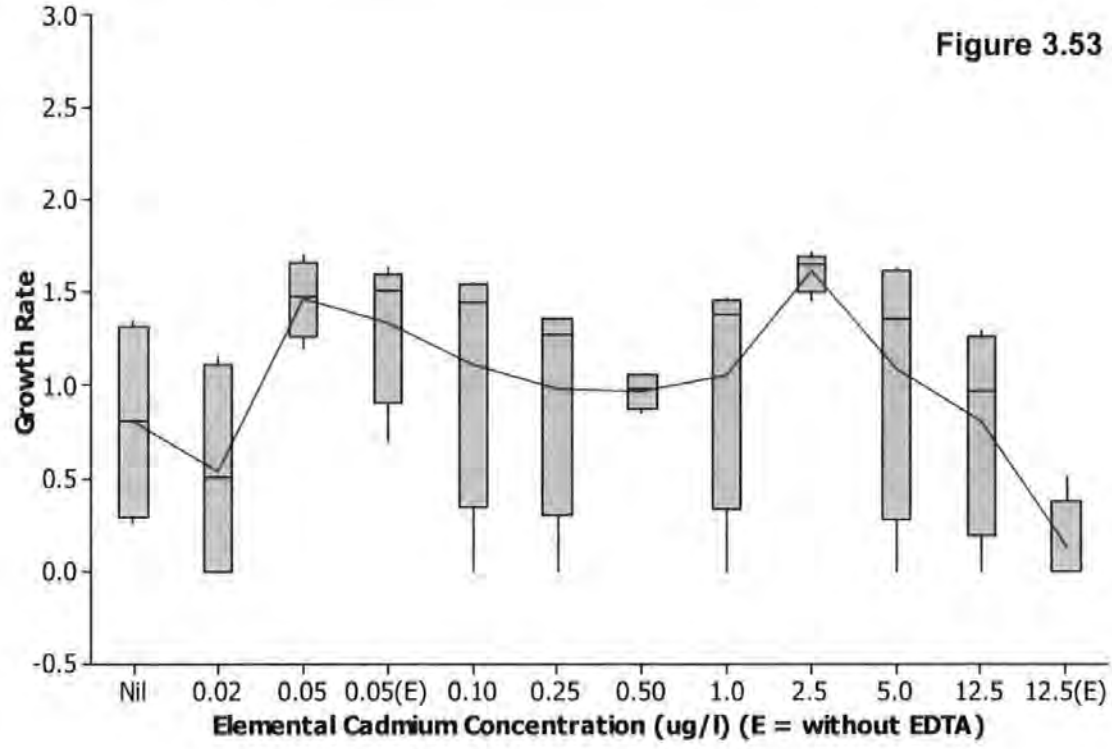
Pairwise comparisons did not indicate any significant differences between the control (Nil cadmium) MEGR and treatment MEGRs. Therefore, all clones appear to be broadly tolerant of high cadmium environments.

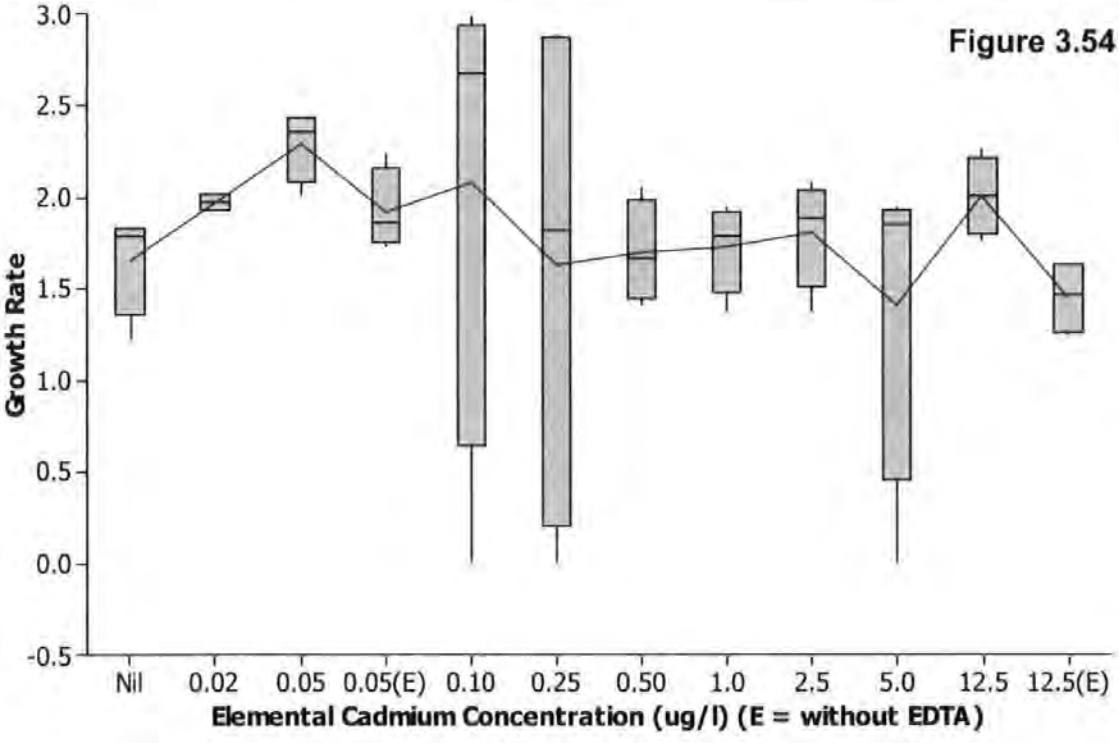




Figures 3.51 and 3.52

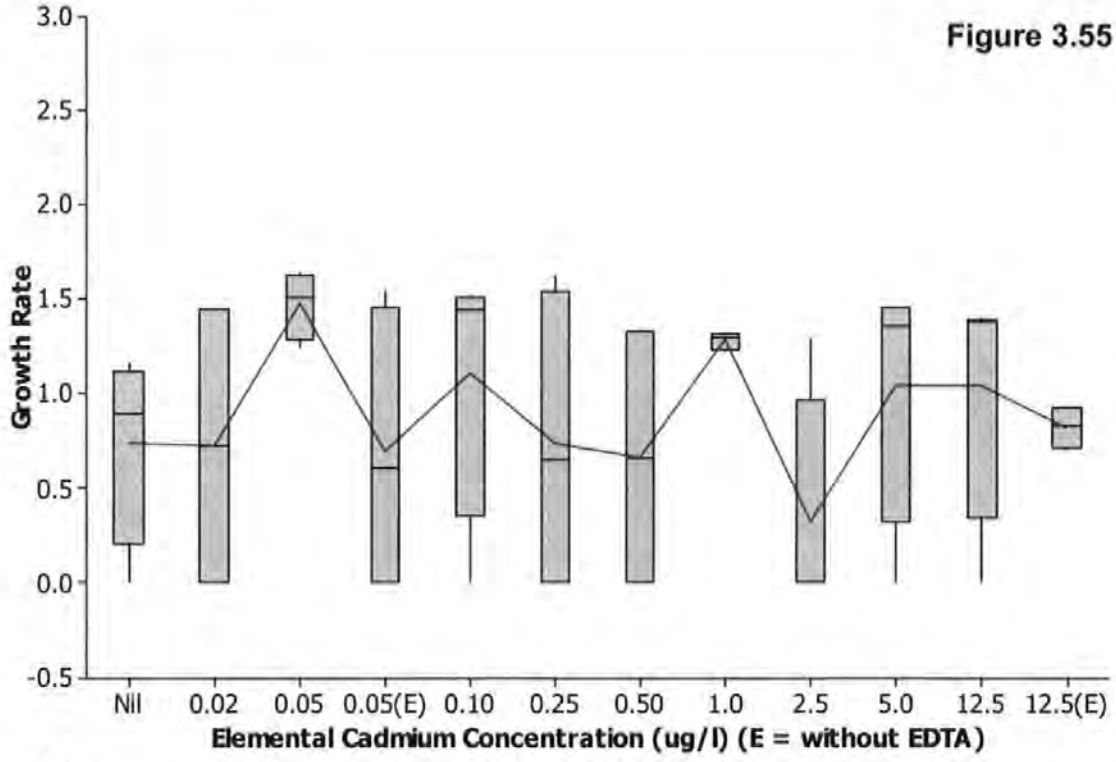
Cadmium Regime, Scion Pond and Ham Gate Pond (G_2) clones respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.

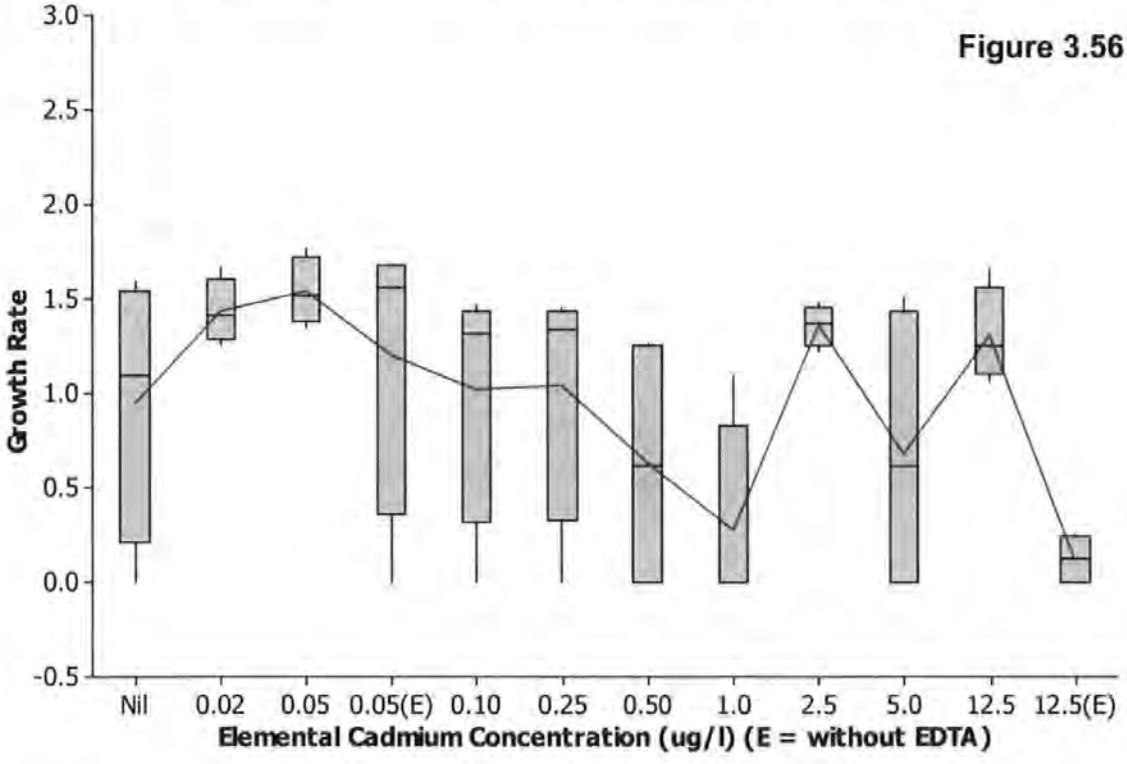




Figures 3.53 and 3.54

Cadmium Regime, Llyn Idwal (G_2) and River Kennet clones respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.





Figures 3.55 and 3.56

Cadmium Regime, Pen-y-Bryn and Parys Mountain (G_1) clones respectively. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.

Figure 3.57

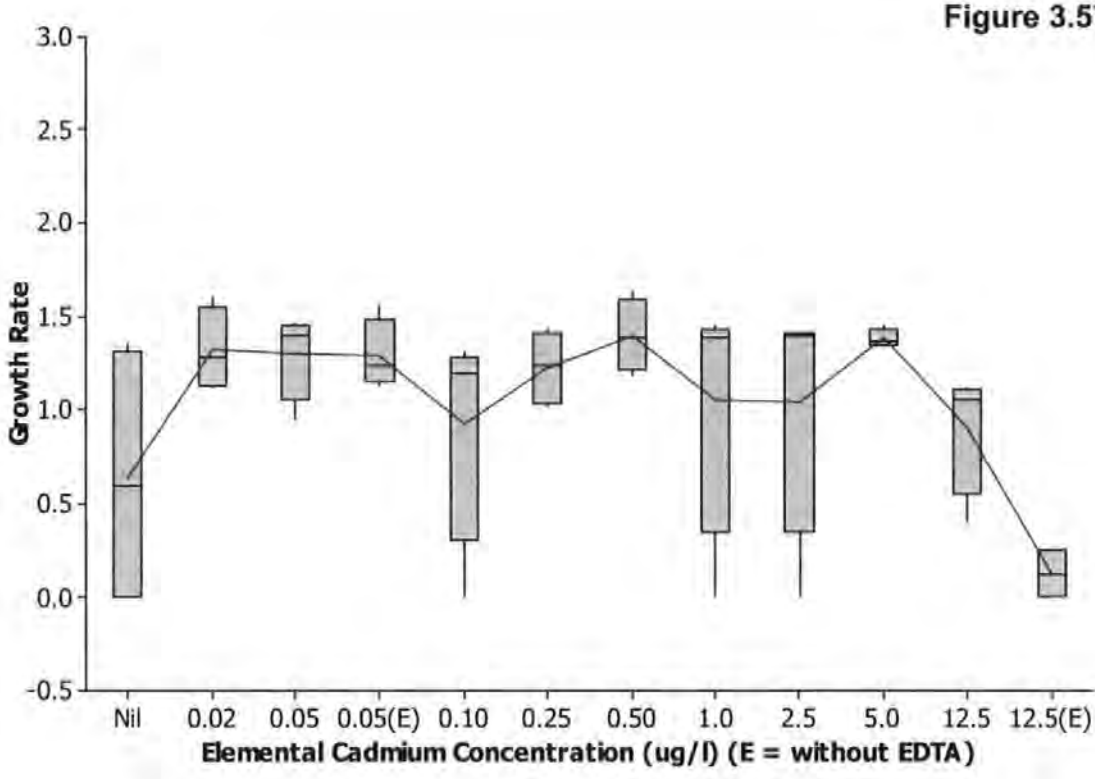


Figure 3.57

Cadmium Regime, Parys Mountain, G_2 clone. Boxplots of mean exponential growth rates (K_e = $\log_{10}$ unit per day) showing variance in replicate growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line) for each treatment group.

3.4 CLONAL PERFORMANCE

Figures 3.59 to 3.64, show MEGRs for each clone under each regime (regardless of treatment), as boxplots. Taking no account of treatment level and comparing only the overall MEGR per regime, it is clear that some clones had overall higher and wider ranging MEGRs, e.g. the River Kennet clone had both the highest MEGR and the widest MEGR range in three regimes (sewage, copper and cadmium), whilst the Lake Ogwen clone was the least productive, having the lowest MEGR in the sewage, copper and zinc regimes. The growth range and optimum for each clone, under each experimental regime, are summarised in **Table 3.3**.

Figure 3.58

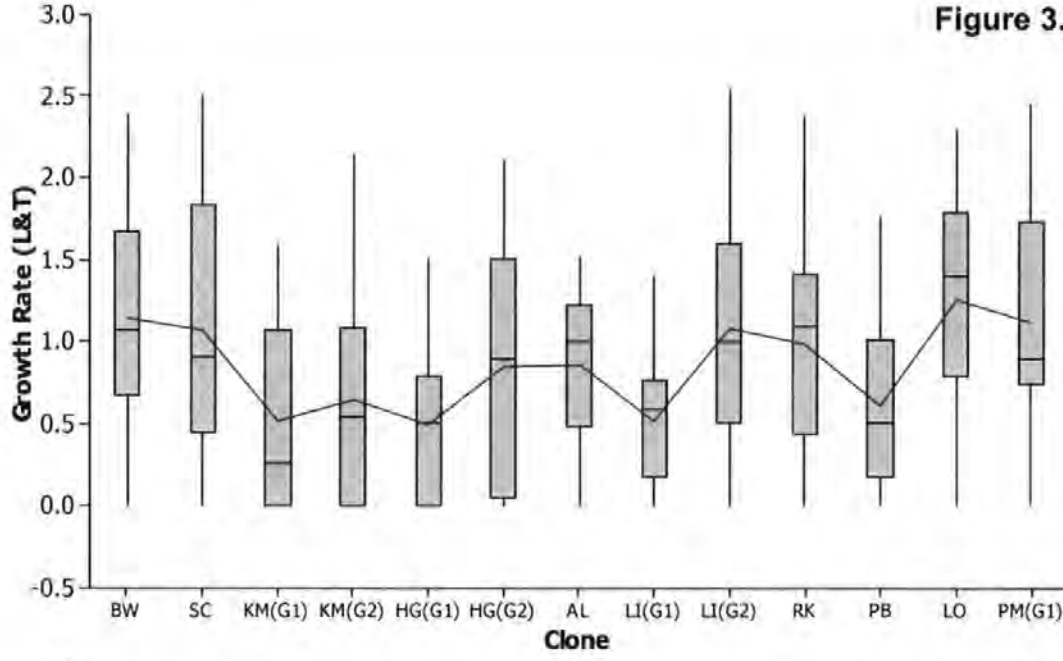


Figure 3.59

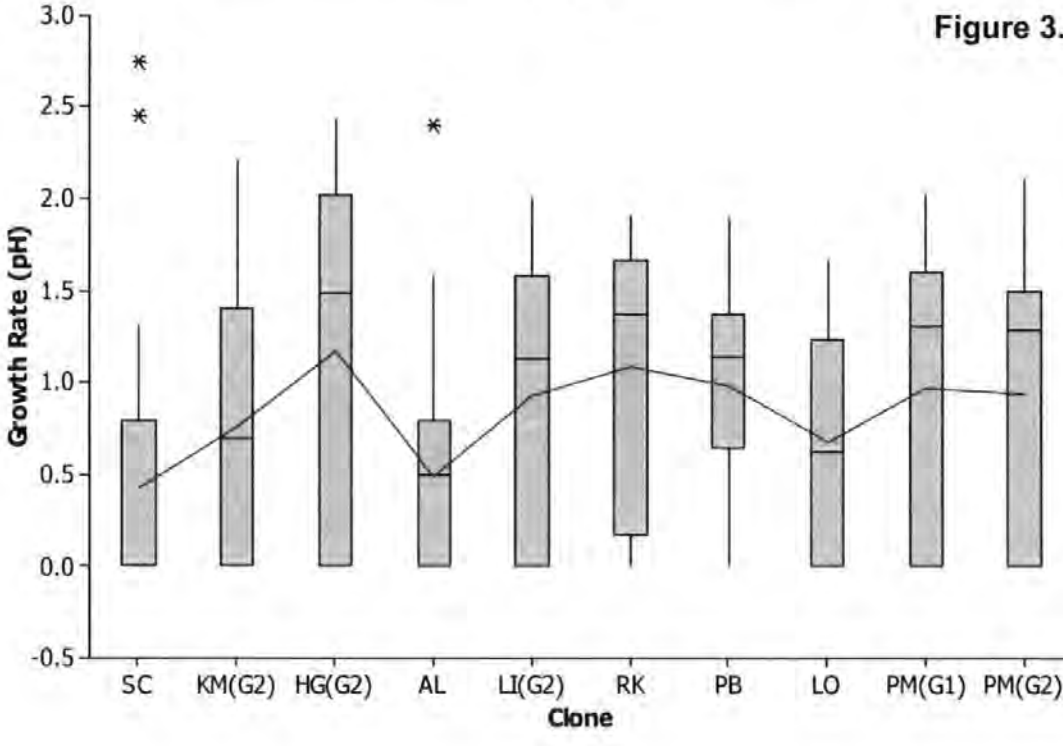


Figure 3.58 and 3.59

Boxplots of overall mean growth rates (K_e = log10 unit per day) for each clone assayed in the L&T and pH regime respectively, showing variance growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line). Stars (*) = outliers. **SC** = Scion Pond, **KM** = Kings Mere, **HG** = Ham Gate Pond, **AL** = Abbey Lakes Pond, **LI** = Llyn Idwal, **RK** = River Kennet, **PB** = Pen-y-Bryn, **LO** =Lake Ogwen, **PM** = Parys Mountain; **G1** = G_1 clone, **G2** = G_2 clone.

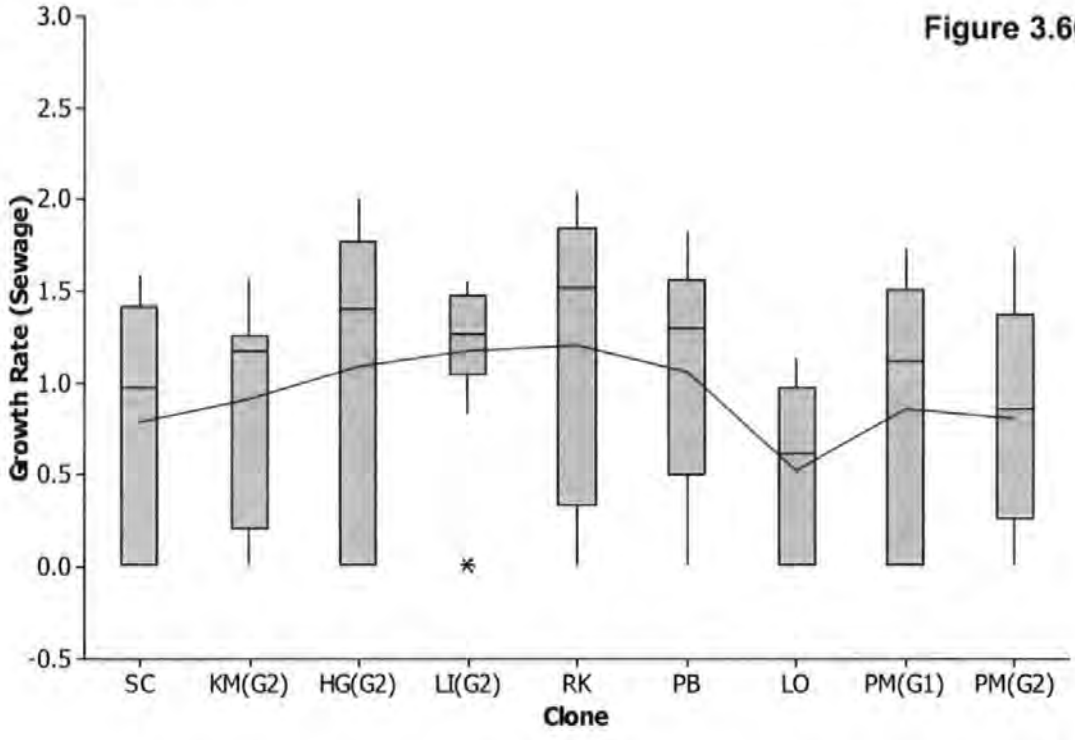


Figure 3.60

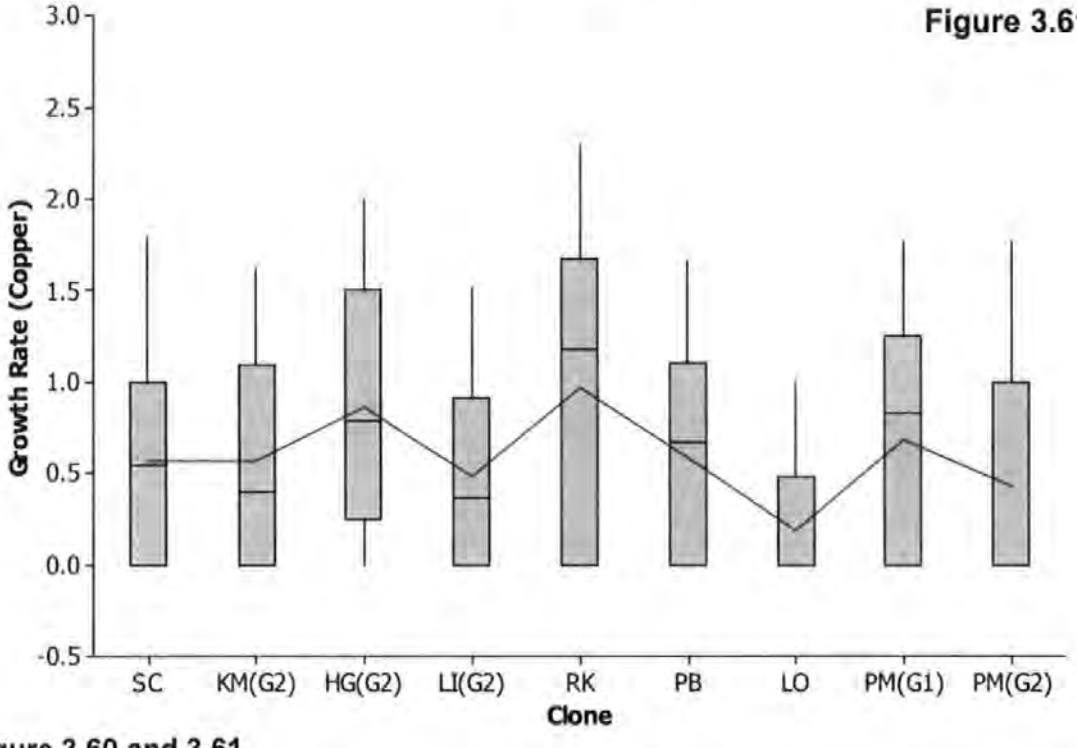
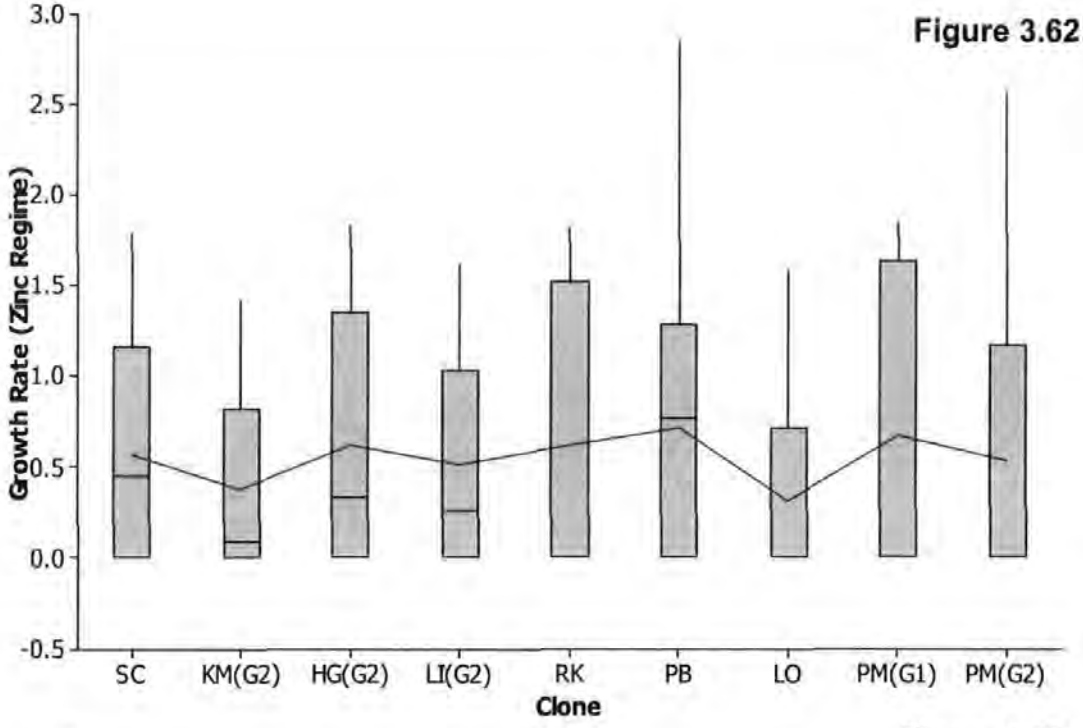


Figure 3.61

Figure 3.60 and 3.61

Boxplots of overall mean growth rates (K_e = log10 unit per day) for each clone assayed in the sewage and copper regimes respectively, showing variance growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line). Stars (*) = outliers. **SC** = Scion Pond, **KM** = Kings Mere, **HG** = Ham Gate Pond, **LI** = Llyn Idwal, **RK** = River Kennet, **PB** = Pen-y-Bryn, **LO** =Lake Ogwen, **PM** = Parys Mountain; **G1** = G_1 clone, **G2** = G_2 clone.



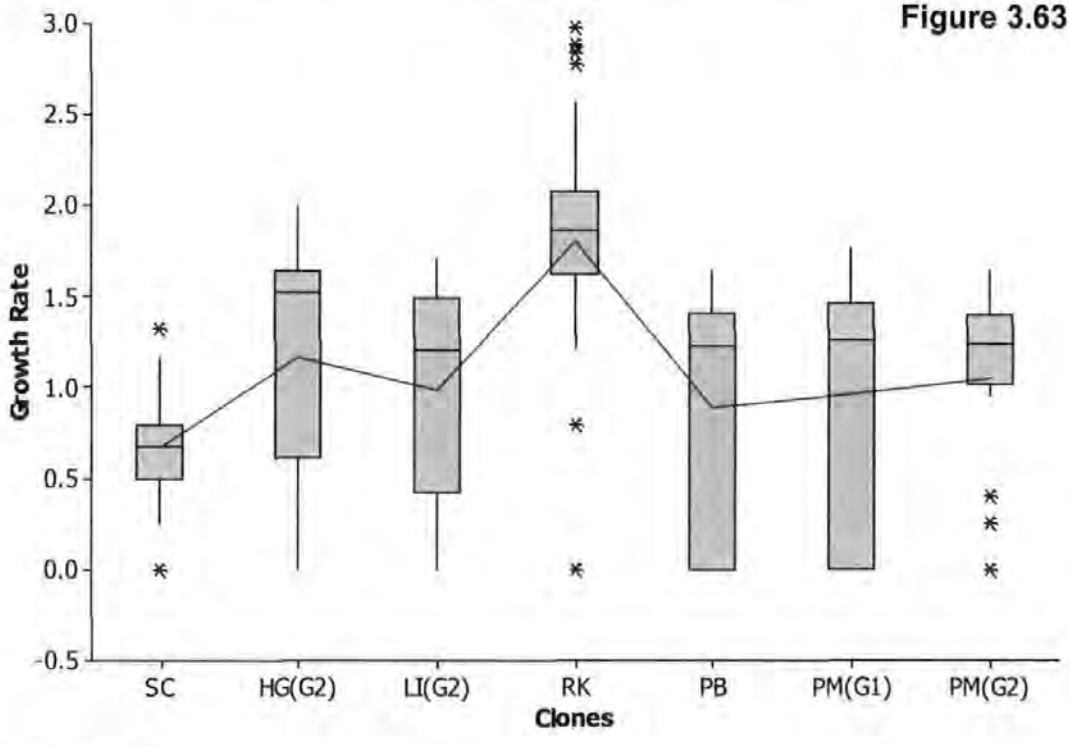


Figure 3.62 and 3.63

Boxplots of overall mean growth rates (K_e = log10 unit per day) for each clone assayed in the zinc and cadmium regimes respectively, showing variance growth rate (including the minimum, maximum and interquartile range), the median and the means (connected line). Stars (*) = outliers. **SC** = Scion Pond, **KM** = Kings Mere, **HG** = Ham Gate Pond, **LI** = Llyn Idwal, **PB** = Pen-y-Bryn, **LO** =Lake Ogwen, **PM** = Parys Mountain; **G1** = G_1 clone, **G2** = G_2 clone.

Table 3.3: part 1

Summary of the tolerance range and optimum for each clone under each experimental regime. **NC** = no culture (culture died prior to experimentation). **H M L** = High, Medium and Low Light. **R** = Range, **O** = Optimum.

Clone		Light and Temperature	pH	Sewage	Copper (µg/l)	Zinc (µg/l)	Cadmium (µg/l)
Barn Wood Pond	R	5-30°C, HML					
	O	25°C, H	NC	NC	NC	NC	NC
Scion Pond	R	5-30°C, HML	pH 3.5-11.0	Nil to full	All conc.	All conc.	All conc.
	O	20-25°C, MH	pH 7.0	1/5th	0.021	0.0026	0.02
Kings Mere (G_1 Clone)	R	5-25 (30)°C, HML					
	O	25°C, L	NC	NC	NC	NC	NC
Kings Mere (G_2 Clone)	R	10-30°C, HL & 5-30°C, M	pH 4.0 - 11.0	Nil to full	Nil to 2.50	Nil to 1.0	NC
	O	30°C, H	pH 6.5-7.0	1/5th	0.00016	0.0053	
Ham Gate Pond (G_1 Clone)	R	5-30°C, HML					
	O	25°C, H	NC	NC	NC	NC	NC
Ham Gate Pond (G_2 Clone)	R	5-30°C, HML	pH 4.0-11.0	Nil to full	Nil to 5.0	All conc.	All conc.
	O	25°C, H	pH 7.0	1/2	0.25	0.00066	0.05
Abbey Lakes River	R	5-30°C, HML	pH 4.5-11.0				
	O	20°C, H & 30°C, M	pH 8.5	NC	NC	NC	NC
Llyn Idwal (G_1 Clone)	R	5-30°C, HML					
	O	30°C, H	NC	NC	NC	NC	NC

Table 3.3: part 2

Summary of the tolerance range and optimum for each clone under each experimental regime. **NC** = no culture (culture died prior to experimentation). **H M L** = High, Medium and Low Light. **R** = Range, **O** = Optimum.

Clone		Light and Temperature	pH	Sewage	Copper (µg/l)	Zinc (µg/l)	Cadmium (µg/l)
Llyn Idwal (G_1 Clone)	R	5-30°C, HML					
	O	30°C, H	NC	NC	NC	NC	NC
Llyn Idwal (G_2 Clone)	R	5-30°C, HML	pH 3.5-11.0	Nil to full strength	Nil to 2.5	Nil to 12.5	All conc.
	O	20°C, M	pH 4.5	1/10th	0.021	0.00066	2.5
River Kennet	R	5-30°C, HML	pH 3.5-11.0	Nil to full strength	Nil to 5.0	Nil to 0.00066	All conc.
	O	20°C, H	pH 9.5	1/5th	0.0053	0.0013	0.05
Pen-y-Bryn	R	5-30°C, HML	pH 3.5-11.0	1/10th to full Strength	Nil to 1.0	Nil to 1.0	All conc.
	O	25°C, M-H	pH 6.5	1/10th	5.0	0.00033	0.05
Lake Ogwen	R	5-30°C, HML	pH 4.0-11.0	Nil to full strength	Nil & 0.05	Nil to 12.5	NC
	O	5 & 20°C, M	pH 7.5	1/10th	0.00033 to 0.00066	0.0013	
Parys Mountain (G_1 Clone)	R	5-30°C, HML	pH 4.0-10.5	Nil to full strength	Nil to 1.0	Nil to 0.25	All conc.
	O	20°C, M	pH 9.0	1/10th	0.00016	0.00066	0.05
Parys Mountain (G_2 Clone)	R		pH 4.0-10.5	Nil to full strength	Nil to 0.25	Nil to 12.5	All conc.
	O	NC	pH 6.0	1/2	0.021	0.0013	5.0

3.5 DISCUSSION

This study looked at seven different factors that are thought not only to affect growth, but also morphology in both micro- and macro-algae. The first two of those factors are light and temperature (L&T). Light and temperature are intricately bound together and therefore must be considered both in unison as well as apart. One expects that at lower light intensities, temperature will be lower (unless heated by another source, e.g. hydrothermal) and there will be less photosynthesis and therefore less growth, as found in a number of investigations for other algae (Fawley, 1984; Foy, 1983 and Meeson & Sweeney, 1982). This can be explained by the fact that light increases temperature, especially in shallow waters, whilst heat causes DNA damage. However, slight rises in temperature can also increase enzyme activity. This study shows that for the *G. parvulum* clones tested, light-limited growth is more susceptible to the effects of temperature change than non light-limited.

One of the main effects of high temperature is the inhibition of enzymes, and the reactions they mediate, however the actual temperature experienced by any single cell is dependant on the conductive and convective properties of the water, which vary both temporally and spatially. As all experimental regimes were carried out in multi-welled dishes, which one could argue are equivalent to small natural pools of water, the influence of irradiance level may have produced higher temperatures than those set by the light-temperature regime, due to the smaller spatial scales involved. Additionally, although cultures were maintained in various different media, the day: night regimes and containers were the same.

The laboratory can never hope to duplicate natural conditions exactly, but it can provide invaluable insights and approximations of tolerance and ecological limits, essential knowledge if diatoms are to be used as bio-indicators. In this study, any potential abiotic variation in the laboratory was kept to a minimum by standardisation of methods. The inherent variation within each clone was minimised by replication. If the temperatures in the L&T regime however, were high enough to have elicited protein denature one might expect a reduction in photosynthetic capacity at higher temperatures and therefore a reduction in growth rate, however that was not always the case. Certainly, a number of clones exhibited a drop in growth rate at 30°C, but this was not always as low as at 5 or 10°C and did not kill the culture nor stop it replicating. It would suggest therefore that whilst higher temperature had a slight inhibitory effect on growth rate, there is no evidence of damage to replication mechanisms.

Living in water and sediments, benthic diatoms are subject to dynamic light regimes and in disturbed areas would be widely tolerant of different light levels. As a biraphid diatom, *G. parvulum* can regulate (to an extent) the amount of light it receives (its phototaxic response), by moving or migrating through the sediment. The light benthic diatoms receive is therefore attenuated by, e.g. terrestrial and aquatic vegetation, water turbidity and the benthic algal community. All the clones used in this study were benthic in origin and many were from disturbed sites, though the type of substratum differed with habitat and sampling site. One would therefore expect *G. parvulum* to be broadly tolerant of differing light regimes and take advantage of optimal conditions. Certainly, for most of the clones in this study, light had less of an effect on growth rate than temperature, but only marginally so.

Interestingly, several studies have shown that diatoms and other algae are able to grow heterotrophically/survive in the dark or in low light levels (Admiraal & Peletier, 1979; Lewin & Lewin, 1960; Peters 1996; Peters & Thomas 1996; Smiley & Darley, 1972). Admiraal & Peletier (1979) suggest that this may be down to a capacity to use one or more organic substrates for growth in the dark, and therefore possibly a metabolism intermediate between exclusive autotrophy and heterotrophy. Such versatility gives great advantage to such diatoms. Whilst this study does not suggest that *G. parvulum* is able to grow in the dark, it is clearly able to compensate for low light levels and more recently, Gould (1994) as cited in Peters & Thomas (1996) has suggested that there may be a genetic basis to dark survival. Little work has been carried out on the physiological implications of dark survival, which diatom taxa are capable of dark survival, or the effect on growth rate recovery. The ecological implications of surviving in low light regimes are clear; benthic diatoms are routinely subject to turbulence either by the nature of the water body or from grazing animals, one consequence of which is a reduction in received light. Additionally, many diatoms form biofilms either with each other or with other algae and competition for space and nutrients in addition to light, can mean that those taxa more able to survive at lower light levels have a greater advantage. *Gomphonema parvulum* however, compensates by being motile and able to form stalks to elevate itself in the water column.

All clones of *G. parvulum* tested were able to grow under all L&T regimes and most had their optimum between 20 and 25°C, medium to high light levels, except clones from Kings Mere (G_1) (25°C, low light), Kings Mere (G_2) and Llyn Idwal (G_1) (both 30°C high light). There were marked differences between G_1 and G_2 cells for Kings Mere and Llyn Idwal Clones, but not for clones from Ham Gate Pond. The cell size difference is large. The G_2 cells are 2-3 times larger than the G_1 cells. Banse (1982), in studies of diatoms

and dinophytes, has shown that for cells of a given class, e.g. Bacillariophyceae or Dinophyceae, maximum specific growth rate decreased with increasing cell size. Admiraal's (1977) study also shows this phenomenon, but only at optimal temperatures; at below optimum temperature there was little difference in growth rate. A general presumption is that smaller cells have higher metabolic rates with faster uptake of nutrients, and due to the smaller surface area: volume ratio may divide more frequently and thus have higher growth rates. There is no evidence in this study that smaller (or conversely larger) cells are more or less sensitive to L&T. However, if a coefficient of variation (CV = standard deviation/mean x 100%) for each clone's dataset is computed, it becomes clear that in all cases the G_1 cells have a larger variability in growth rate than the G_2 cells. Additionally, in all cases, within clone growth rate varied considerably more than between clone growth rate.

Acid tolerant forms are well documented in the diatom literature (Hustedt, 1937-1939; Moss, 1973 and Patrick & Reimer, 1966). However, there is little in the literature on the specific pH optimum or tolerance of *G. parvulum*. From this study, it is clear that different clones within the same complex exhibit slightly different preferences, but are generally tolerant of extremes of temperature, light, pH, eutrophication and metal concentrations.

There were marked differences in optima between the G_1 and G_2 cells for the Parys Mountain clone, the former growing better under alkaline conditions and the latter under slightly acidic conditions. This suggests that different stages in the life cycle have different tolerances, which leaves us with a problem. How does the diatom cell at either end of the size continuum change is habitat to suit a particular stage in its life cycle? Does it need to change habitat, or is the physiology of the cell sufficiently robust to tolerate any pH

regardless of life cycle stage? The fact that both the G_1 and the G_2 clones are able to grow at pH 4.0 through to pH 10.5, suggests that clonal physiology is sufficiently robust. However, it is hypothetically possible (though has not been tested) for benthic and other attached diatoms to "detach" themselves from one undesirable habitat and float freely either in the water column or with water currents, and float down stream to a more suitable habitat. This may seem somewhat random, but plenty of land plants exhibit the same "behaviour" with wind/animal borne pollen and seeds. Sufficient cells make it to a more desirable habitat to ensure survival of the population. That the smaller G_1 cells in this study should prefer conditions that are more alkaline, does not however agree with published data that suggest smaller cells are generally found in conditions that are more acid. The CVs for each clone show that growth rate for all clones in the pH regime was highly variable, though unlike the L&T regime there was less of a difference between G_1 and G_2 cells for the Parys Mountain clone. These data therefore agree with published data, in that *G. parvulum* appears to be generally pH indifferent.

In diatoms, the study of organic pollutant uptake generally focuses on the role of elemental phosphorus, nitrogen and carbon or their compounds, e.g. NH_4, NH_3, PO_4. *Gomphonema parvulum* is generally considered broadly tolerant of high organic pollution as shown in Palmer's (1969) composite rating. *Gomphonema parvulum* is also found in organically enriched waters the world over as one of the most pollution tolerant diatoms (Dakshini & Soni, 1982; Kelly & Wilson, 2004; Lange-Bertalot, 1979; Lobo *et al.*, 1996 and van Dam, 1982).

Phosphate uptake and metabolism has been shown to be affected by pH in some studies with other algae (Healey, 1979). Boström *et al.*, (1988) have shown that assay

procedures in the laboratory may be unfavourable for certain P-mobilisation processes and that the bioavailability of phosphorus will vary according to other biochemical and physical conditions, such as pH and temperature (high pH favours P-mobilisation). The most important form of phosphorus for diatoms is dissolved inorganic phosphorus, e.g. orthophosphate. In this study however, nutrients were present in sufficient quantities over the short assay period, as the medium was regularly topped up, so it is unlikely that nutrient deficiency at low pH would reduce growth. In the sewage regime however, phosphate compounds are considerably higher, mimicking moderate to high organic pollution, and *G. parvulum* clones generally grew less well at full-strength sewage than at lower concentrations. These results agree with Admiraal's (1977) study, which show that nitrate and orthophosphate are either not at all, or only slightly, inhibitory to benthic diatoms at concentrations of 0.9 mg/l to 16.9 mg/l.

All transfer of material and media were conducted under aseptic conditions nonetheless, the sewage regime inevitably became infected by bacteria. In the sewage regime, both algae and bacteria will therefore compete for phosphorus just as they would in nature. Diatoms are known to perform luxury accumulation of phosphorus to enable survival in times of limited availability however, Currie & Kalff (1984a, 1984b) suggest that bacteria unlike diatoms, are not phosphorus limited but carbon-limited. Most of the carbon is supplied by phytoplankton. Bacteria have a higher affinity for phosphorus than algae so there is the possibility that they could starve diatoms of this resource. In a review of literature on bacterial and algal phosphate utilisation and uptake however, Jansson (1988), suggested that the main source of phosphorous for algae (in a mixed population) was organic excretory products from bacteria in phosphorus-rich waters (usually ortho-phosphate) in a feedback mechanism. If this were true for this study, it would account for the ability of *G. parvulum* clones to grow well at all sewage concentrations despite

increasing concentrations of bacteria. Jansson (1988) also suggests that some algae may have the same ability as bacteria, so that when phosphorus concentrations are low, both have a mechanism to ensure continuous access to a limiting nutrient.

Ammonia and nitrate however, have more deleterious effects in both long-term exposure culture experiments and short-term exposure photosynthesis measurements. Nitrate has been shown to retard growth, and ammonia is known to retard growth and inhibit photosynthesis (Admiraal, 1977). Yet *Gomphonema* species are generally associated with higher nitrate (NO_3^-) (and dissolved oxygen) in organically polluted drains (Dakshini & Soni, 1982), and, using clay pots (Fairchild *et al.*, 1985) dosed with specific ions to study the growth responses of algae in a lake, found *Gomphonema* species were nitrate-limited. Whilst this study does not distinguish between different elements making up the artificial sewage, the constituent ingredients suggest high phosphorus and nitrogenous compounds will be present. Certainly ammonia and urea will be broken down into nitrate by the bacteria, however nitrate becomes limiting in the presence of plentiful phosphorus.

In the metals regimes, the pattern of growth rate was more straightforward and largely as expected, i.e. growth rate decreased with increasing metal concentration, although there were slight differences between the clones. In this study, the addition of EDTA as well as Tris buffers, had the potential to detoxify metals by lowering the concentration of free metal-ions. In the experiments presented here, EDTA and Tris buffer were retained as constituents of the metals regime media so that they were comparable to the clonal stock cultures and the L&T, pH and sewage regimes. However, at the lowest and highest metal concentrations, clones were also inoculated into EDTA-free media for comparison, none of which grew well, if at all, suggesting that EDTA does not have a strong inhibitory effect.

Any effect that EDTA and Tris may have however was possibly short-lived. Fábregas *et al.*, (1993) report that Tris buffer has been implicated in increasing bacterial populations in non-axenic algal cultures if sodium phosphate is also present. In this study however, $NaPO_4$ was not used to make the medium, but both Na^+ and PO_4^{2-} were present in solution and consequently may have contributed to the bacterial problem in the sewage regime.

Leland & Carter (1984) and Denisegar *et al.*, (1986) showed that metal adaptation differs between clones of the same species based on history of exposure, e.g. Ivorra *et al.*, (2002) observed clones of *G. parvulum* with a natural history of chronic Zn and Cd exposure were more tolerant to metals than a clone isolated from a markedly less contaminated site (though not to Cu). In short-term studies Medley & Clements (1998) found greater sensitivity of *G. parvulum* to metals compared to other diatom taxa, and Monteiro *et al.*, (1995) show that *G. parvulum* was strongly tolerant to Cu, Zn and Cd (concentration 0.088mg/l, 1.8mg/l and 0.03mg/l respectively). Conversely, Rushforth *et al.*, (1981) and Sabater (2000) assessed *G. parvulum* as metal intolerant. Oliveira's (1985) study, showed that Cu mine effluent enabled the development of a Cu-tolerant group of species, of which *G. parvulum* had the highest population.

Metal tolerance was exhibited to varying degrees by all clones in this study. In particular, the Parys Mountain (G_2) clone in the Zn and Cd regimes, though less so for the Parys Mountain (G_1) clone. If we compare the Parys Mountain clones, (from a known metal contaminated site), with the clone from Llyn Idwal (from an oligotrophic lake), the Parys Mountain clone (both G_1 and G_2 cells) had a wider tolerance and generally higher MEGRs than the Llyn Idwal clone in the Cu regime, though not in the Zn regime, where the Parys

Mountain G_1 clone did less well. There was little difference between the two clones in the Cd regime. This suggests a genetic basis for the difference. On the other hand, Joux-Arab *et al.*, (2000) showed that cell size in the marine diatom *Haslea ostrearia* (Gaillon) Simonsen did not affect metabolic rate in the absence of Cu, although significant differences in Cu sensitivity were found between all three size ranges during cell size reduction, with the largest cells showing the greatest sensitivity, suggesting tolerance changes throughout the life cycle of a cell.

Ivorra *et al.*, (1999) showed that the percentage abundance of *G. parvulum* (among others) increased in polluted sites, compared to reference sites that contained less pollution (in particular Zn) in spring and autumn, but decreased slightly in winter. In Ivorra *et al.'s*, (2002) study on *G. parvulum*, Zn induced tolerance showed significant difference between clones and treatment. In particular, they showed that clones isolated from waters subject to chronic Zn (and Cd) loading, were more tolerant to Zn than clones originating from waters with markedly lower Zn loadings, despite pre-incubation in a metal-free medium. This tolerance was still evident 2 years later, suggesting a genetic basis. Further evidence of *G. parvulum's* zinc tolerance is given in Loez *et al.*, (1995) where *G. parvulum* is one of only a few diatom species still surviving at 25mg/l. This was similar to results obtained by Say & Whitton (1978), Say & Whitton (1981) and Takamura *et al.*, (1989). The literature therefore suggests that *G. parvulum* should be strongly tolerant to zinc, and in this study, this was largely true, though some clones were slightly less tolerant than others, and the Parys Mountain G_1 clone was more sensitive than the G_2 clone, or any other clone.

Like many heavy metals, cadmium is also known to have inhibitory effects at low concentrations. Rushforth *et al.*, (1981) found that *G. parvulum* was one of the diatom species occurring at high concentrations of Cd for at least three seasons in the Uintah Basin, USA. The Cd range was 0.1-5.7 mg/l, much higher than in this study, suggesting that *G. parvulum* is Cd tolerant. In fact, *G. parvulum* in the Uintah study showed a consistent preference for high metal concentration, especially Cd and Al. In this study, Cd had little effect on growth rate, generally in line with Rushforth *et al.'s*, (1981) study.

3.6 CONCLUSIONS

Although *G. parvulum* is recorded as a common taxon and abundant in polluted sites, this study has shown that this is not always the case. *Gomphonema parvulum* can be difficult to find and does not occur in great numbers. In over 100 samples collected, *G. parvulum* was only isolated from 10.

Results show that different clones of the same species have similar broad tolerances to several physico-chemical measures, regardless of genetic and environmental exposure history, though they vary in their optima. This supports the reported adaptability of *G. parvulum*. *Gomphonema parvulum* is able to grow and reproduce in a wide spectrum of habitats and is especially tolerant of pH and heavy metals. However, this study also highlights that it may not be as tolerant of organic pollution as has been reported, growing better in standard MBL than in media laced with artificial sewage. From this study, it is clear that different clones exhibit different preferences and, and although responses overall were very similar, there are indications of a genetic basis for the encountered differences.

4 MORPHOLOGY

4.1 INTRODUCTION

The inherent morphological variability of *G. parvulum* suggests that a number of taxa constitute the species complex. This seems at odds with its use as an indicator of organic pollution in diatom indices of water quality. The separation of either ecotypes or morphotypes however, may rely on subtle differences. A case in point is the morphologically variable, freshwater diatom *Sellaphora pupula* (Kützing) Mereschowsky. Mann (1989a, 1999) and Mann *et al.*, (2004) have established that slight differences in valve shape, size and striation pattern and density are markers for non-interbreeding populations. Although the differences are subtle, these morphological characters have been demonstrated as stable in culture, and are supported by descriptive and diagrammatic references and quantitative analyses. The fact that these morphotypes remain distinct even within the same water body, suggests that they represent separate lineages that are reproductively isolated (Mann & Droop, 1996). The same may be true for *G. parvulum*. It may be that different morphotypes are separated by habitat, ecological niche or season, and therefore no gene flow occurs, or that morphologies are induced by environmental conditions. As Mann & Droop (1996) point out however, if a sexual organism shows discontinuity in its pattern of morphological variation between populations that live together, the biological species concept may serve as a test of significance where molecular analysis is not feasible.

Variability in shape, stria density and size, and the lack of intermediate morphologies, make *G. parvulum* a highly heterogeneous taxon. Ideally, a good biological indicator has the wide distribution of *G. parvulum*, however it should also have a well-defined

ecological range and be easily and reliably identifiable (Geissler, 1982). That does not appear to be the case for this taxon insofar as many morphologies make up the species, few varieties are attributable to defined environmental conditions, and it can be confused with other *Gomphonema* spp. In particular, it is very similar to *G. angustatum*, which has somewhat coarser and more radiate striae. However, these are matters of degree, and there are no standard measures of "radiate" or "coarseness" for striae. How certain are we that, given is similarity to *G. parvulum*, *G. angustatum* is a separate species and not simply a morphological variety linked to a particular environment? What about other similar taxa? Under what conditions does morphology change?

The rare occurrence of auxospores and initial cells in samples or cultures can also confuse the taxonomy of a taxon unless a direct link is made to a known species. Morphology disparate from the *sensu stricto* however, would cause such a taxon to be identified as either a separate species or a new taxon. The usefulness of diatom cell wall morphology as the sole criterion on which to classify diatoms is therefore questioned.

Experimental work however, can help clarify some of these issues, and several authors (Cox, 1995; Fisher *et al.*, 1981; Geissler, 1970a, 1970b, 1982; Gensemer, 1990; Jahn, 1986; Schultz,1971) have shown that changes in environmental factors can modify diatom valve morphology or induce sexual reproduction, though little work has been done on freshwater pennates. In some cases, these modifications have been shown to be characteristic of described taxa (Holmes & Reimann, 1966; Schultz, 1971), bringing into question the taxonomy of those species in addition to the taxon under evaluation.

The purpose of the analyses in this chapter, apart from exploring the variation in *G. parvulum* morphology, is to establish a baseline data set that can be used for future morphometric comparisons of *G. parvulum* and one that can, alongside ecophysiological data and observations on live material, inform the separation of taxa from the complex. This study aims to determine the effects of particular environmental conditions (pH, sewage, copper, zinc and cadmium) on the morphology of several isolated clones of *G. parvulum*, testing whether morphological variability is a response to changes in these conditions, and assess whether clones can be differentiated into ecomorphs or ecotypes.

4.2 CLONAL IDENTITY

Initial inspection of clonal material provided clonal identity to species. Clones were then assigned to variety or form where possible **(Table 4.1)**.

LM examples of each clone at the start of subculture are provided in **Figures 4.1 to 4.5**. Krammer and Lange-Bertalot's, Süßwasserflora von Mitteleuropa (1986-1991) is one of the main identification flora's used to identify European freshwater diatoms, therefore beneath each set of figures, a comparison with equivalent taxa in the Süßwasserflora von Mitteleuropa is made, to illustrate if these clones agree with Krammer and Lange-Bertalot's descriptions and micrographs.

Table 4.1

Taxonomic identity of each of the ten clones under study.

Clone	Taxonomic identity of clone	Taxonomic Authority
Barn Wood Pond	*G. parvulum Kützing* var. *parvulum* fo. *parvulum*	Kützing
Pen-y-Bryn,	*G. parvulum* var. *exilissimum* (morph #1)	Grunow
Parys Mountain	*G. parvulum* var. *exilissimum* (morph #2)	Grunow
Kings Mere Ham Gate Pond Lyn Idwal	*G. parvulum* var. *parvulum* fo. *saprophilum* (morph #1)	Lange-Bertalot & Reichardt
Abbey Lakes River	*G. parvulum* var. *parvulum* fo. *saprophilum* (morph #2)	Lange-Bertalot & Reichardt
Scion Pond	*G. parvulum* (morph #1)	Kützing
River Kennet	*G. parvulum* (morph #2)	Kützing
Llyn Ogwen	*Gomphonema gracile* / fo. *saprophilum*	Ehrenberg, Lange-Bertalot & Reichardt

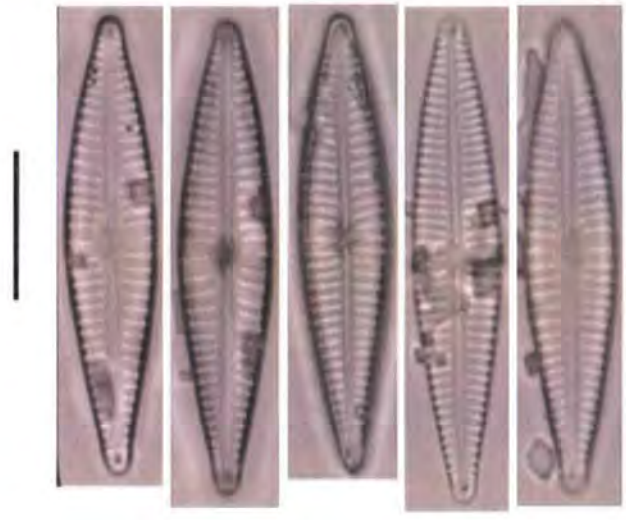


Figure 4.1

Barnwood Pond clone, *G. parvulum* fo. *parvulum*. This clone agrees well with Krammer & Lange-Bertalot's, (1991b) descriptions and LM plates, specifically their Plate 76: Figs1-2. Scale Bar = 10µm.

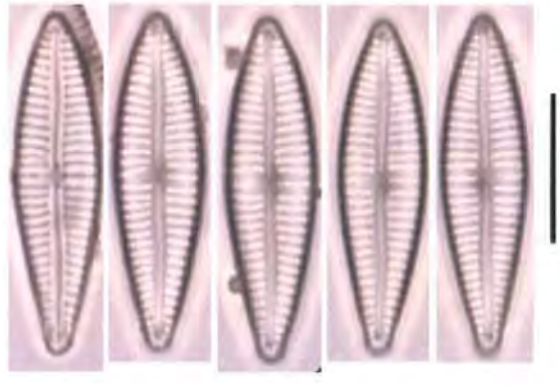


Figure 4.2

Llyn Ogwen clone, *G. gracile / G. parvulum* var. *parvulum* fo. *saprophilum*. This clone is very similar in size and shape to the Kings Mere clone (*G. parvulum* var. *parvulum* fo. *saprophilum* #1), however the striae appear finer and the upper part of the valve has slightly straighter valve margins. In the literature, *Gomphonema gracile* is noted to resemble *G. parvulum* though the size range is larger than that of *G. parvulum*, and it has been suggested that *G. gracile* sensu Hustedt (non Reichert & Fricke), may form part of the *G. parvulum* complex (Krammer & Lange-Bertalot, 1986). Scale Bar = 10µm.

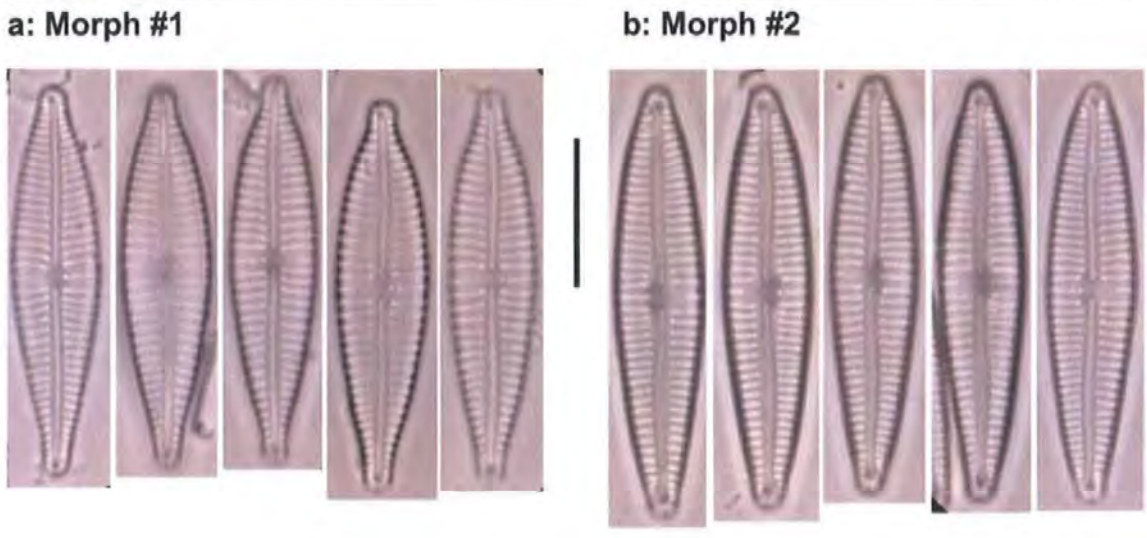


Figure 4.3: a-b

(a) Pen-y-Bryn clone and **(b)** Parys Mountain Clone, both identified as *G. parvulum* var. *exilissimum*. Note the clear demarcation with respect to valve and pole shape. Morph #1 (clone from Pen-y-Bryn) has a more attenuated rostrate head and foot pole than morph #2 (Clone from Parys Mountain), and morph #2 has a less rounded, slightly rhombic valve shape compared to morph #1, which is more rounded. This demarcation agrees with the flora of Krammer & Lange-Bertalot, (1991b). Specifically, morph #1 agrees with Krammer & Lange-Bertalot's, (1991b) Plate 77, Fig 2, and morph #2 with Plate 76, Fig 14. The authors suggest that very narrow individuals are generally characteristic of oligotrophic waters and certainly, both clones in this study originate from low nutrient waters in Wales. Scale bar = 10µm.

a: Morph #1 **b: Morph #2**

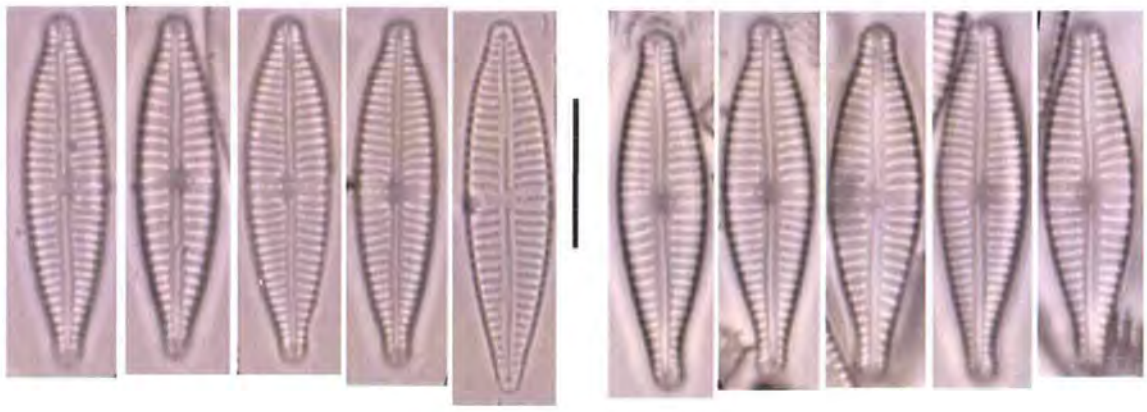
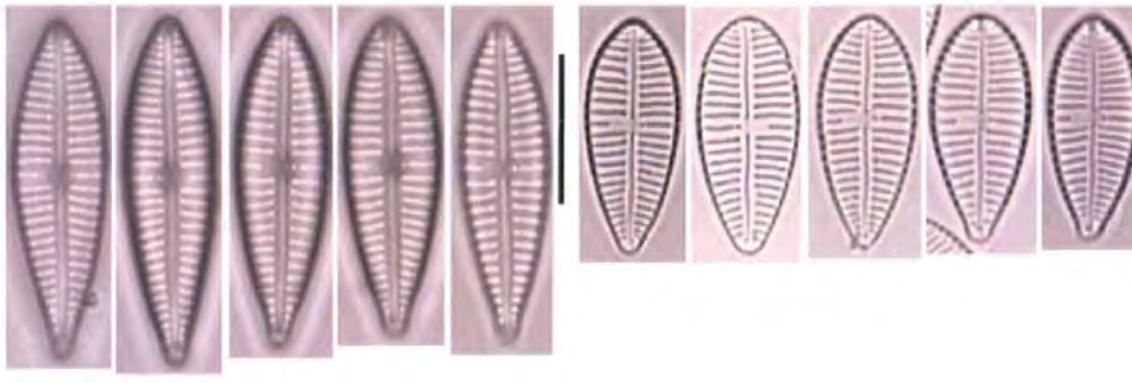


Figure 4.4: a-b

Clones from **(a)** Scion Pond and **(b)** River Kennet are identified as *G. parvulum* (morphs #1 and #2 respectively) without further taxonomic refinement. They both have a broadly similar lanceolate valve outlines, though the Scion Pond clone is slightly narrower in appearance. The foot pole in morph #2 is slightly more attenuated than morph #1 and the head pole marginally wider. Scale bar = 10µm.

a: Morph #1

b: Morph #1



c: Morph #1

d: Morph #2

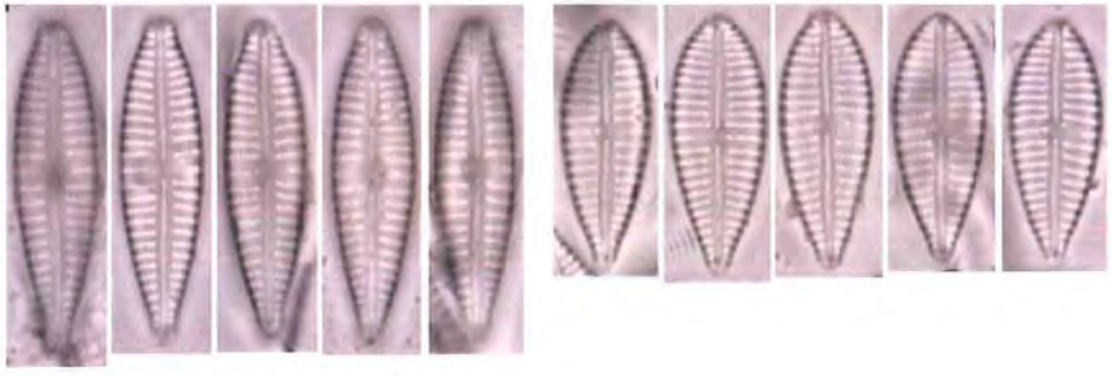


Figure 4.5:a-d

Clones designated *G. parvulum* var. *parvulum* fo. *saprophilum*. Morph #1 from **(a)** Kings Mere, **(b)** Ham Gate Pond and **(c)** Llyn Idwal **(d)** Morph #2 from Abbey Lakes River. All scale bars = 10μm. Of the four clones identified as *G. parvulum* var. *parvulum* fo. *saprophilum*, the Kings Mere, Ham Gate Pond and Llyn Idwal clones are the most similar (morph #1) with rounded head poles and attenuated foot poles. The Abbey lakes River clone (morph #2) however, despite being almost the same size as the clone from Kings Mere, is narrower, and has a slightly less broad rostrate head pole. This is also in agreement with Krammer & Lange-Bertalot, (1991b) figures. Specifically Plate 76: Figs 8-13 is akin to morph#1 in this study, and Plate 77: Figs 5-9, to morph#2.

4.3 GENERAL OBSERVATIONS

4.3.1 Morphology of Clonal Stock Cultures

Clonal subcultures were grown in stock culture for between 8 and 21 months. LM examination of clones grown in long-term culture reveal that size, valve and apical pole shape and stria density are the characters that change most significantly with time **(Figures 4.6 to 4.15)**. Curiously, there is a slight increase in length with time in the River Kennet clone, despite there being no evidence of auxosporulation. Despite the highly variable valve shape in all clones except River Kennet and Llyn Ogwen, a principal form was apparent, i.e. the form produced by >50% of the population. For the Barnwood Pond and Parys Mountain clones, it was lanceolate. In the Scion Pond and Abbey Lakes River clones, it was ovate-clavate, whilst the Kings Mere, Ham Gate Pond and Pen-y-Bryn clones were lanceolate-clavate, and in the Llyn Idwal clone lanceolate to lanceolate-clavate. Observations of clonal stock cultures also found that Janus cells occurred in some populations, but rarely. One exception was the Kings Mere clone, in which one subculture population consisted of circa 25% Janus cells. Janus cells often had the same overall shape and size as the rest of the population, but the striae were more numerous. There is often a concave/convex aspect to the valve profile when in girdle view (Epitheca = convex and Hypotheca = concave) **(Figure 4.16).** Apical pole shape varies with size and degree of attenuation, and the Kings Mere clone was the most variable in this respect. The propensity for morphological abnormality increases as cells get smaller. The smallest cells often have rounded profiles in girdle view. Abnormalities rarely occur at more than 3% in a population.

Striae density between the stigma and non-stigma side of a valve rarely differed by more than a mean value of 1 stria, although individually, the differences could be 2 and in some

cases, they are equally numbered. The ratio between the stigma side and non-stigma side striae was constant, irrespective of size or clone. Differences in striae density on each side of a single valve are generally due to the common absence of a shortened stria opposite the stigma, and occasionally where there is more than one shortened striae. Patterns of variation in central stria density on both sides of the valve cluster around 10-15 /10μm, with the exception of the clone from Scion Pond, which is 14-17/10μm on the stigma-side and 11-16/10μm on the non-stigma side. The occurrence of non-central short striae and inserted striae occurs in all clones except Barnwood Pond, which was not observed to have other short striae, and the Llyn Idwal clone in which no inserted striae were observed. Striae direction is generally radial throughout all the clones, often becoming almost parallel at the lower end of the size range. There is also variation to the degree of curvature of the striae approaching the central area both within and between clones.

The loss of morphological features, particularly at the apices, as size reduction occurs, is of particular importance. Size reduction occurs more rapidly in length than in breadth, and consequently cells often appear 'fatter' when they are smaller. In particular, the apices of the valve become less attenuated and/or rostrate and instead become more rounded, and the frustule in valve view becomes more ovate. The reduction in size and loss of morphological features is so great that different stages in the life cycle of a single clone are identified as different taxa. This clearly undermines the use of diatom valve morphology as the sole criterion with which to identify taxa in water quality indices, and therefore may invalidate existing analyses that have used diatom indices.

Although difficult to separate, differences in the valve morphology of *G. parvulum* clones are demonstrated.

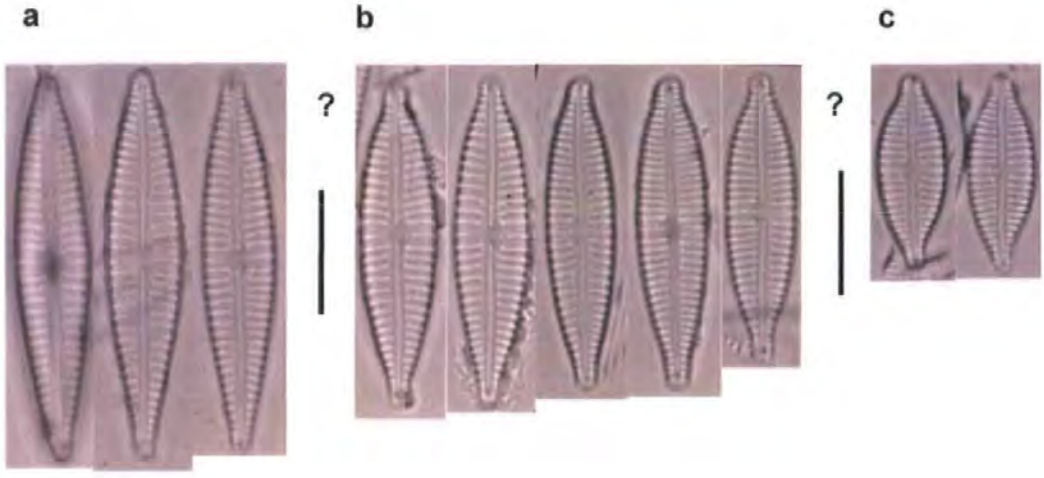


Figure 4.6: a-b

LMs of the Barn Wood Pond clone, grown in long-term culture (December 2001 to July 2002) illustrating changes in valve size and morphology with time. Initially identified as **(a and b)** *Gomphonema parvulum* var. *parvulum* fo. *parvulum*, smaller cells would be identified as **(c)** *Gomphonema* cf. *lagenula*?. Scale bars = 10μm.

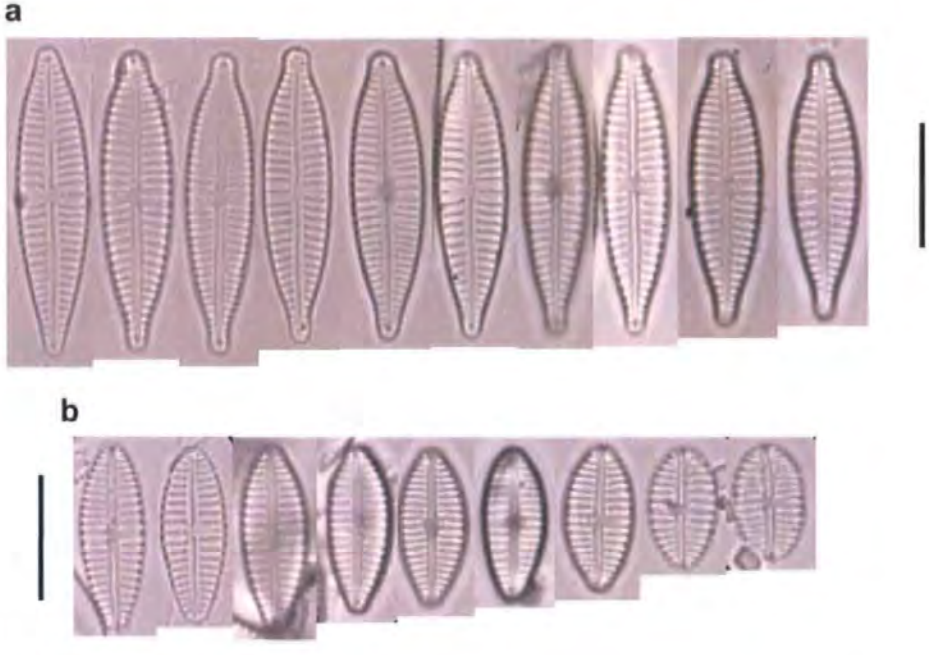


Figure 4.7: a-b

LMs examples of the Scion Pond clone, grown in long-term culture (April 2002 to January 2003) illustrating changes in valve size and morphology with time. Initially identified as **(a)** *Gomphonema parvulum* var. #1, smaller cells of the same clone would be identified as **(b)** *Gomphonema* cf. *innocens*?. Scale bars = 10μm.

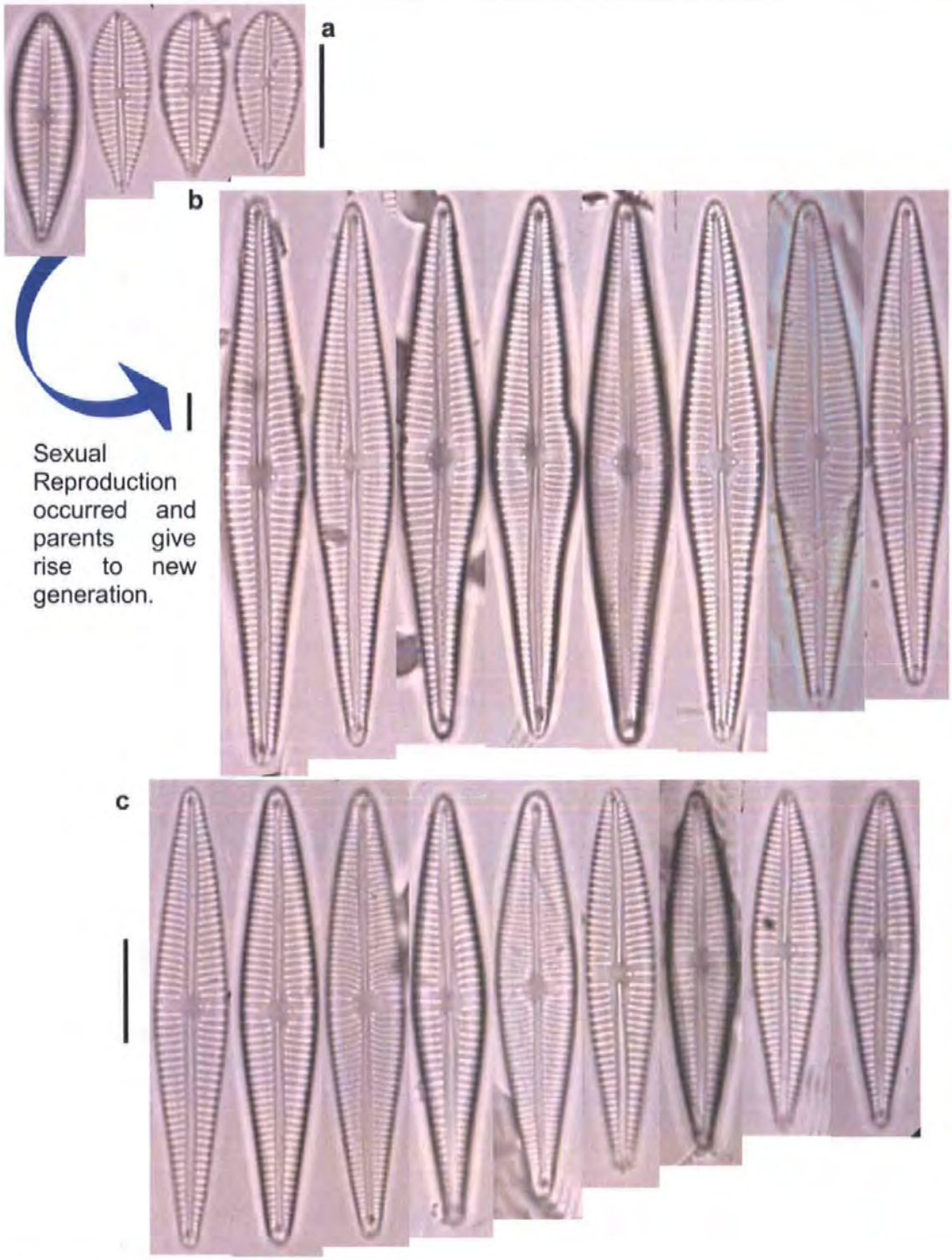


Figure 4.8: a-c

LMs of the Kings Mere clone, grown in long-term culture (December 2001 to November 2002), illustrating changes in valve size and morphology with time and sexual reproduction. Initially identified as **(a)** *Gomphonema parvulum* var. *parvulum* fo. *saprophilum* #1 Lange-Bertalot & Reichardt, larger post auxospore cells would be identified as **(b-c)** *Gomphonema gracile* Ehrenberg. Scale bars = 10μm.

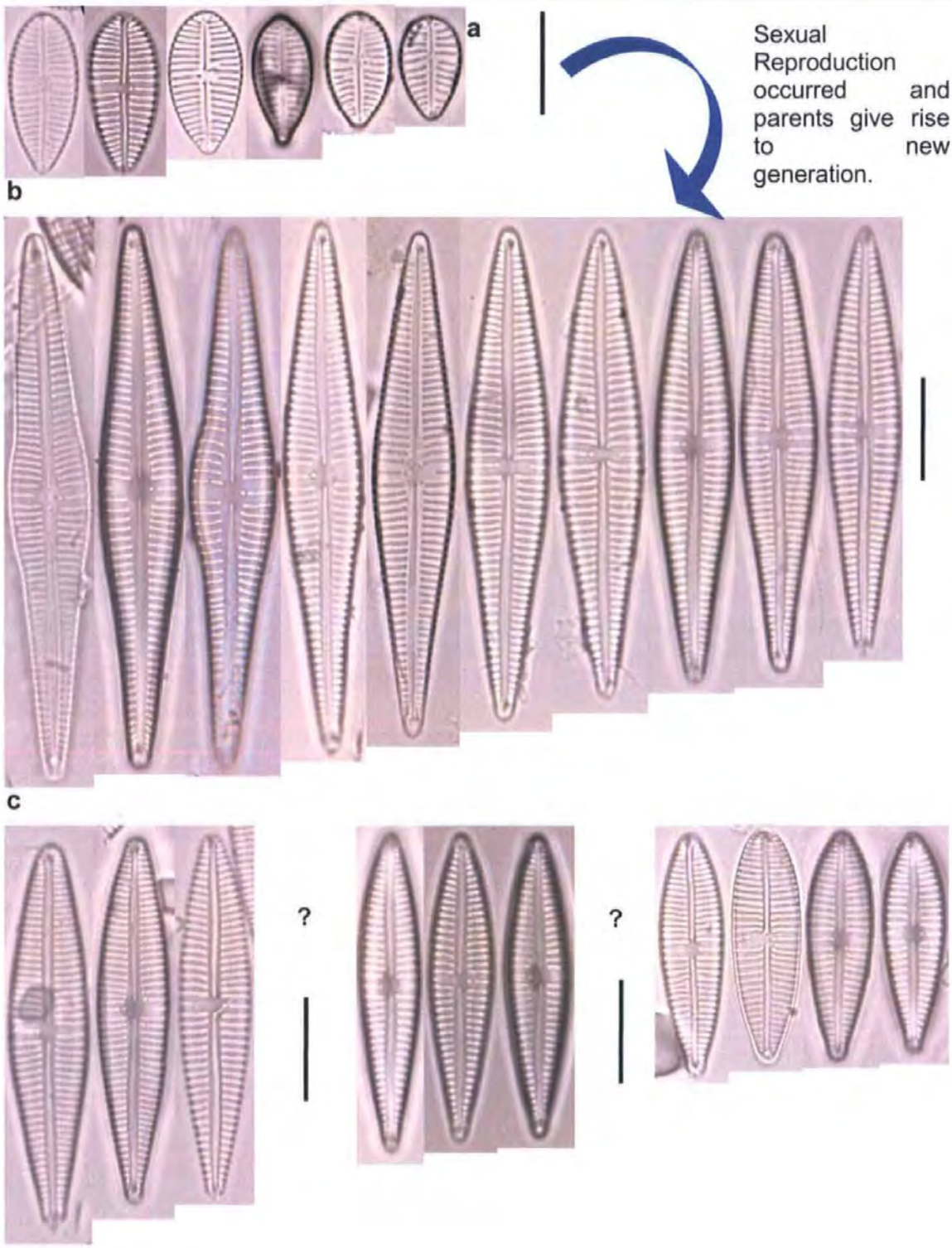


Figure 4.9: a - d

LMs of the Ham Gate clone, grown in long-term culture (February 2002 to April 2003), illustrating changes in valve size and morphology with time and sexual reproduction. Initially identified as **(a)** *Gomphonema parvulum* var. *parvulum* fo. s*aprophilum* Lange-Bertalot & Reichardt, larger post auxospore cells would be identified as **(b-c)** *Gomphonema gracile* Ehrenberg. Scale bars = 10μm.

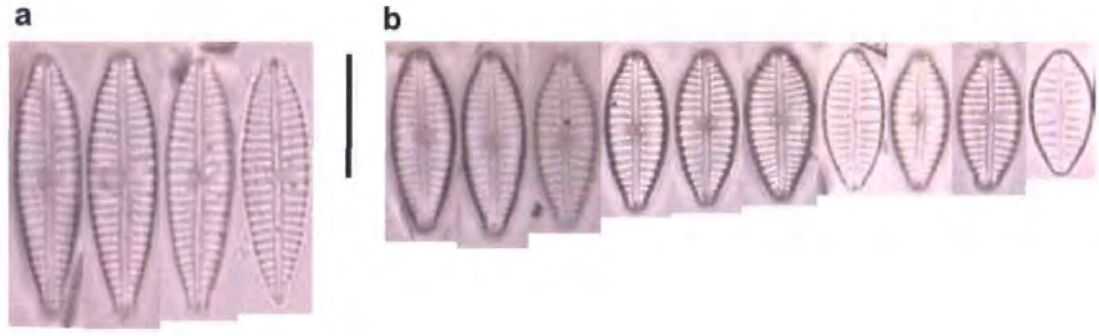


Figure 4.10: a-b

LMs of the Abbey Lakes Pond clone, grown in long-term culture (December 2001 to July 2002), illustrating changes in valve size and morphology with time. Initially identified as **(a)** *Gomphonema parvulum* var. *parvulum* fo. s*aprophilum* #2 Lange-Bertalot & Reichardt, smaller cells would be identified as **(b)** *Gomphonema parvulum*. Scale bar = 10μm.

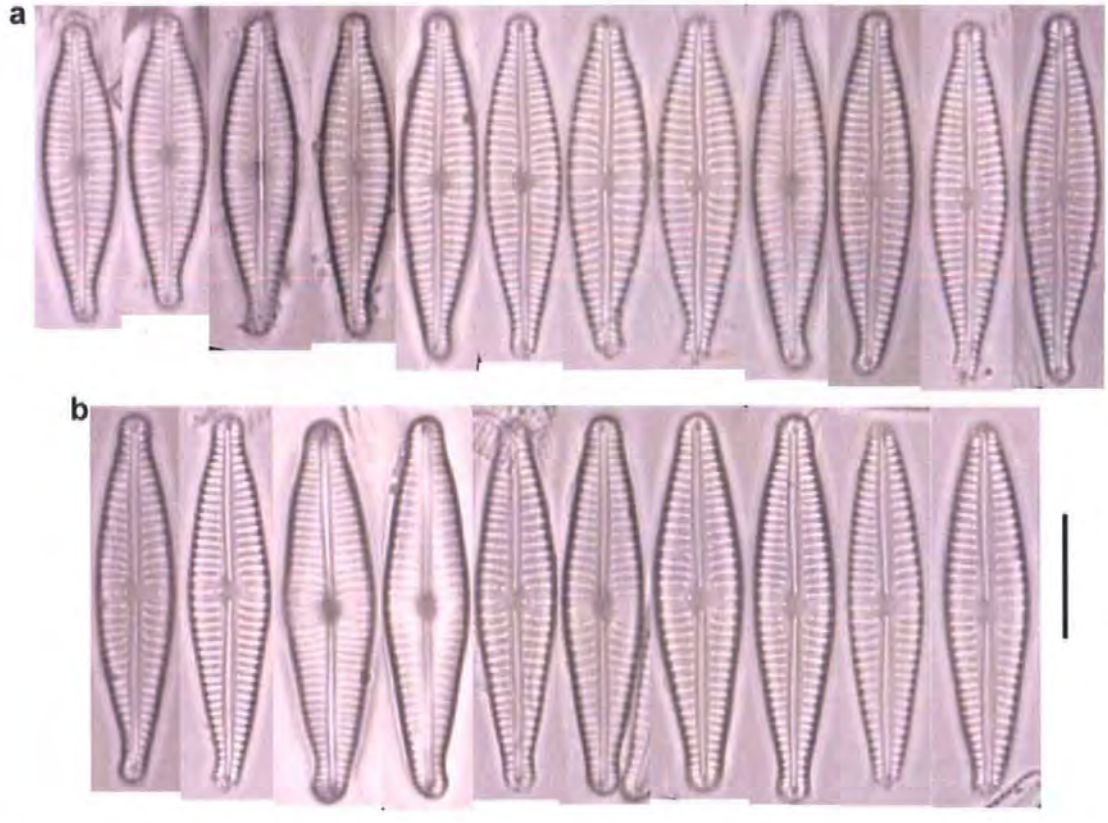
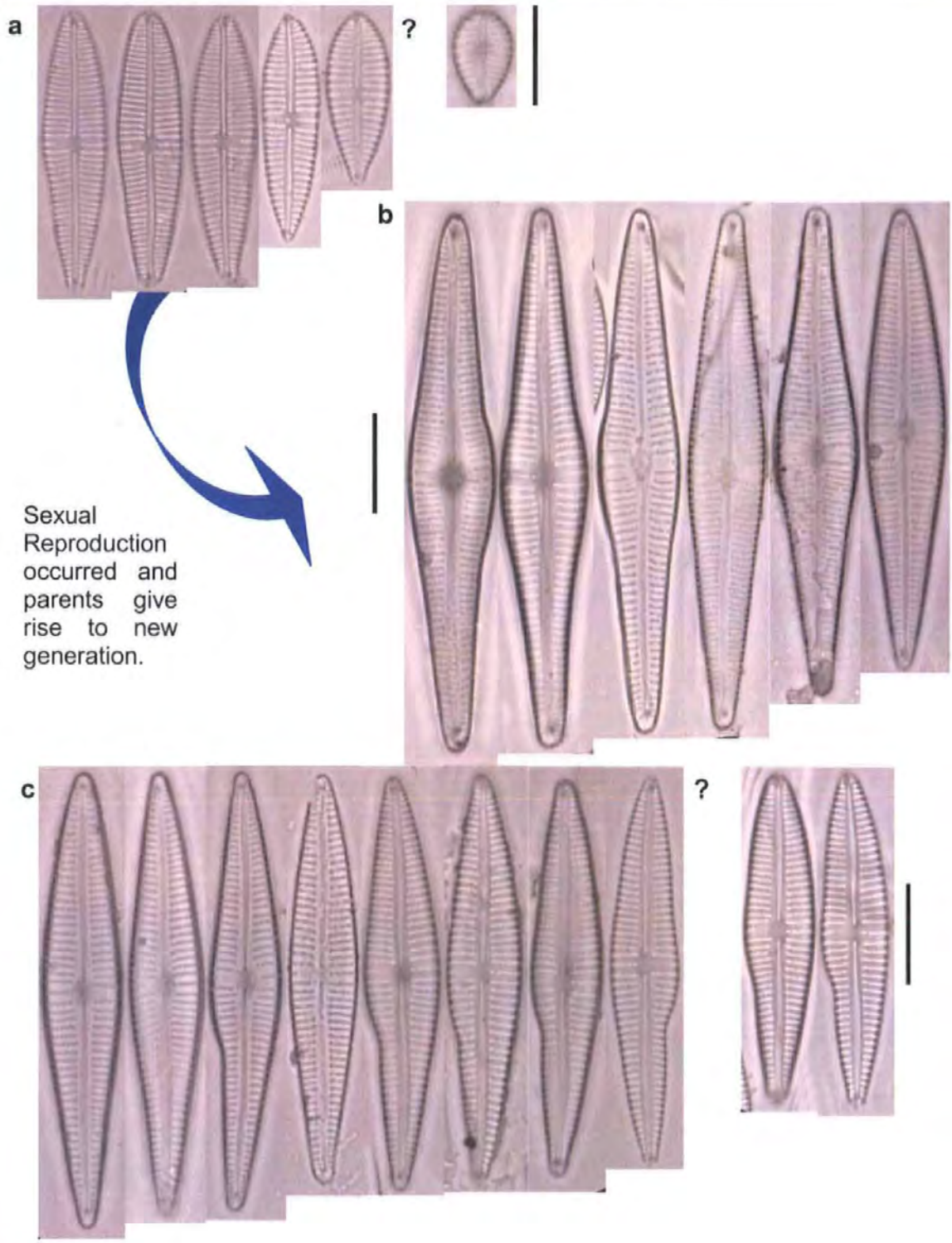


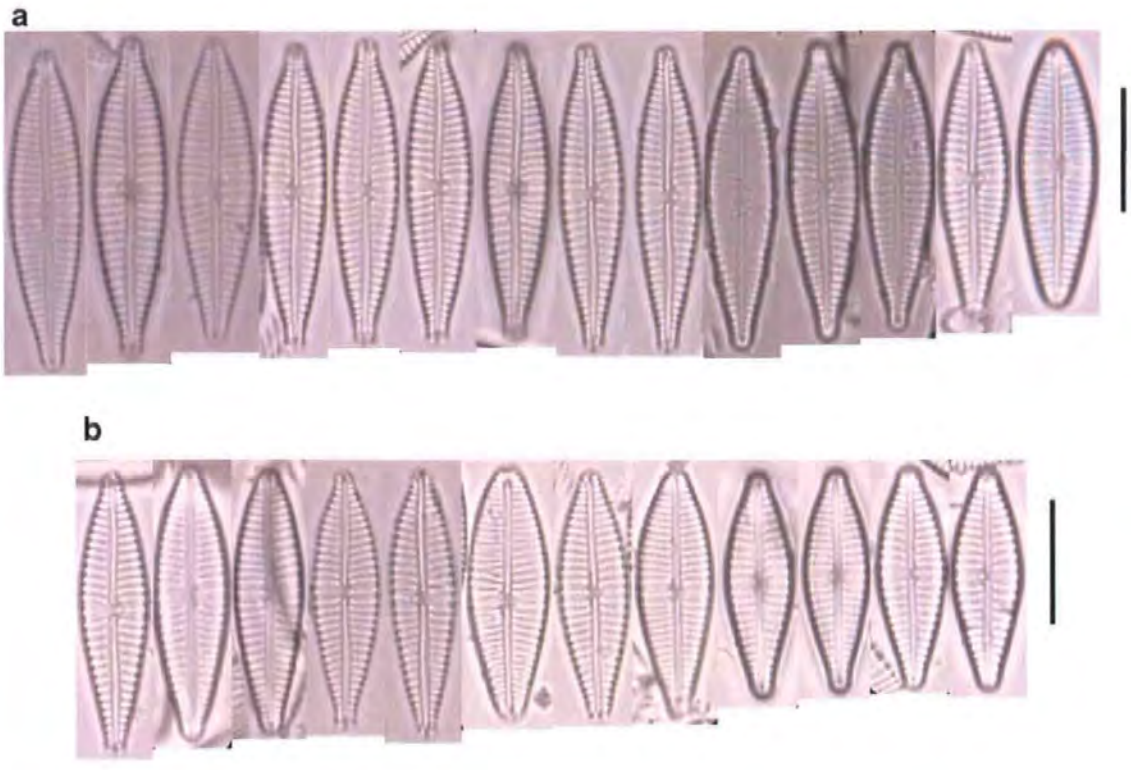
Figure 4.11: a-b

LMs of the River Kennet clone, grown in long-term culture (April 2002 to December 2002), illustrating changes in valve size and morphology with time. Initially identified as **(a)** *Gomphonema parvulum* #2. This identity was maintained throughout culture. Scale bar = 10μm. **NB:** this clone apparently increased slightly in size without undergoing sexual reproduction.



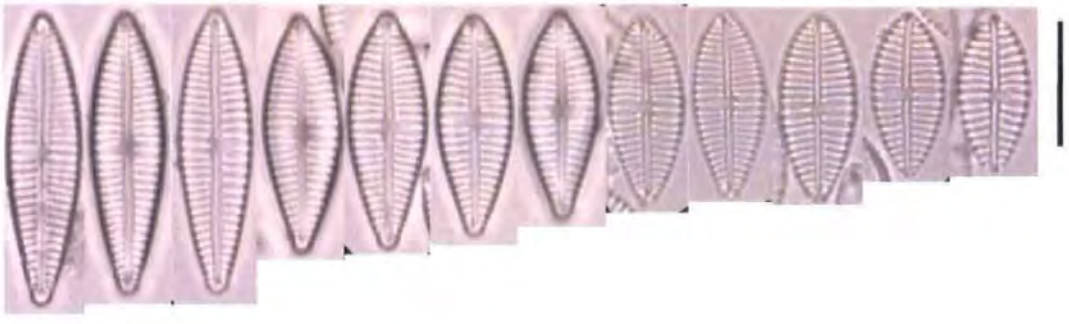
Figures 4.12: a-b

LMs of the Llyn Idwal clone, grown in long-term culture (May 2002 to April 2003), illustrating changes in valve size and morphology with time and sexual reproduction. Initially identified as **(a)** *Gomphonema parvulum* var. *parvulum* fo. *saprophilum* #1 Lange-Bertalot & Reichardt, larger post auxospore cells would be identified as **(b-c)** *Gomphonema gracile* Ehrenberg. Scale bars = 10μm.



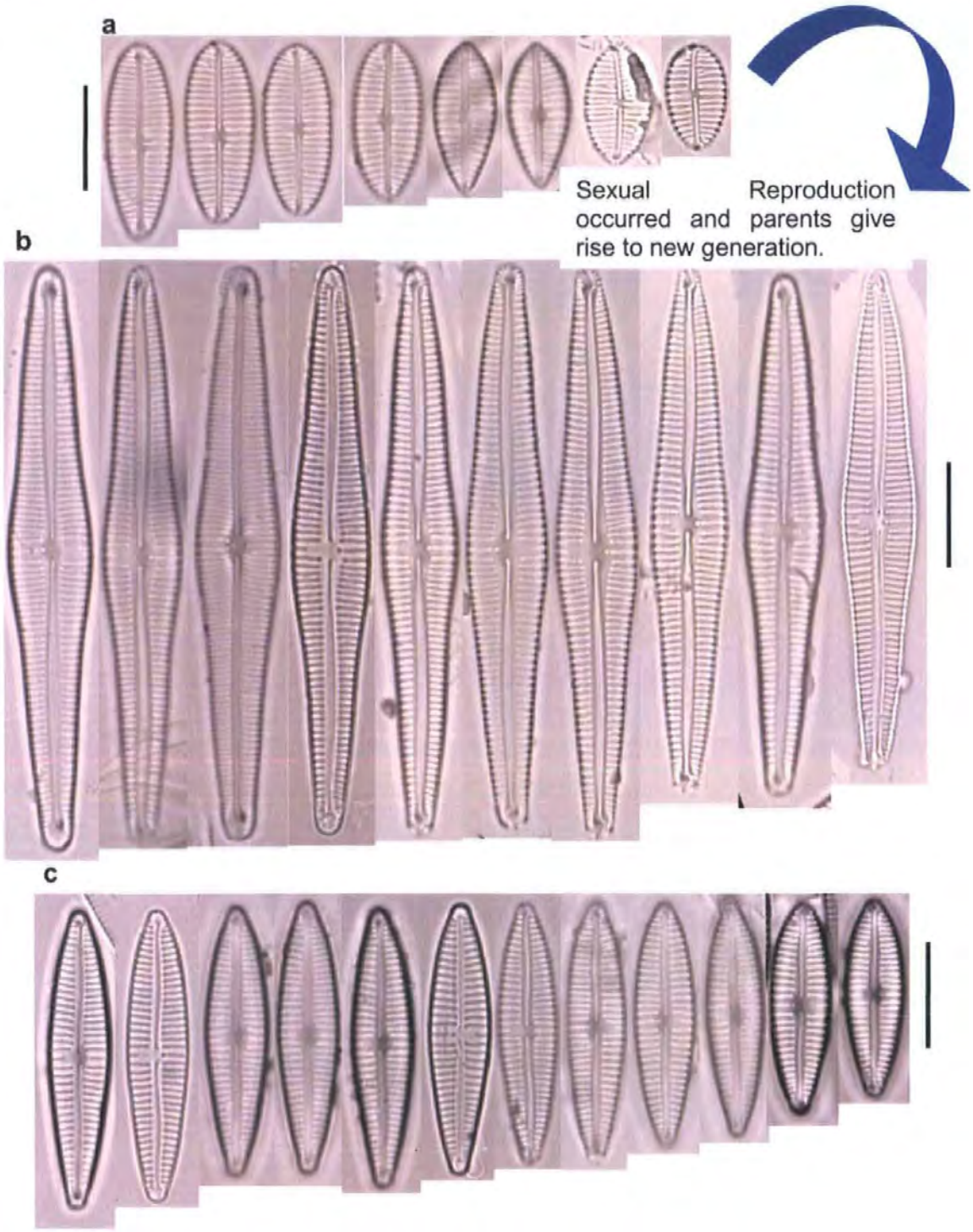
Figures 4.13: a-b

LMs of the Pen-y-Bryn clone, grown in long-term culture (May 2002 to February 2003), illustrating changes in valve size and morphology with time. Initially identified as *Gomphonema parvulum* var. *exilissimum* #1 Grunow, this identity was maintained with reduction in size. Scale bars = 10μm.



Figures 4.14: a-b

LMs of the Llyn Ogwen clone, grown in long-term culture (May 2002 to July 2002), illustrating changes in valve size and morphology with time. Initially identified as *Gomphonema gracile* / *G. parvulum* var. *parvulum* fo. *saprophilum*, this identity was maintained with reduction in size. Scale bar = 10μm.



Figures 4.15: a-b

LMs of the Parys Mountain clone, grown in long-term culture (May 2002 to February 2003), illustrating changes in valve size and morphology with time and sexual reproduction. Initially identified as **(a)** *Gomphonema parvulum* var. *exilissimum* #2, larger post auxospore cells were identified as **(b)** *Gomphonema hebridense* Gregory Grunow and intermediated sized cells as **(c)** *Gomphonema parvulum* var. *exilissimum* #2. Scale bars = 10μm.

Figure 4.16

SEM of Barnwood Pond clone showing convex/concave profile in girdle view. Scale bar = 10μm.

SEM examination of clonal stock cultures reveal that the basic features of *G. parvulum* are present in all clones, and do not reveal features that allow taxonomic separation of clones. However, in cells at the smallest end of the size range, some features were not as marked as in larger specimens, e.g. in the Scion Pond clone, some valves had central raphe endings that were not as strongly hooked **(Figure 4.17)**, whilst in the Ham Gate Pond clone, girdle band pores were more elongate **(Figure 4.18)** and thicker cell walls were observed, as well as a number of stria/pore disruptions **(Figure 4.19).** Additionally, G_1 clones from Ham Gate Pond and Llyn Idwal had less well-developed areolae **(Figure 4.20)**. However these are matters of degree and further work is needed to determine how useful or stable these slight differences are. In girdle view, all clones had very shallow mantles **(Figure 4.21)**, girdle bands with uneven edges **(Figure 4.22)**, and were either barely wedge-shaped or rectangular in profile. No valve under SEM was observed to have more than one stigma.

The clone from Llyn Idwal was also observed to be asymmetrical about the apical axis with a convex bulge (attained during/after auxosporulation) on the non-stigma side of the valve, which was maintained in all G_2 cultures.

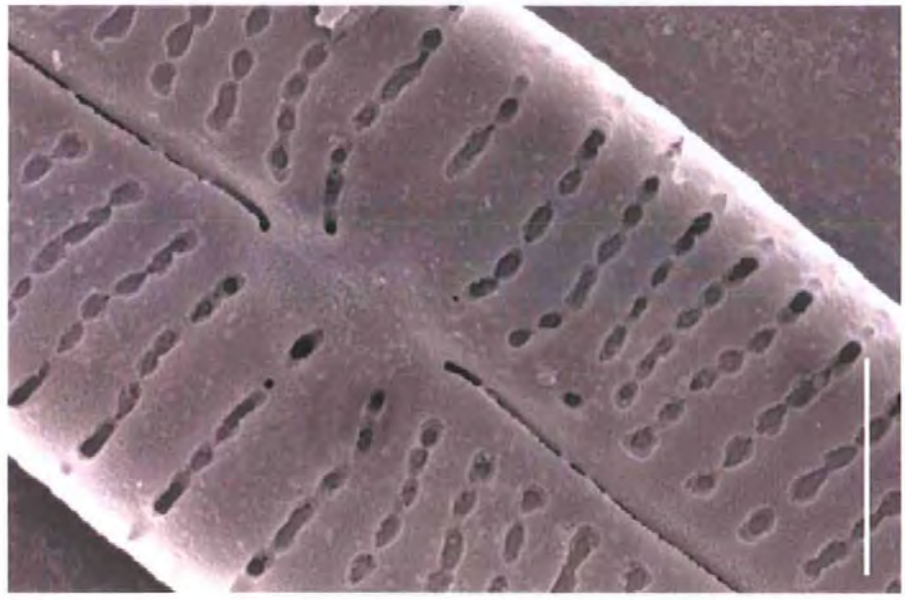


Figure 4.17

SEM of the Scion Pond clone, internal view in which the central raphe endings are less hooked than in other clones. Scale bar = 5µm.

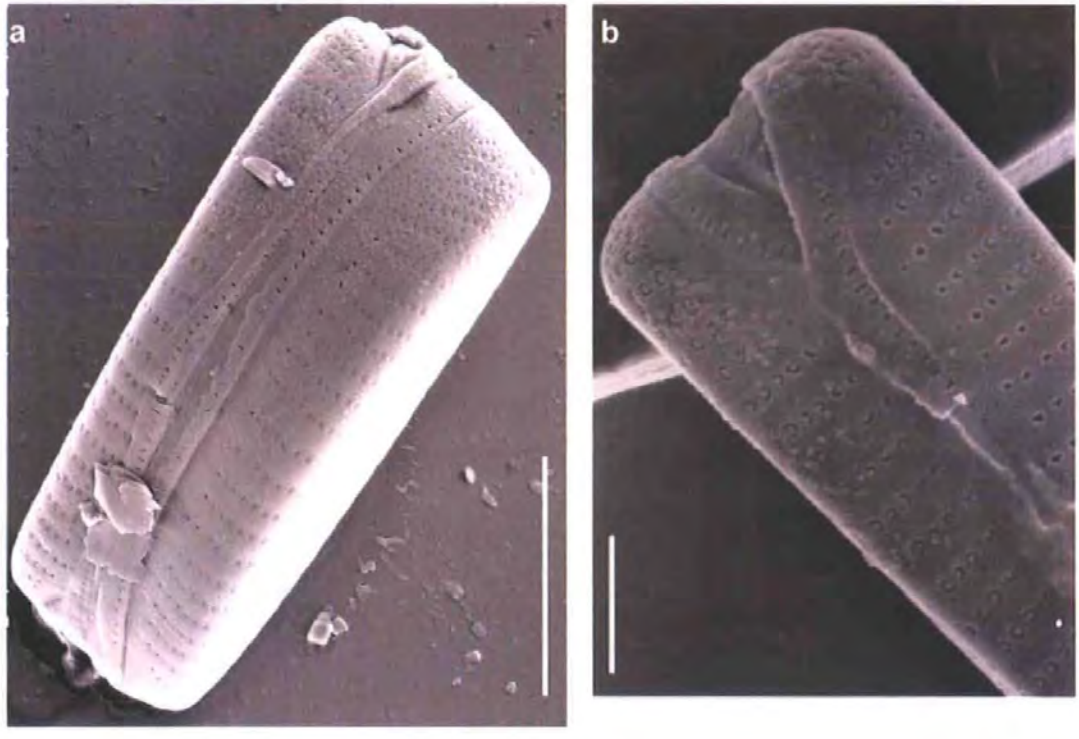


Figure 4.18: a-b

SEM of the **(a)** Ham Gate Pond clone and **(b)** Scion Pond clone, external views showing elongate girdle band pores. Scale bars **(a)** = 5µm and **(b)** = 2µm.

Figure 4.19

SEM of pore and stria disruption in the Ham Gate Pond clone. Scale bar = 5µm.

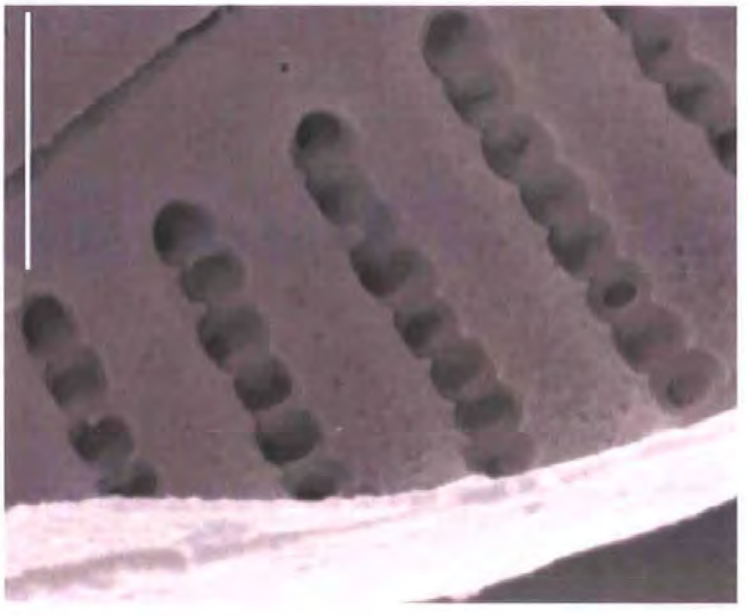


Figure 4.20

SEM of underdeveloped areolae in the Ham Gate Pond clone. Scale bar = 1µm.

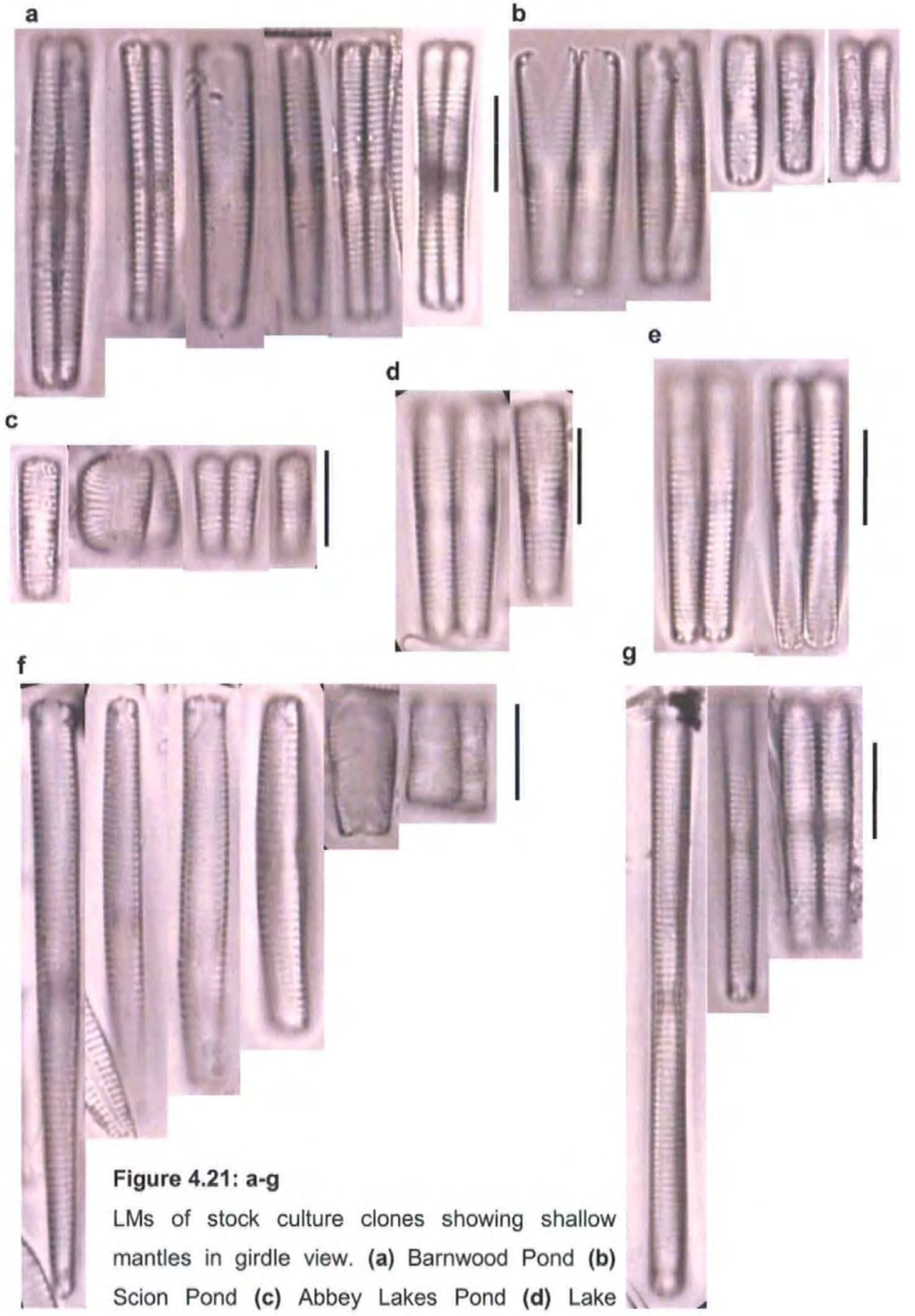


Figure 4.21: a-g

LMs of stock culture clones showing shallow mantles in girdle view. **(a)** Barnwood Pond **(b)** Scion Pond **(c)** Abbey Lakes Pond **(d)** Lake Ogwen **(e)** Pen-y-Bryn **(f)** Llyn Idwal **(g)** Parys Mountain. All scale bars = 10μm.

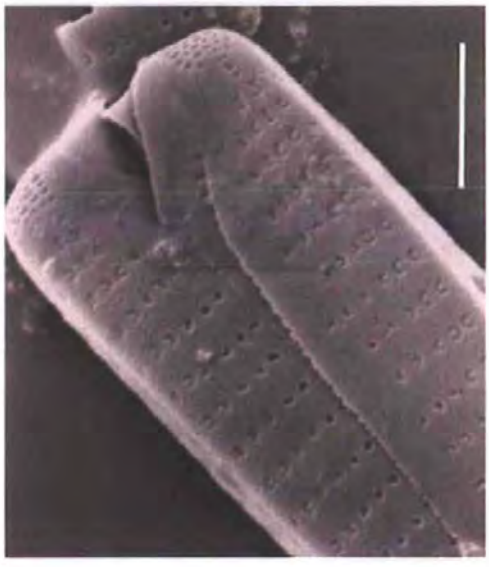


Figure 4.22

SEM of Scion Pond clone showing uneven edge of girdle bands. Scale bar = 2μm.

4.3.2 Morphology and Experimental Regime

Observations under LM reveal that under certain conditions, valves of some clones often appeared thicker (noted as denser, darker valve perimeter/mantle), e.g. clones from Scion Pond (Nil and 0.00016µg/l zinc concentration, initial run), Ham Gate Pond (pH 8.5 and 9.5), Llyn Idwal (pH 4.0 and 4.5), River Kennet (0.25, 1.0 and 2.5µg/l zinc concentration, extended run) and Pen-y-Bryn (0.00033µg/l zinc concentration, initial run). Additionally, pH caused shortening and rounding of shape in some clones and in some cases, transapical asymmetry. The Ham Gate Pond clone often appeared more rounded and squat at pH 5.0 to pH 8.0, but more rhombic in shape at pH 8.5. Whereas the Kings Mere clone became more squat at pH 7.5 to 9.0, and was often asymmetrical about the apical axis with the stigma side of the valve slightly wider. Striae were also much denser. However, both the Kings Mere and the Ham Gate Pond clones had much more attenuated foot poles compared to head poles, regardless of assay or size. Whereas the apical poles in the River Kennet clone became less pronounced in the cadmium regime.

The clone from Llyn Idwal maintained the convex bulge on the non-stigma side of the valve, regardless of assay. Although normal shape was restored after the next auxosporulation, or became less pronounced at the smaller end of the size range. However, in the zinc assay, the apical asymmetry was often enhanced and the valve appeared thicker at 0.00033µg/l in the initial run and 0.10µg/l in the extended run. This clone also maintained a more attenuated foot pole compared to head pole.

Sewage concentration had the greatest effect on valve morphology, modifying length and central striae density in several clones. The Parys Mountain clone was the least affected

by any assay except sewage. From the data it is clear that the Scion Pond clone is tolerant of sewage, clones from Llyn Idwal, River Kennet and Parys Mountain (G_1) are tolerant of pH, the Pen-y-Bryn and Parys Mountain (G_2) clones are tolerant of copper, clones from Scion Pond, River Kennet and Parys Mountain (G_1 and G_2) are tolerant of zinc, and clones from Ham Gate Pond, Llyn Idwal, River Kennet, Pen-y-Bryn and Parys Mountain (G_2) are tolerant of cadmium.

Although the sample size for the assays is smaller than that of the stock cultures, there are nonetheless between 3 and 7 times more abnormalities occurring in certain assayed clones.

Observations in SEM generally agreed with those found under LM, although stria and raphe disruption were more readily identifiable, and other developmental abnormalities observed. In the copper regime, a few valves of the Llyn Idwal clone had less prominently hooked central raphe endings. Underdeveloped pores were observed in a few valves of clones from Llyn Idwal, River Kennet and Pen-y-Bryn in the copper, zinc and cadmium regimes **(Figure 4.23)**, whilst non-reniform and completely occluded pores were observed in the River Kennet clone in the copper regime **(Figure 4.24)**.

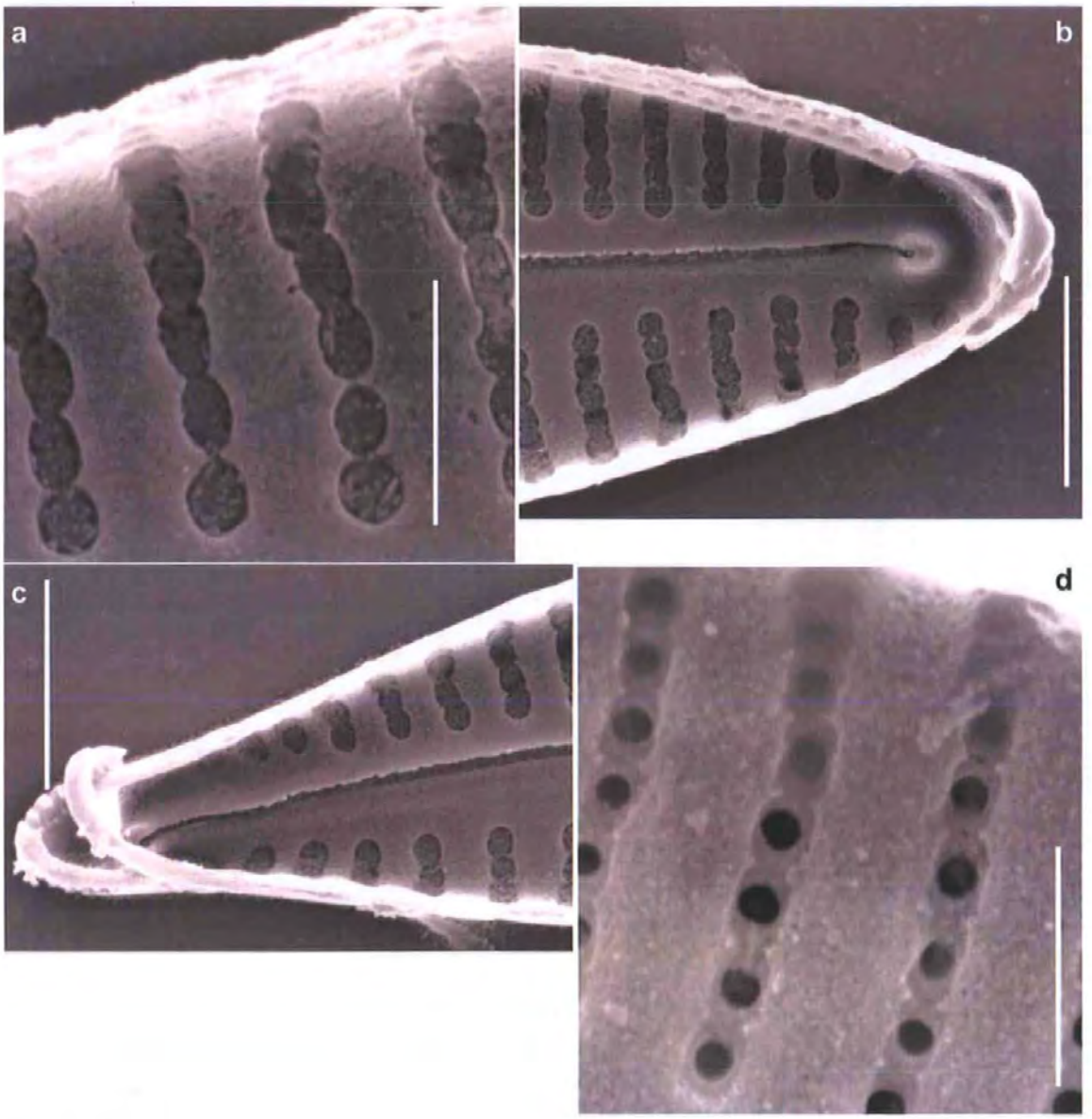


Figure 4.23: a- d

SEM examples of underdeveloped pores produced by clones in the metals regimes. Scale bars = (a) 1μm (b) 2μm (c) 2μm (d) 1μm.

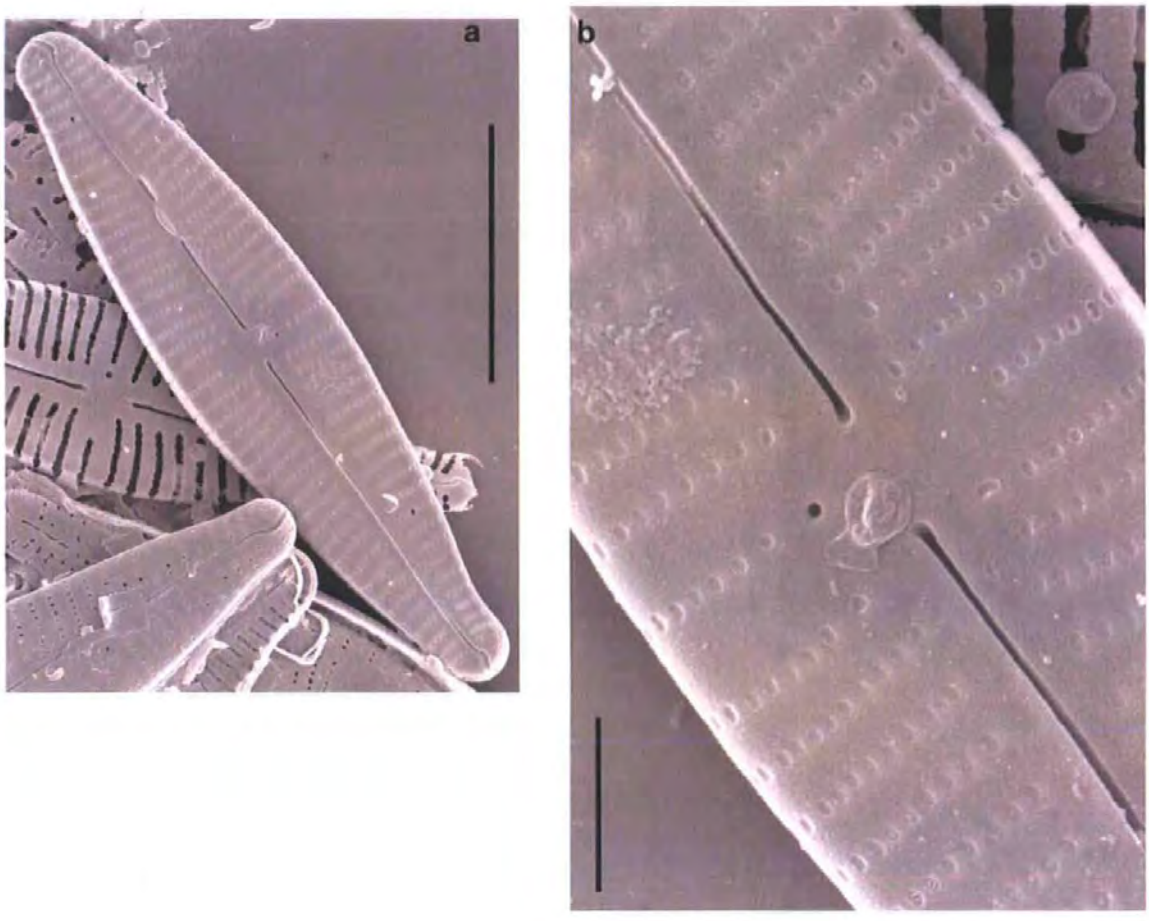


Figure 4.24: a- b

SEM of occluded non-reniform areolae in the River Kennet clone subject to the copper regime. Scale bars (a) 10μm (b) 5μm.

4.4 MORPHOMETRIC ANALYSIS

4.4.1 Morphometric Analysis of Clonal Stock Cultures

Results tables for Kruskal-Wallis tests and multiple pairwise comparison tests (Bonferroni, 95% Confidence Level) can be found on the enclosed CD along with Coefficients of Variation (CV) and descriptive statistics.

Figures 4.25 to 4.27 are boxplots summarising the measurements for morphological characters of length, breadth, striae density, raphe length, valve shape and apical pole shape for each clone. **Figures 4.28 to 4.37** are line graphs showing how mean values for these later characters change with each subsequent subculture for each clone, and **Table 4.2** provides the percent occurrence of each morphological character per clone.

Results of the Kruskal-Wallis tests indicate highly significant differences between clones for the morphological characters of length, breadth, striae density, raphe length, valve shape and apical pole shape. Additionally, 90% of the pairwise comparisons for the later characters were significantly different. Multiple pairwise comparisons of paired characters (upper and lower raphe, primary and secondary side central striae and head and foot poles), show 66% as significantly different. The results suggest that these characters may be useful in diagnosing different forms of *G. parvulum*. However, there are overlaps in the length and width ranges as shown in the scatterplot for all clones **(Figure 4.38)** and therefore no clear separation between clones using length and breadth.

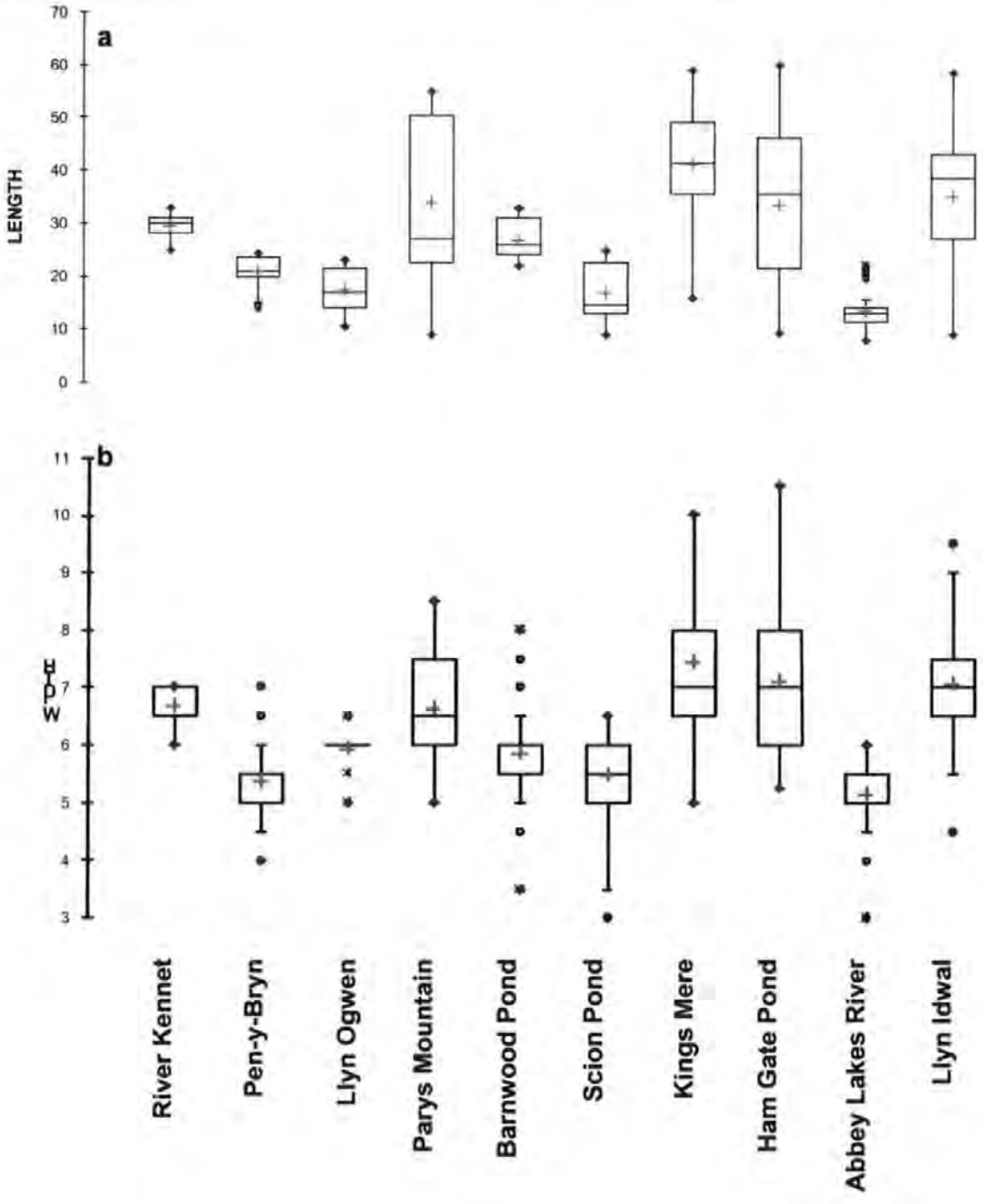


Figure 4.25: a-b

Boxplots of **(a)** valve length and **(b)** valve breadth (μm), for each clone.

Key

Boxes = enclose interquartile range
Horizontal line with box = median
+ = Mean
◆ = Minimum and maximum values in range
* = Extreme outliers
O = moderate outliers

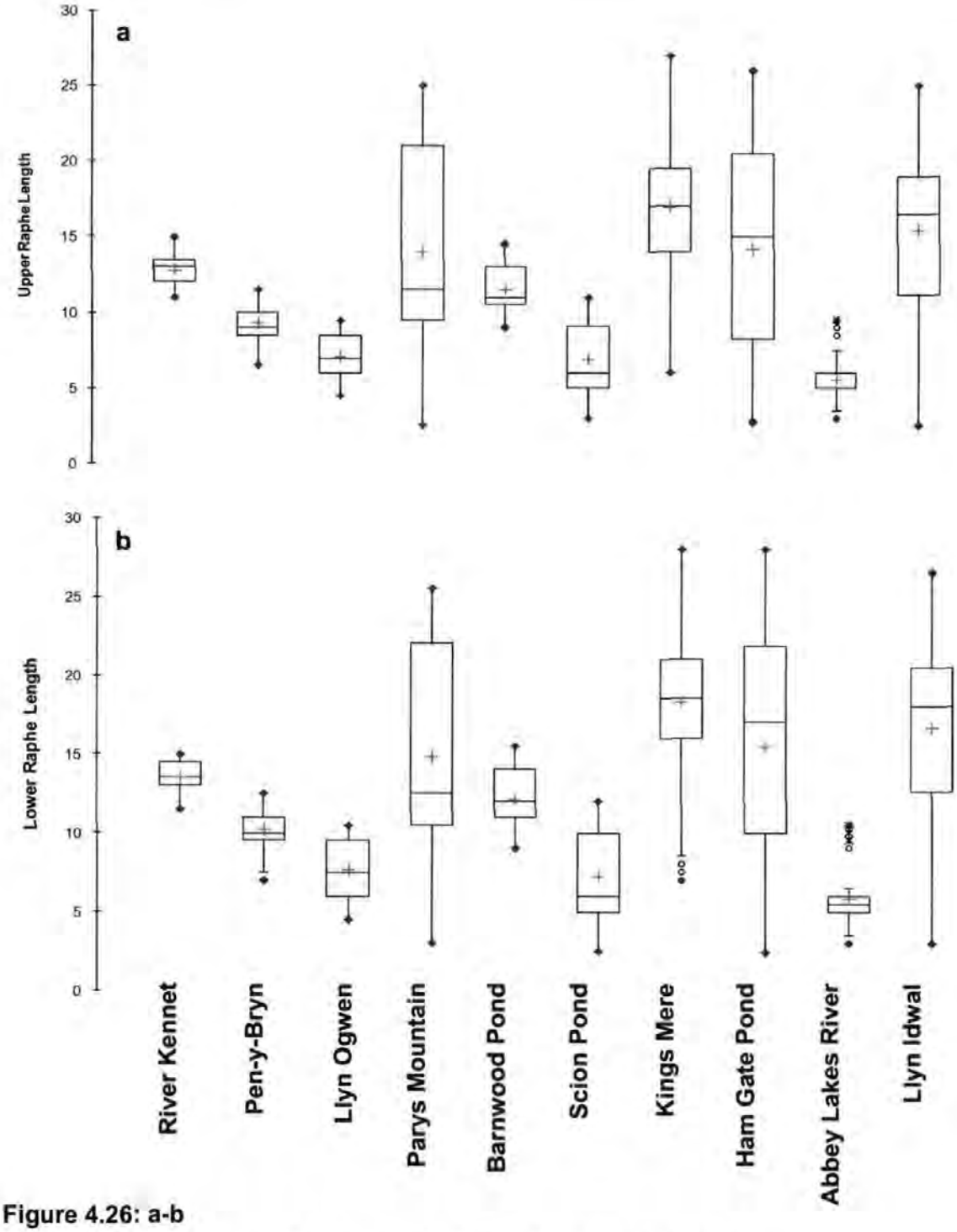


Figure 4.26: a-b

Boxplots of **(a)** upper and **(b)** lower raphe length (µm), for each clone.

Key

Boxes = enclose interquartile range
Horizontal line with box = median
+ = Mean
◆ = Minimum and maximum values in range
* = Extreme outliers
O = moderate outliers

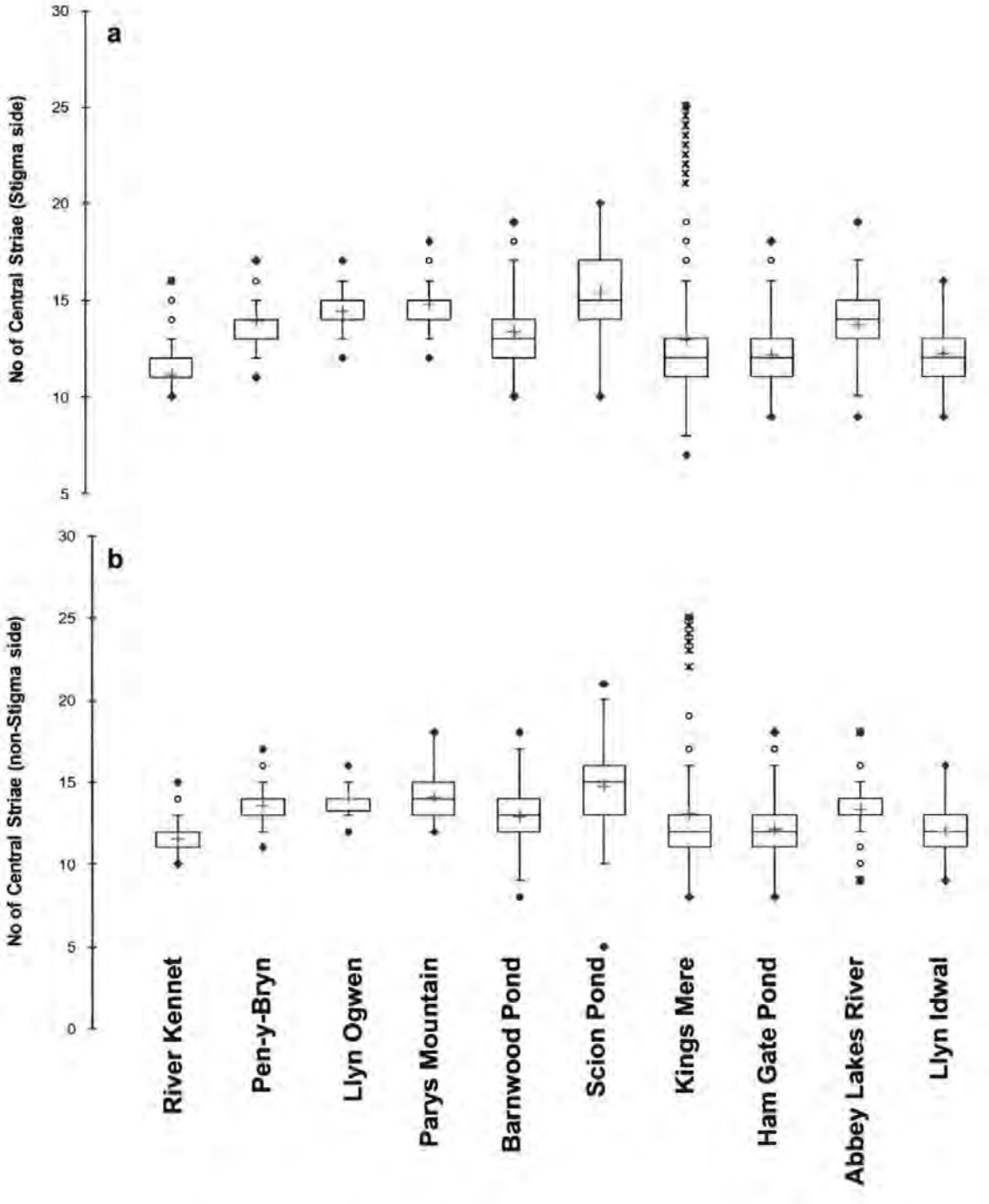
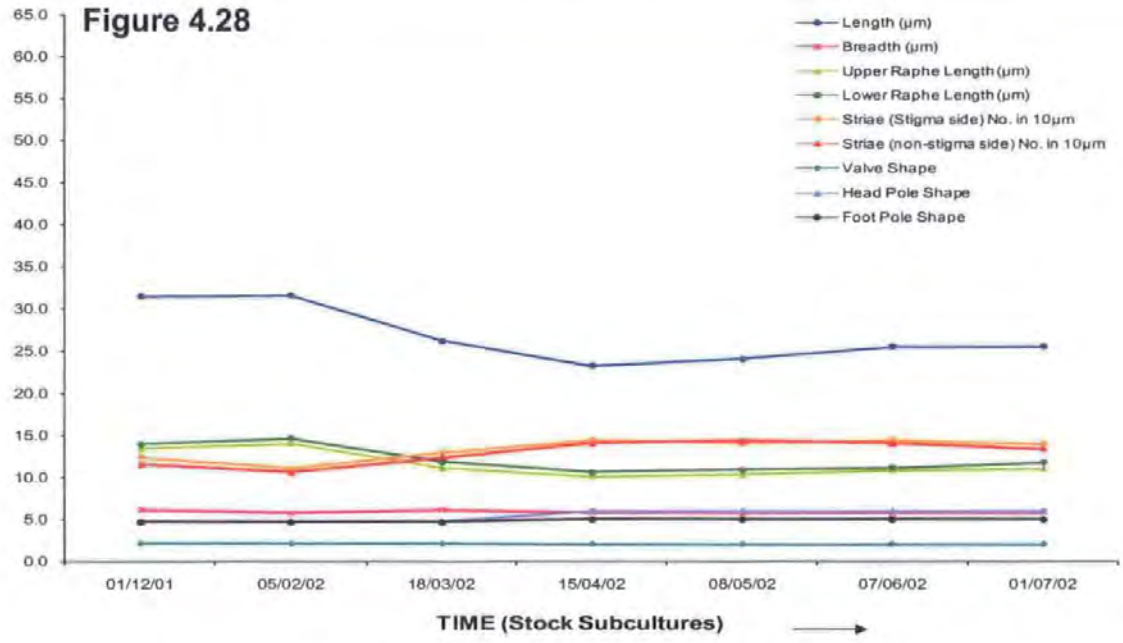


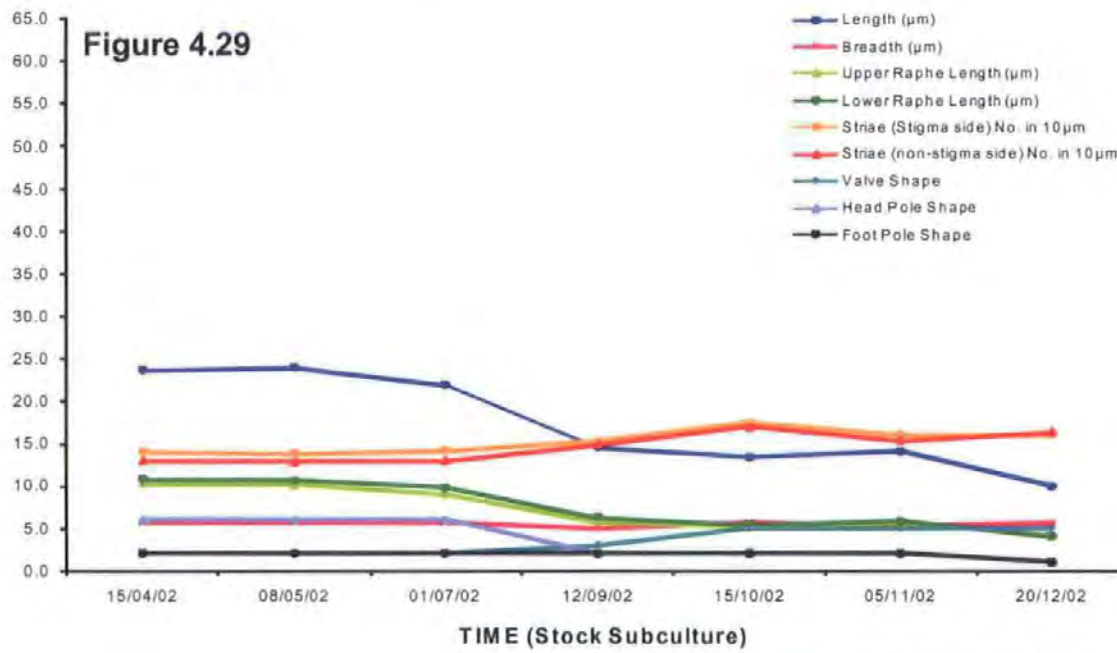
Figure 4.27: a-b

Boxplots of central striation density in 10µm on **(a)** stigma side of the valve and **(b)** the non-stigma side of the valve, for each clone.

Key

Boxes = enclose interquartile range
Horizontal line with box = median
+ = Mean
◆ = Minimum and maximum values in range
* = Extreme outliers
O = moderate outliers

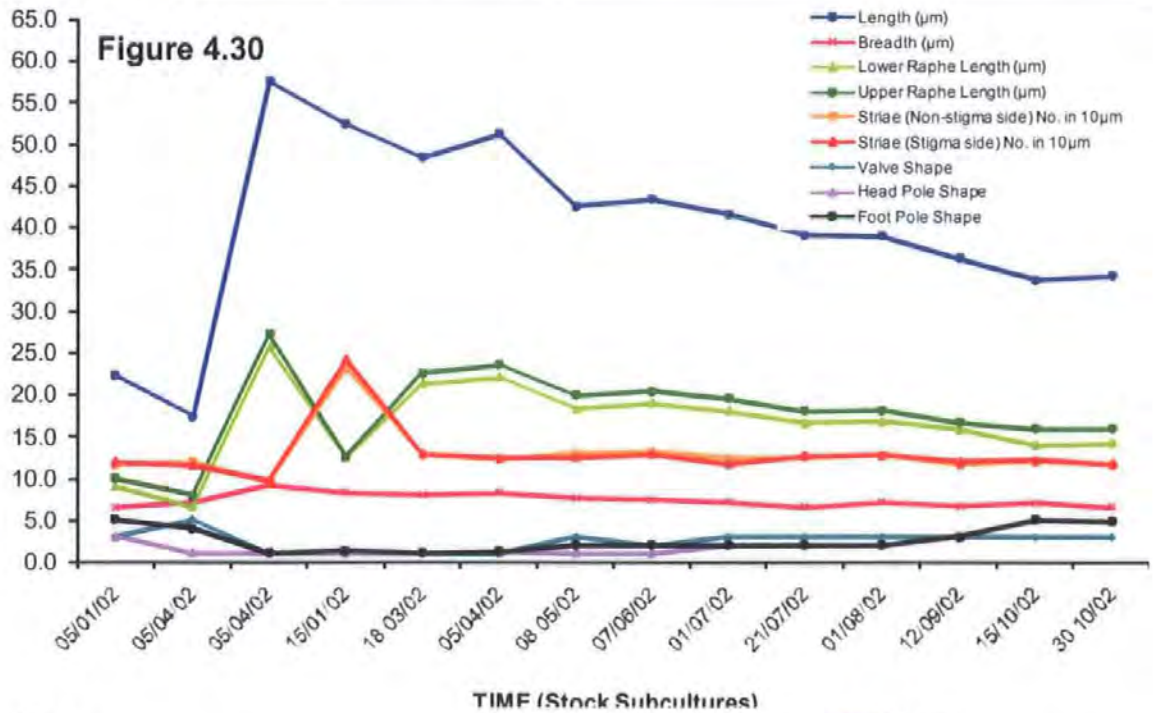


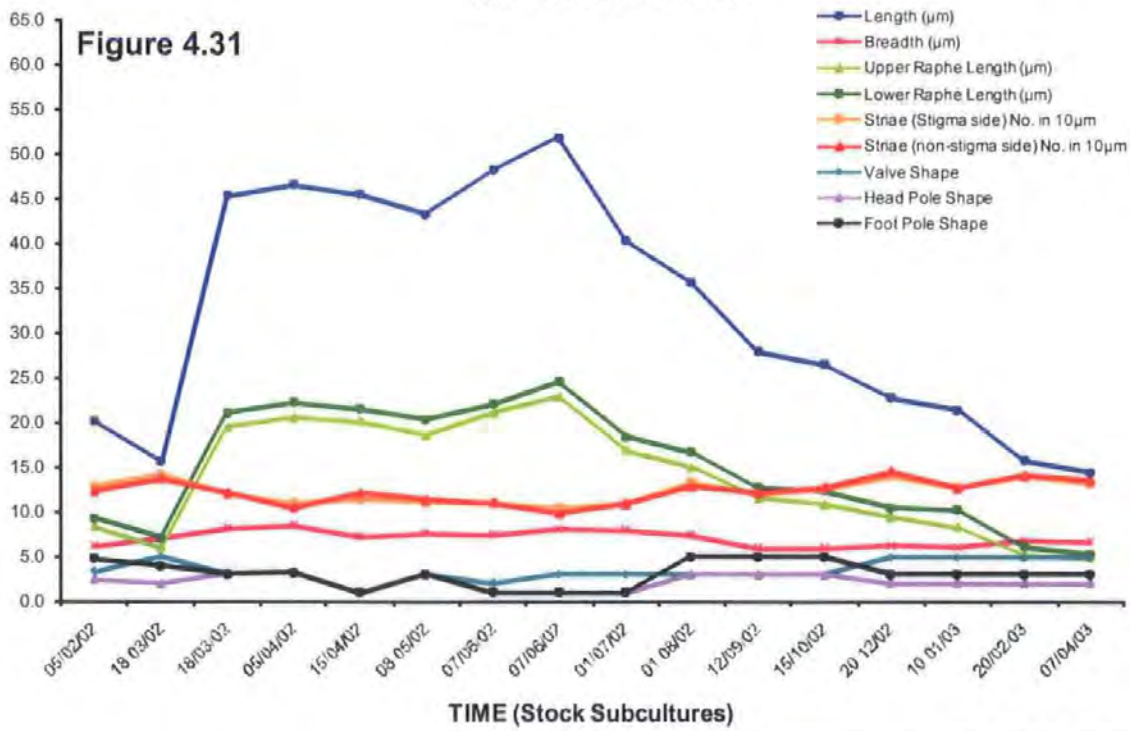


Valve Shape	Head / Foot Pole Shape
1 Narrow lanceolate	1 Rounded
2 Lanceolate	2 Rounded-subrostrate
3 Lanceolate-clavate	3 Subrostrate
4 Ovate	4 Subrostrate-rostrate
5 Ovate-clavate	5 Rostrate
6 Elliptic	6 Rostrate-subcapitate
	7 Subcapitate

Figures 4.28 and 4.29

Line Graphs for Barnwood Pond and Scion Pond clones respectively. **NB:** The y-axis is a multiunit scale and the values for the characters of valve and pole shape are given in the table bottom right.

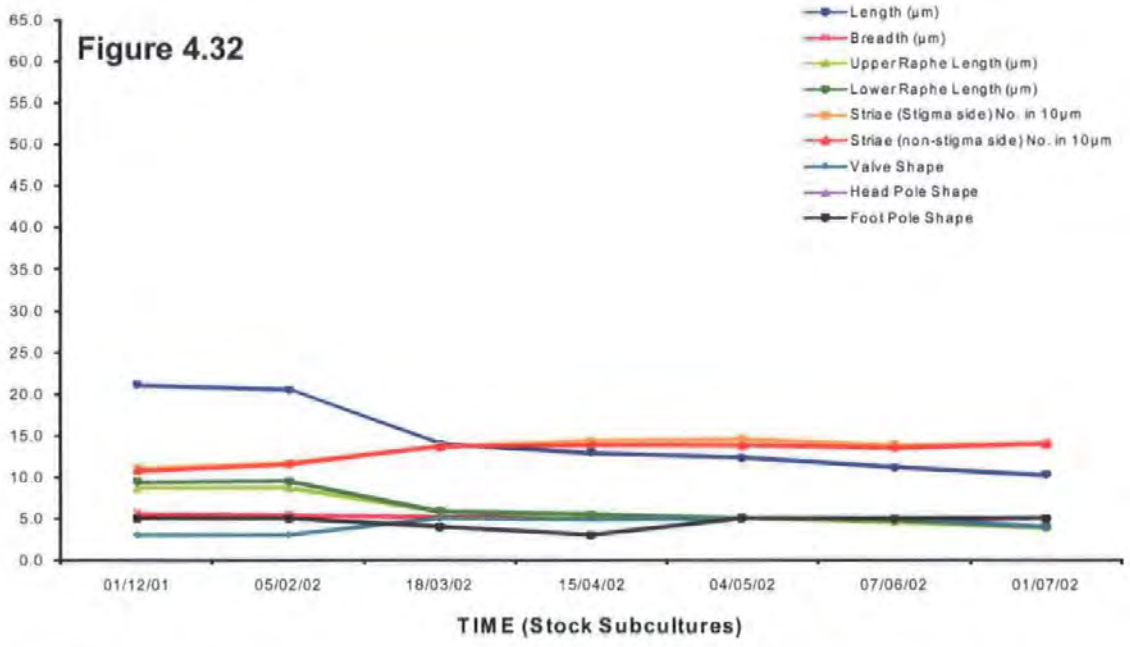


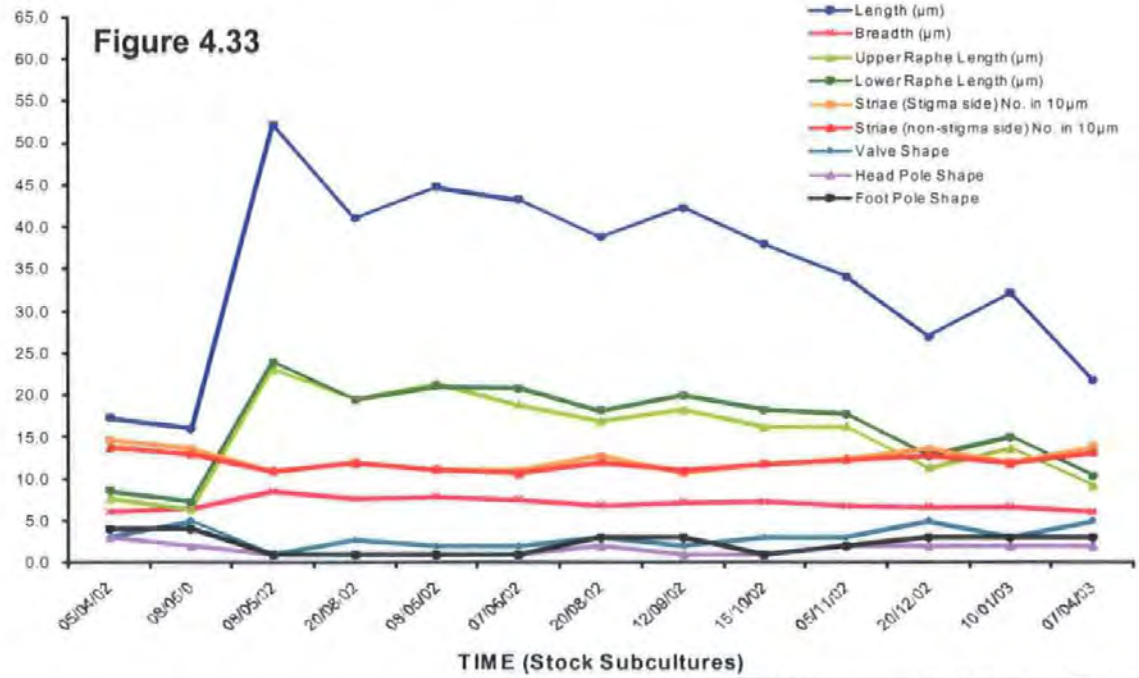


Valve Shape	Head / Foot Pole Shape
1 Narrow lanceolate	1 Rounded
2 Lanceolate	2 Rounded-subrostrate
3 Lanceolate-clavate	3 Subrostrate
4 Ovate	4 Subrostrate-rostrate
5 Ovate-clavate	5 Rostrate
6 Elliptic	6 Rostrate-subcapitate
	7 Subcapitate

Figures 4.30 and 4.31

Line Graphs for Kings Mere and Ham Gate Pond clones respectively. **NB:** The y-axis is a multiunit scale and the values for the characters of valve and pole shape are given in the table bottom right. Note the second auxosporulation at 07/06/02 for Ham Gate Pond.

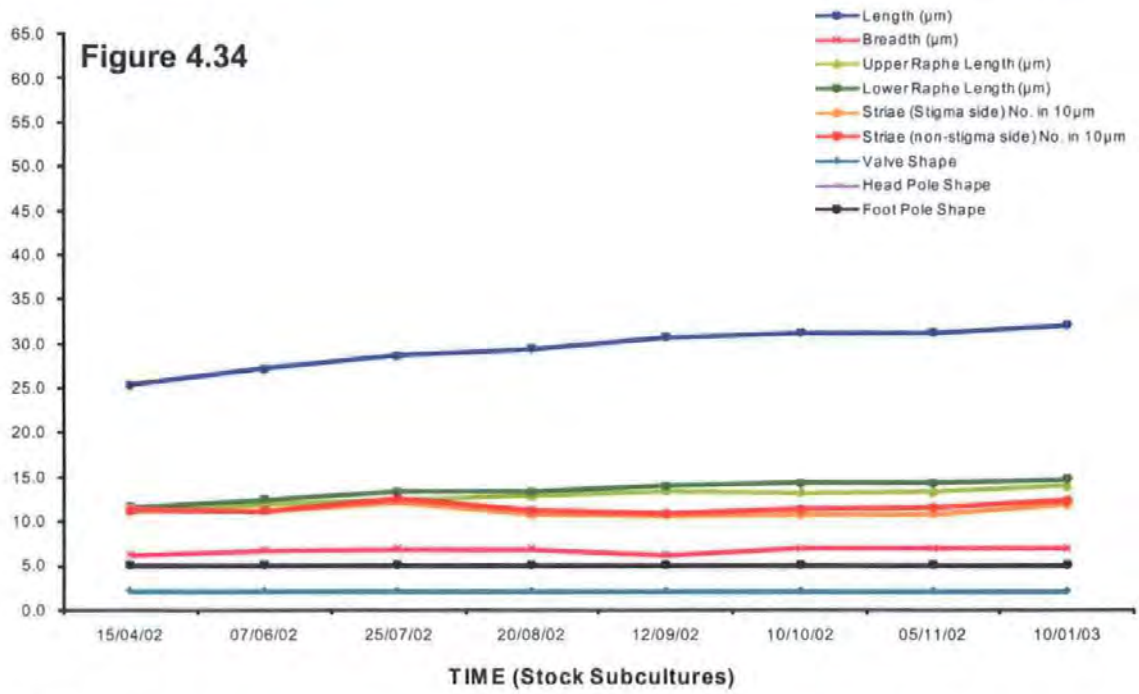


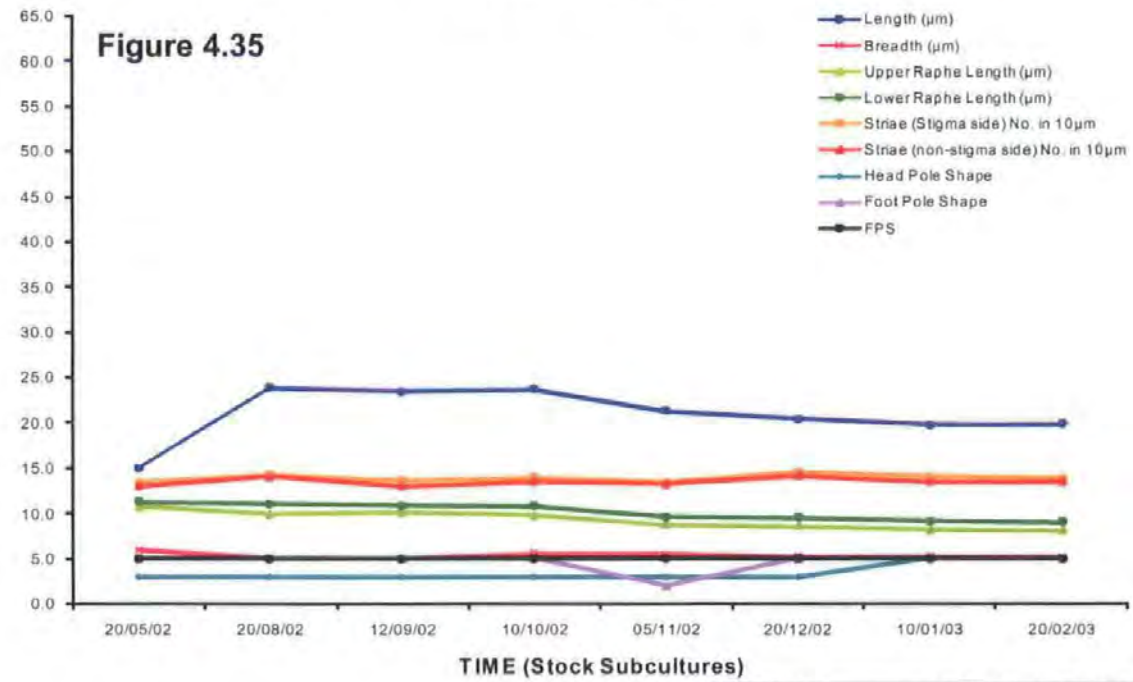


Valve Shape	Head / Foot Pole Shape
1 Narrow lanceolate	1 Rounded
2 Lanceolate	2 Rounded-subrostrate
3 Lanceolate-clavate	3 Subrostrate
4 Ovate	4 Subrostrate-rostrate
5 Ovate-clavate	5 Rostrate
6 Elliptic	6 Rostrate-subcapitate
	7 Subcapitate

Figures 4.32 and 4.33

Line Graphs for Abbey Lakes Pond and Llyn Idwal clones respectively. **NB:** The y-axis is a multiunit scale and the values for the characters of valve and pole shape are given in the table bottom right. Note the second auxosporulation at 08/05/02 for Llyn Idwal.

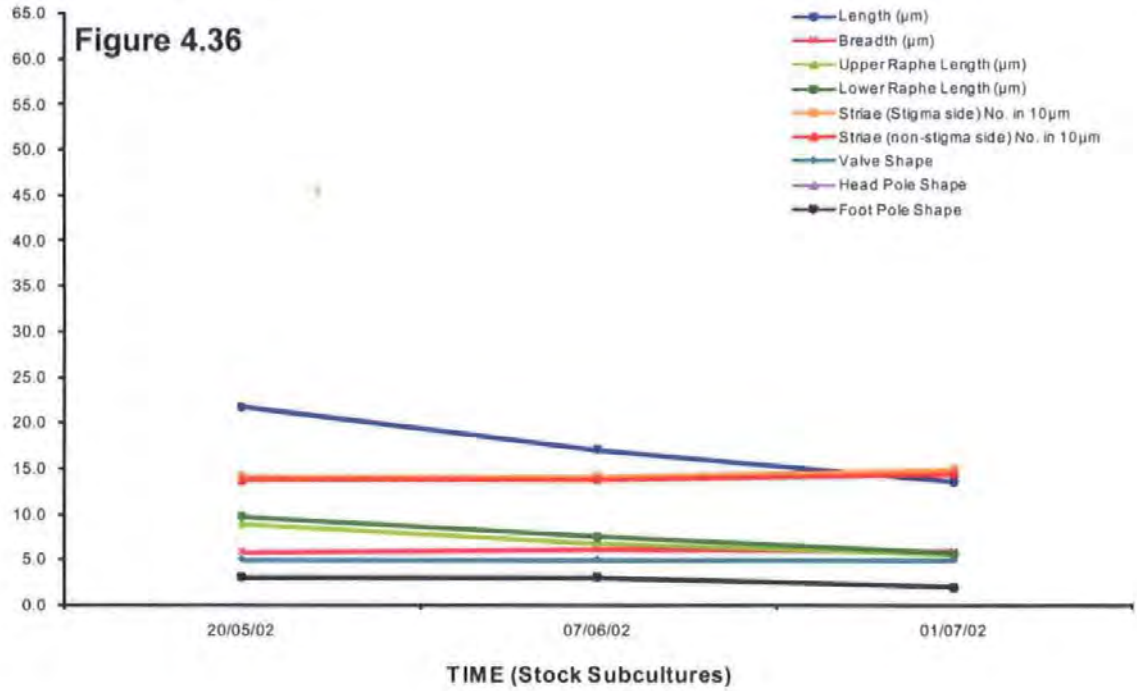


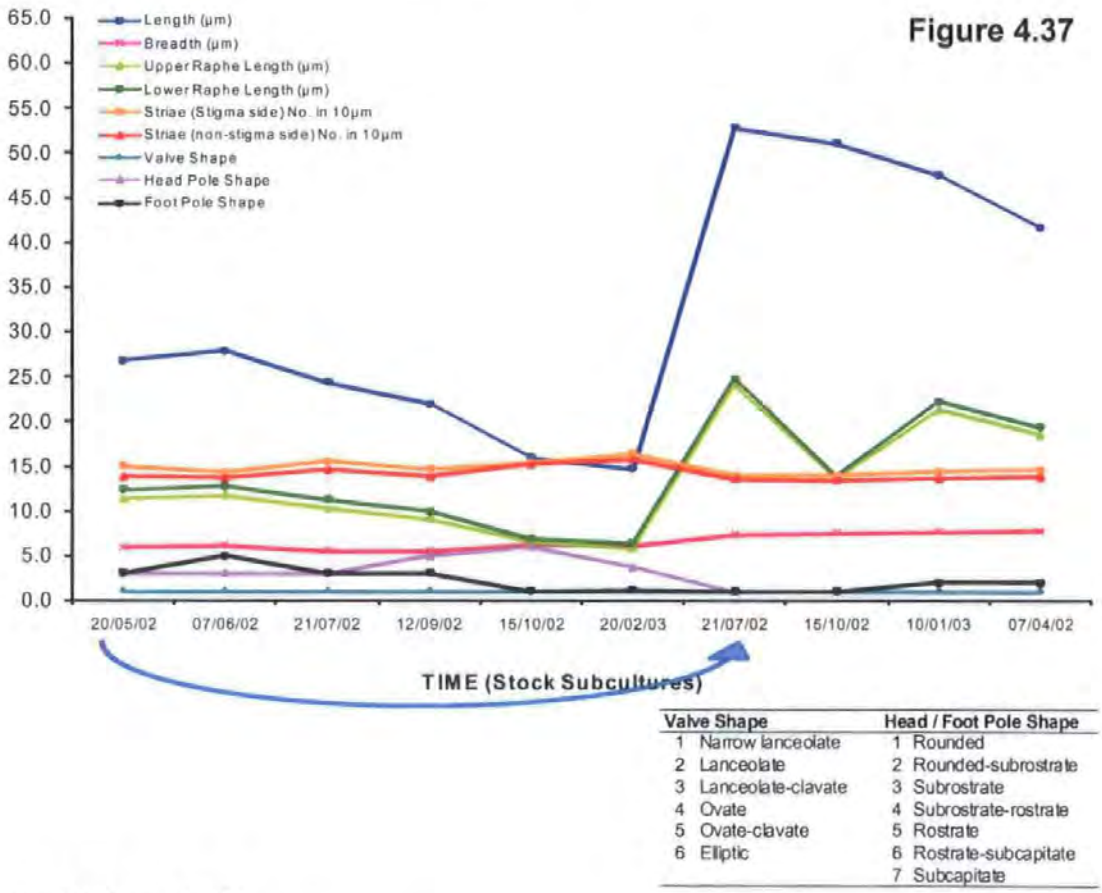


Valve Shape	Head / Foot Pole Shape
1 Narrow lanceolate	1 Rounded
2 Lanceolate	2 Rounded-subrostrate
3 Lanceolate-clavate	3 Subrostrate
4 Ovate	4 Subrostrate-rostrate
5 Ovate-clavate	5 Rostrate
6 Elliptic	6 Rostrate-subcapitate
	7 Subcapitate

Figures 4.34 and 4.35

Line Graphs for River Kennet and Pen-y-Bryn clones respectively. **NB:** The y-axis is a multiunit scale and the values for the characters of valve and pole shape are given in the table bottom right. Note that the apparent auxosporulation at 20/08/02 for Pen-y-Bryn was not observed and other than a change in length there was no evidence that sexual reproduction had taken place.





Valve Shape	Head / Foot Pole Shape
1 Narrow lanceolate	1 Rounded
2 Lanceolate	2 Rounded-subrostrate
3 Lanceolate-clavate	3 Subrostrate
4 Ovate	4 Subrostrate-rostrate
5 Ovate-clavate	5 Rostrate
6 Elliptic	6 Rostrate-subcapitate
	7 Subcapitate

Figures 4.36 and 4.37

Line Graphs for Llyn Ogwen and Parys Mountain clones respectively. **NB:** The y-axis is a multiunit scale and the values for the characters of valve and pole shape are given in the table bottom right. The arrow points to the parent clone which gave rise to the G_2 generation.

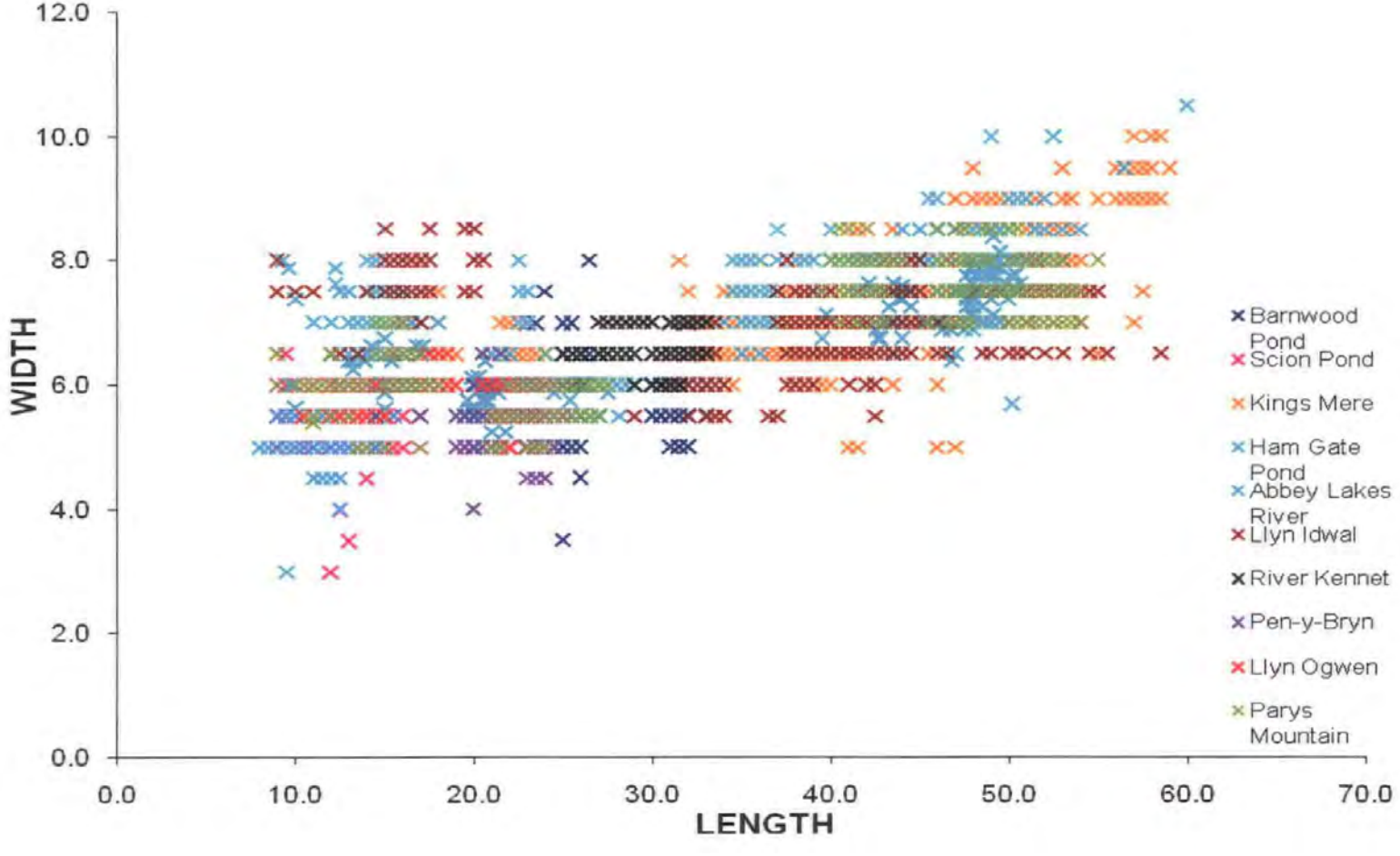
Table 4.2

Percent occurrence of qualitative characters and character states for each *G. parvulum* clone in stock culture. All values are percentage occurrences. * = no value.

	Barnwood Pond	Scion Pond	Kings Mere	Ham Gate Pond	Abbey Lakes River	Llyn Idwal	River Kennet	Pen-y-Bryn	Lly Ogwen	Parys Mountain
Non-central short stria										
Absent 0	100.00	93.50	90.95	96.89	99.65	96.66	98.75	95.75	97.33	98.36
Present 1	*	1.50	9.05	3.11	0.35	3.34	1.25	4.25	2.67	1.64
Inserted striae (IS)										
Absent 0	97.71	97.25	95.71	93.91	98.96	95.48	97.75	95.75	88.67	94.73
Present 1	2.29	2.25	4.29	5.97	1.04	4.52	2.25	4.25	10.67	5.27
Interrupted striae (BS)										
Absent 0	99.43	98.75	99.05	97.73	100.00	99.50	99.75	99.75	94.67	98.18
Present 1	0.57	1.25	0.95	2.27	*	0.50	0.25	0.25	5.33	1.82
Striae Direction (SD)										
Radiate 1	100.00	100.00	99.05	100.00	100.00	99.50	100.00	100.00	100.00	100.00
Convergent 5	*	0.00	0.95	*	*	0.50	*	*	*	*
Valve shape										
Narrow lanceolate 1	*	0.00	30.48	5.97	*	8.36	*	*	*	18.91
Lanceolate 2	95.71	37.50	7.94	11.47	0.35	28.86	100.00	*	*	26.73
Lanceolate-clavate 3	4.29	12.50	57.94	51.37	16.61	36.79	*	75.00	*	27.27
Ovate 4	*	*	*	*	14.19	*	*	*	*	8.36
Ovate-clavate 5	*	50.00	3.65	30.82	68.86	26.09	*	*	*	9.64
Elliptic 6	*	*	*	0.36	*	*	*	25.00	100.00	9.09
Head pole shape (HPS)										
Rounded 1	*	12.50	50.00	31.30	*	46.49	*	*	*	44.18
Rounded-subrostrate 2	4.29	50.00	20.63	29.75	*	50.17	*	12.50	33.33	18.18
Subrostrate 3	*	*	16.35	38.47	16.96	3.34	*	*	66.67	28.55
Subrostrate-rostrate 4	*	*	*	*	17.30	*	*	*	*	*
Rostrate 5	38.57	*	13.02	0.48	65.74	*	100.00	87.50	*	9.09
Rostrate-subcapitate 6	57.14	37.50	*	*	*	*	*	*	*	*
Subcapitate 7	*	0.00	*	*	*	*	*	*	*	*
Foot pole shape (FPS)										
Rounded 1	*	12.50	30.48	30.35	*	38.13	*	*	*	44.18
Rounded-subrostrate 2	4.29	87.50	36.51	*	*	8.36	*	*	33.33	18.18
Subrostrate 3	*	*	7.94	41.34	16.96	41.81	*	*	66.67	28.55
Subrostrate-rostrate 4	*	*	3.65	5.85	17.30	11.71	*	*	*	*
Rostrate 5	95.71	*	21.43	22.46	65.74	*	100.00	100	*	9.09
Rostrate-subcapitate 6	*	*	*	*	*	*	*	*	*	*
Subcapitate 7	*	*	*	*	*	*	*	*	*	*
Abnormalities (ABN)										
Absent 0	99.71	97.75	98.41	97.01	94.81	99.50	99.50	99.75	100.00	98.18
Present 1	0.29	2.25	1.59	2.99	5.19	0.50	0.50	0.25	*	1.82

Figure 4.38

Scatterplot of Length versus Breadth (µm) for all clones.



Apart from length and breadth, which were expected to decrease over time, stria density increased in tandem with a reduction in length in clones from Barnwood Pond, Scion Pond, Abbey Lakes River, River Kennet and Llyn Ogwen, and less markedly so in clones from Ham Gate Pond, Llyn Idwal and Pen-y-Bryn. Two clones (Kings Mere and Llyn Idwal) exhibited non-radiate (convergent) striae in some valves, occurring in less than 1% of the population. The number of short central striae varied considerably from none to four, although the majority of valves were observed to have one. The propensity to produce two or three short central striae opposite the stigma was common among all the clones, although the Scion Pond clone was the most variable in this respect with a quarter of all valves exhibiting no short striae, and three quarters one short stria. Clones from Abbey Lakes River and the River Kennet never had more than one short central stria, though they may also have been absent.

Rarely more than 5% of a population of any clone exhibited either other non-central short striae or inserted striae, with the exception of the Kings Mere (9%, other short stria), Ham Gate Pond (6%, inserted striae) and Llyn Ogwen (11%, inserted striae) clones. The presence of interrupted striae occurred in $\leq 2\%$ of any clonal population with the exception of the Llyn Ogwen clone in which occurrence was 5%.

Clones from River Kennet and Llyn Ogwen did not vary in shape, maintaining lanceolate and ovate-clavate shapes respectively, regardless of size or culture age. The most variable clone was from Parys Mountain, in which all six valve shapes occurred across the full size range, though lanceolate was the dominant form, occurring in 45% of the population. Only clones from Ham Gate Pond and Parys Mountain produced elliptic

valves, and only at the lower end of the size range. All listed pole shapes, except subcapitate, occurred at both apices. Clones from Barnwood Pond, Scion Pond and Llyn Idwal were unlike the other clones, having rostrate-subcapitate, rounded-subrostrate and rounded to rounded-subrostrate head poles, respectively.

Abnormalities rarely occurred in more than 3% of any clonal population. Generally, abnormalities occurred between 1.6 and 3.0 % of a population, with the exception of the clone from Abbey Lakes River in which 5% abnormalities were recorded, and the Llyn Ogwen clone in which no gross abnormalities were observed. The types of abnormality observed are illustrated in **Figures 4.39 and 4.40**, and include major disruptions to stria configuration, buckling of the raphe, and convergent striae. There are no significant differences in the percent occurrence of abnormalities between any paired clones.

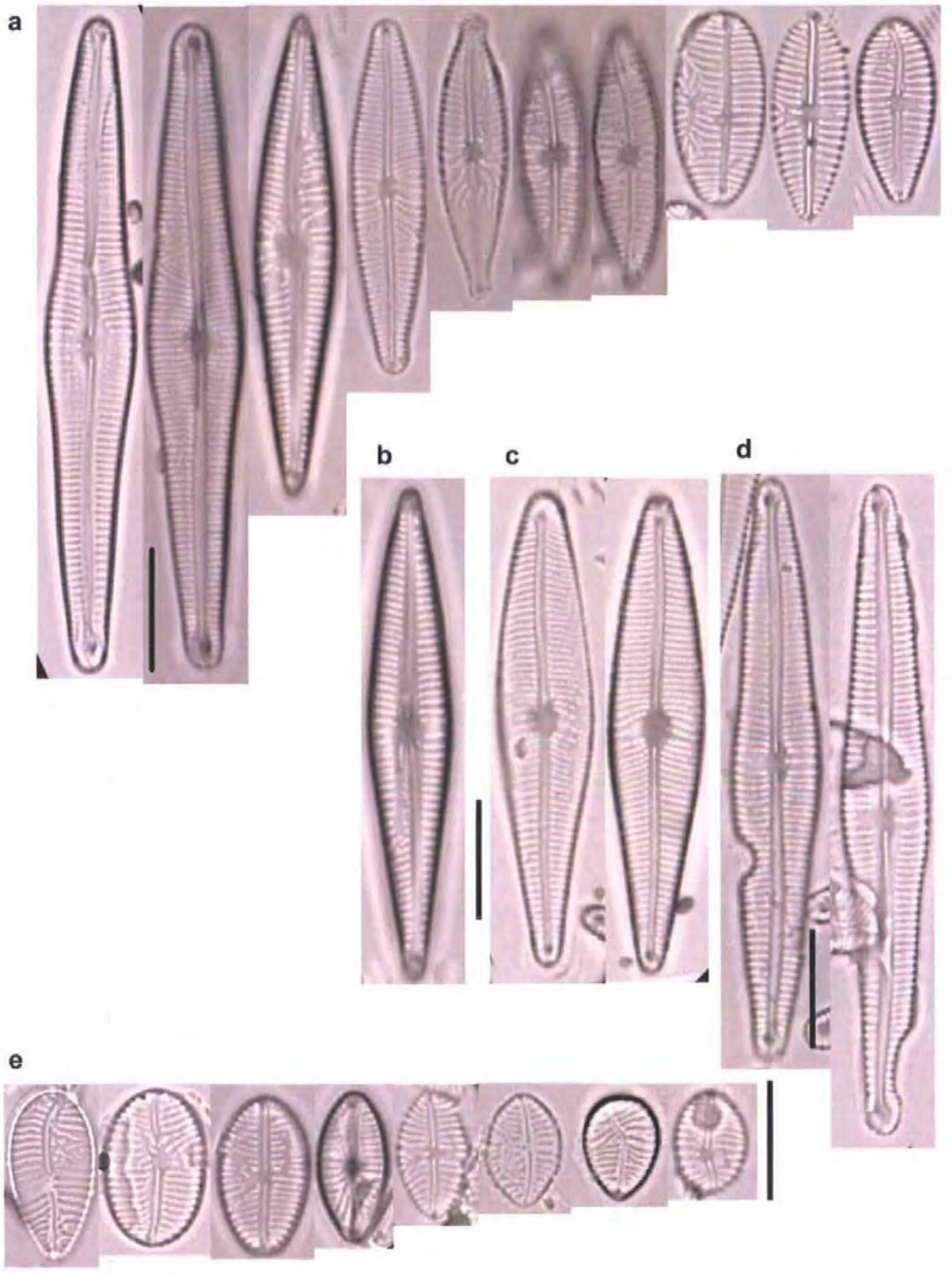


Figure 4.39: a- e

Examples of abnormalities in *G. parvulum* clones. **(a)** disruption to striae pattern **(b)** disruption both striae and raphe, note that the lower raphe is flanked by two shorter pseudo-raphes **(c)** convergent stria, possibly Janus cells **(d)** "dents" to valve shape **(e)** striae and raphe disturbances at the lower end of the size range, note the buckling of the raphe in some. All scale bars = 10μm.

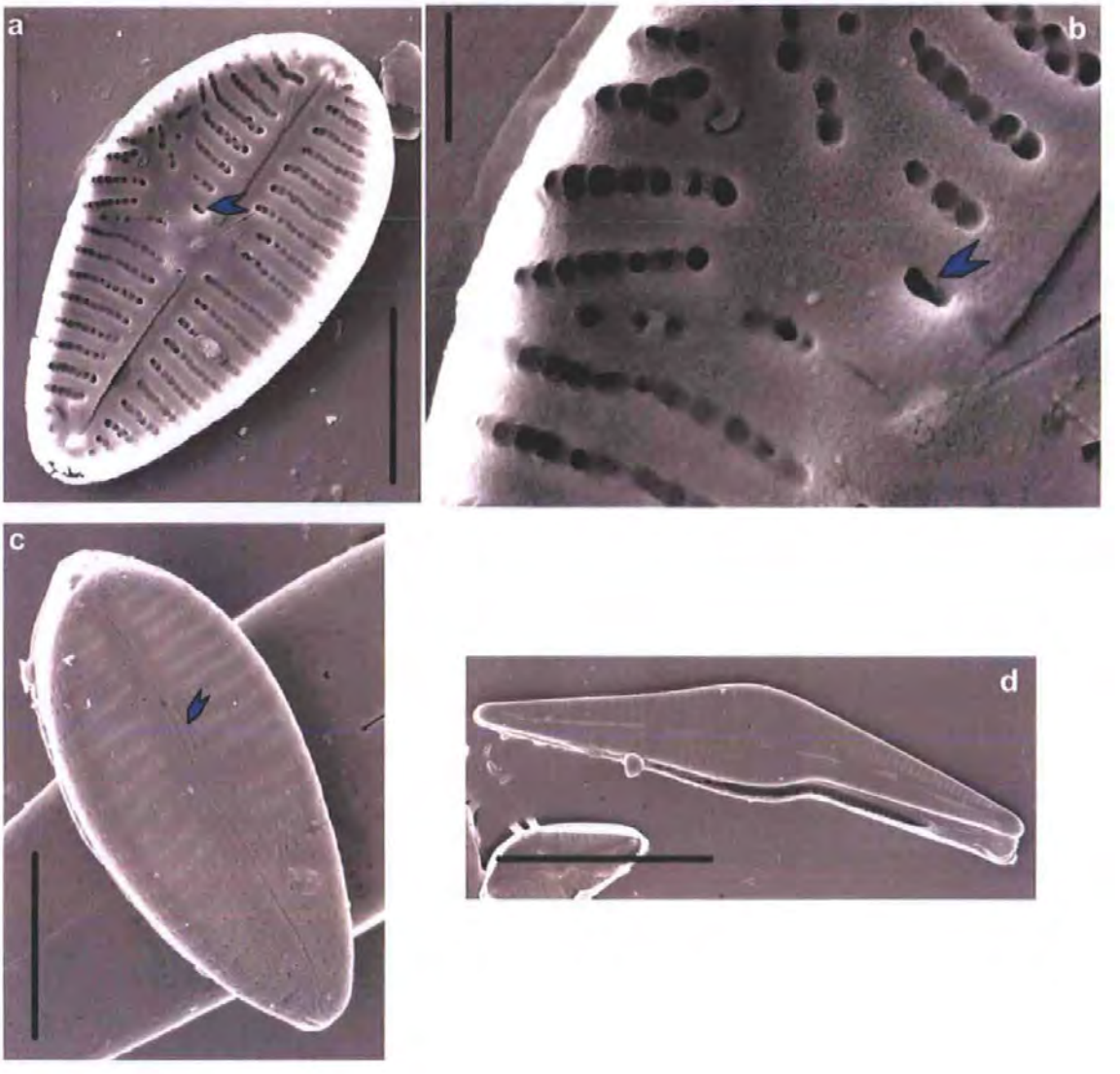


Figure 4.40: a- d

Examples of abnormalities in *G. parvulum* clones. **(a)** and **(b)** internal view showing disruption to striae pattern. Note the raised nature of the areolae in (a) and that the stigma has been repositioned (arrow) **(c)** disruption to the raphe system. Note that the upper raphe is flanked by a shorter pseudo-raphe **(d)** abnormal valve shape. Note the "cymbelloid" aspect and expanded central area. This possibly an initial cell. Scale bars = (a) 5μm, (b) 1μm, (c) 5μm and (d) 20μm.

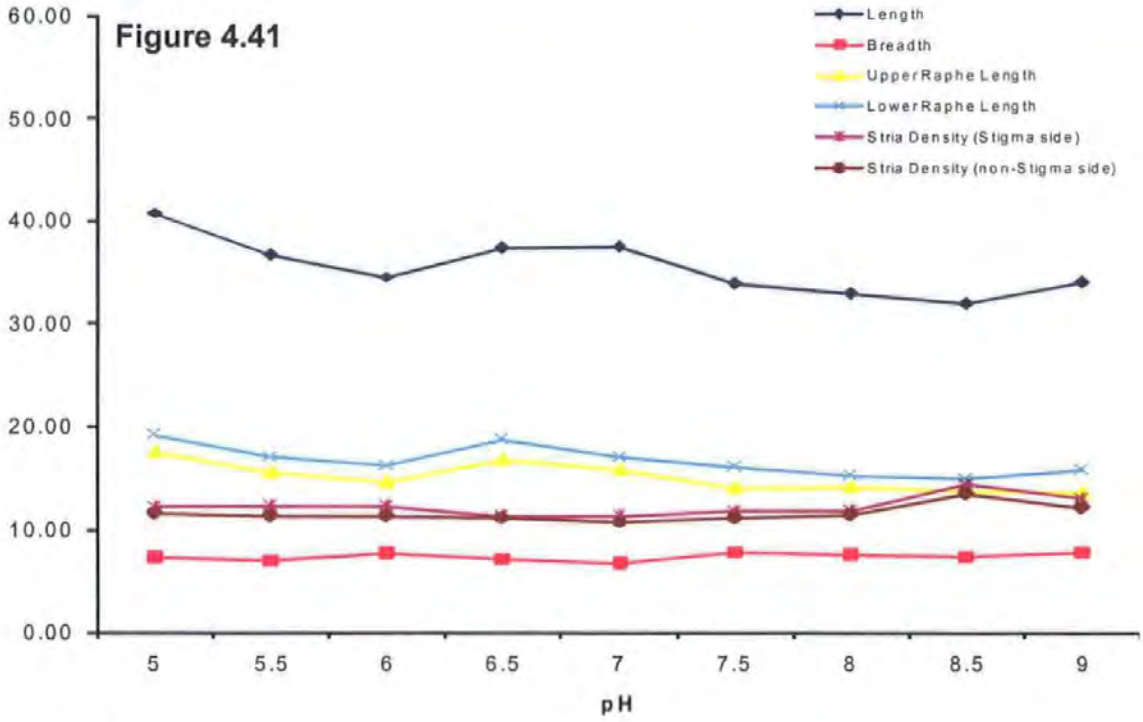
4.4.2 Morphometric Analysis of Experimental Assays

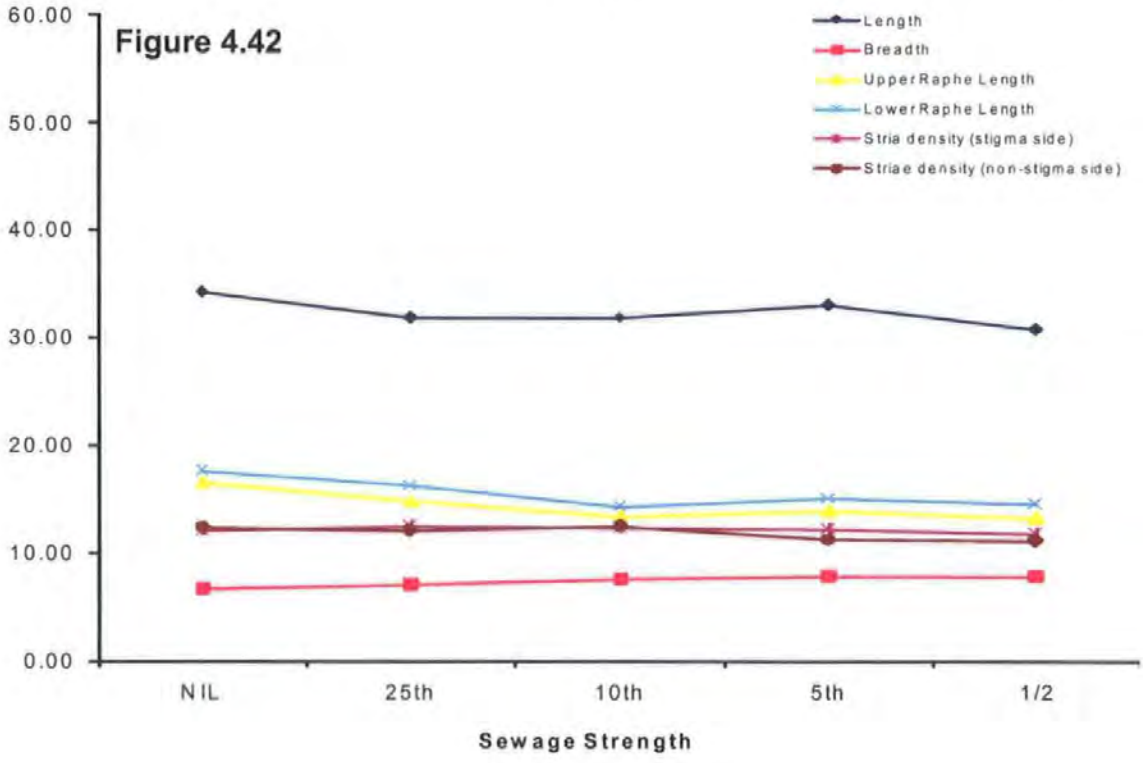
Assayed subcultures were grown for 4-8 weeks. Descriptive statistics for quantitative measures and percent occurrences for each character are provided on the enclosed CD. Mean valve length and breadth, raphe length and central stria density for each assay level are presented as a series of line graphs, where relevant, for each clone in **Figures 4.41 to 4.52**. are also on the enclosed CD.

There was very little morphological change in any clone for the quantitative characters of breadth, raphe length and striae density. Raphe length as expected, increased or decrease in tandem with length, and striae density varied little other than when auxosporulation occurred or another unexplained change in length occurred. Length was the only character to change significantly, but only in a few clones and some assays. There was a decrease in length with increasing assay concentration and/or pH in clones from Kings Mere (pH regime) and Llyn Idwal (sewage, copper – initial and extended runs)**(Figures 4.41 and 4.43)**. In the initial run of the copper regime however, the River Kennet clone showed a sudden decrease in size at concentration of 0.00016µg/l **(Figure 4.44)**. There was no evidence of auxosporulation in this clone.

Auxosporulation occurred in clones from Scion Pond (copper concentration = 000.16µg/l), Ham Gate Pond (pH 8.5-9.0, $1/5^{th}$ strength sewage), Llyn Idwal (zinc concentration 0.0053µg/l) and Llyn Ogwen (pH 6.5) **(Figures 4.45 to 4.49)**. There were also other sudden increases in valve length not associated with auxosporulation in clones from Scion Pond (pH 9.0), Pen-y-Bryn (pH 5.5 onwards) and Parys Mountain (G_1) (copper concentration 0.0026 and 0.0053µg/l) **(Figures 4.50 to 4.52)**. The sudden increases

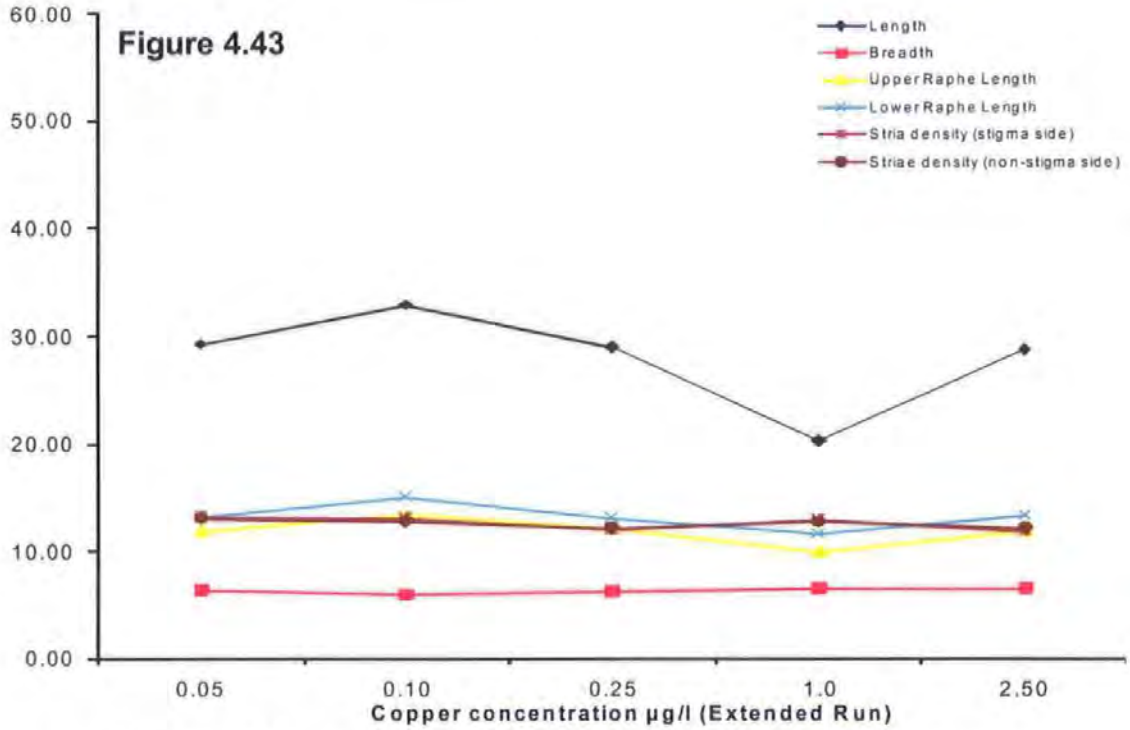
were not so large that they might be considered post auxospore cells, as these are usually > 45µm long, particularly in the Parys Mountain clone, though it is possible that the Scion Pond and Pen-y-Bryn clones produce smaller auxospores. However, there was no evidence to suggest that auxosporulation had taken place, e.g. mix of parent, auxospore and initial cell valves.

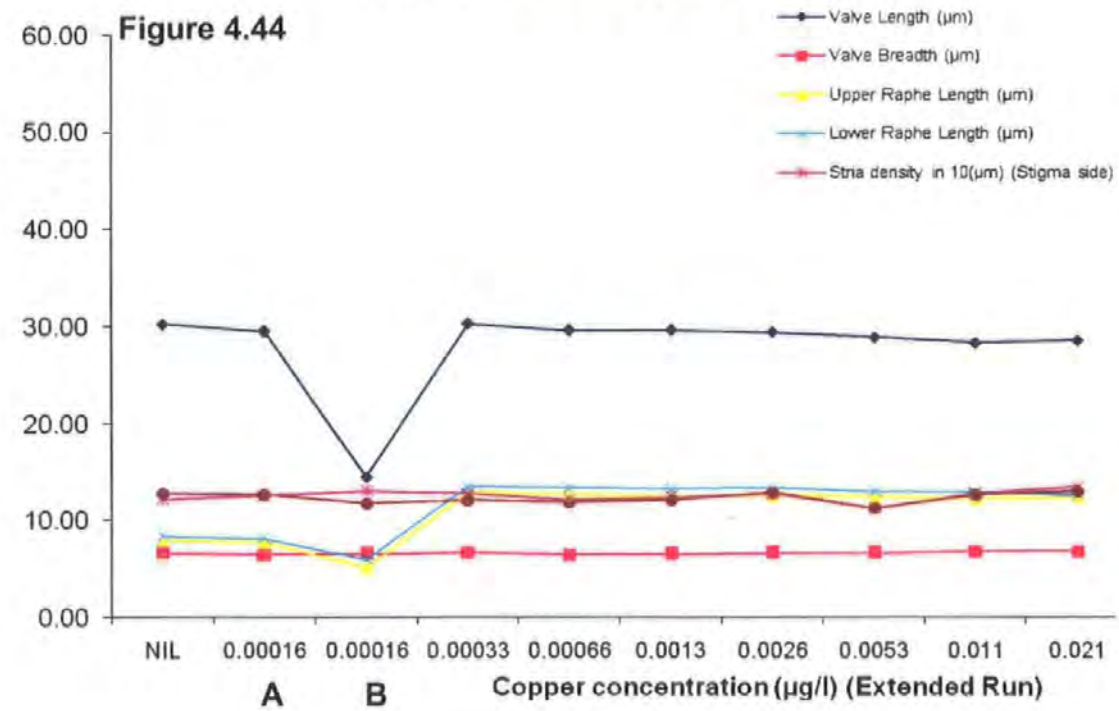




Figures 4.41 and 4.42

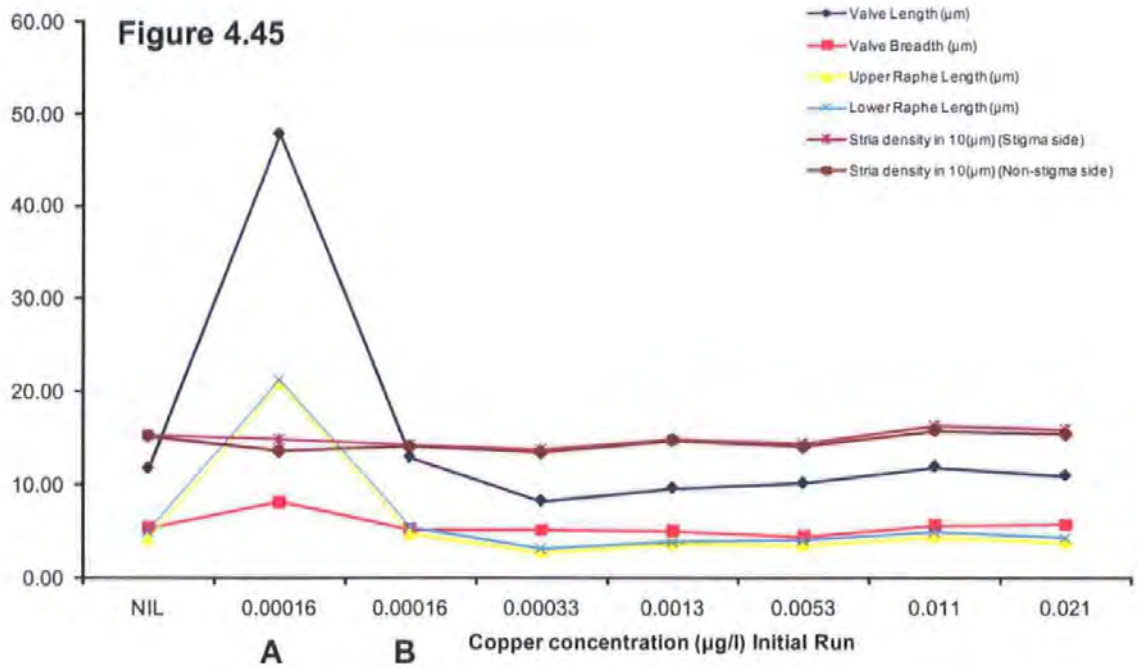
Line graphs for Kings Mere (pH Regime) and Llyn Idwal (Sewage Regime) clones respectively, showing mean length, breadth, raphe length and central striae density in 10µm.

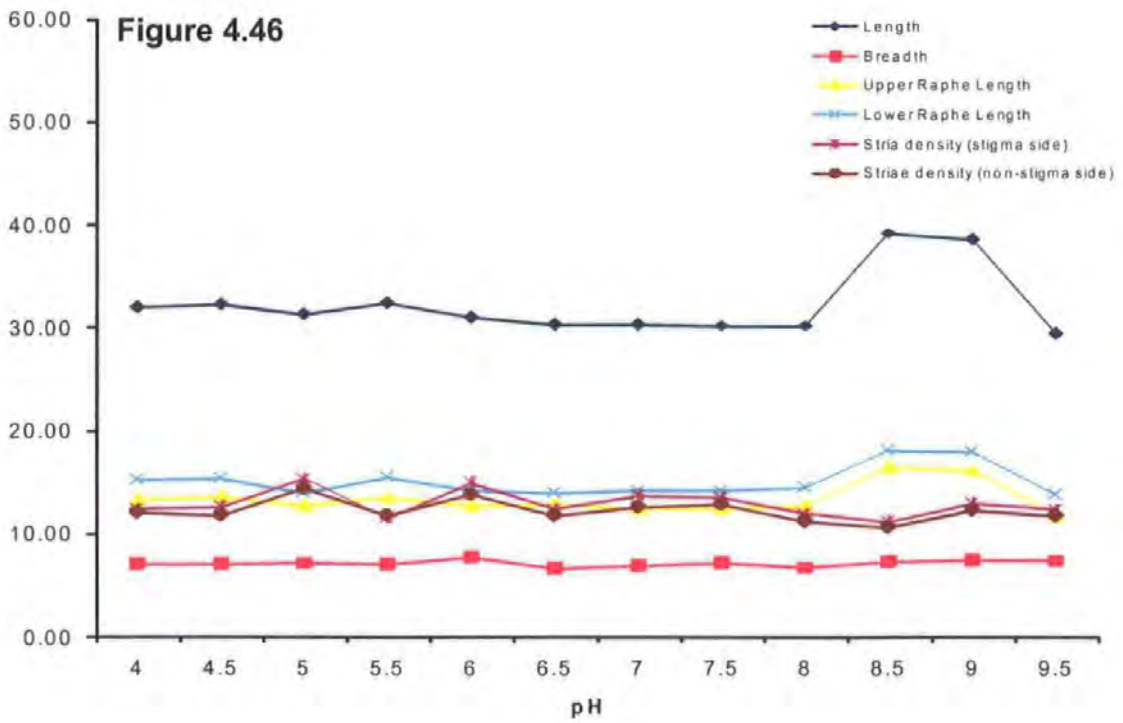




Figures 4.43 and 4.44

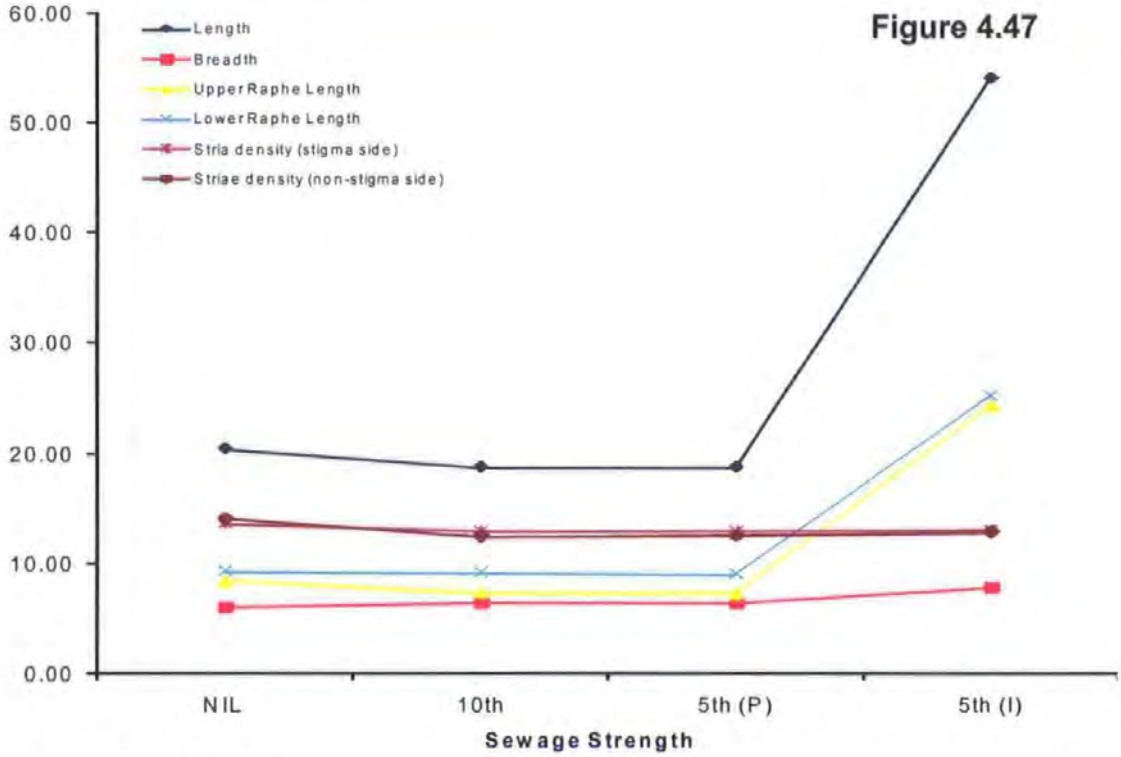
Line graphs for Llyn Idwal (Copper Regime) and River Kennet (Copper Regime) clones respectively, showing mean length, breadth, raphe length and central striae density in 10µm. **NB:** A and B at .00016µg/l indicate that there were two sets of valve length in this clone but no evidence of auxosporulation.

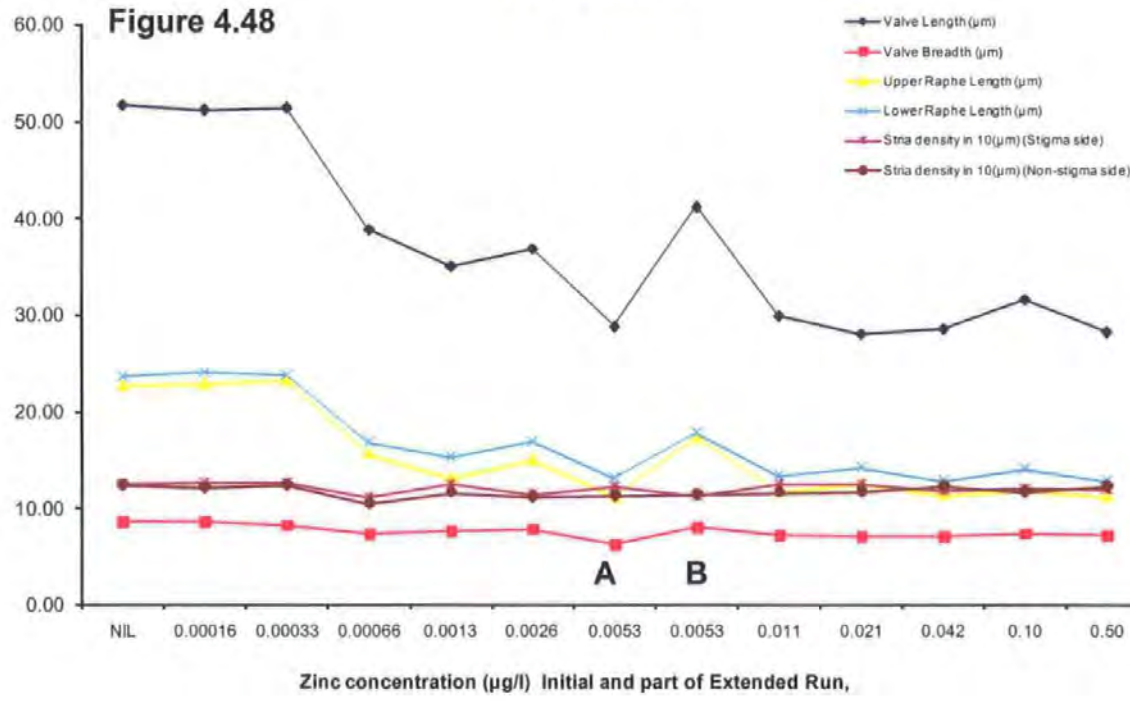




Figures 4.45 and 4.46

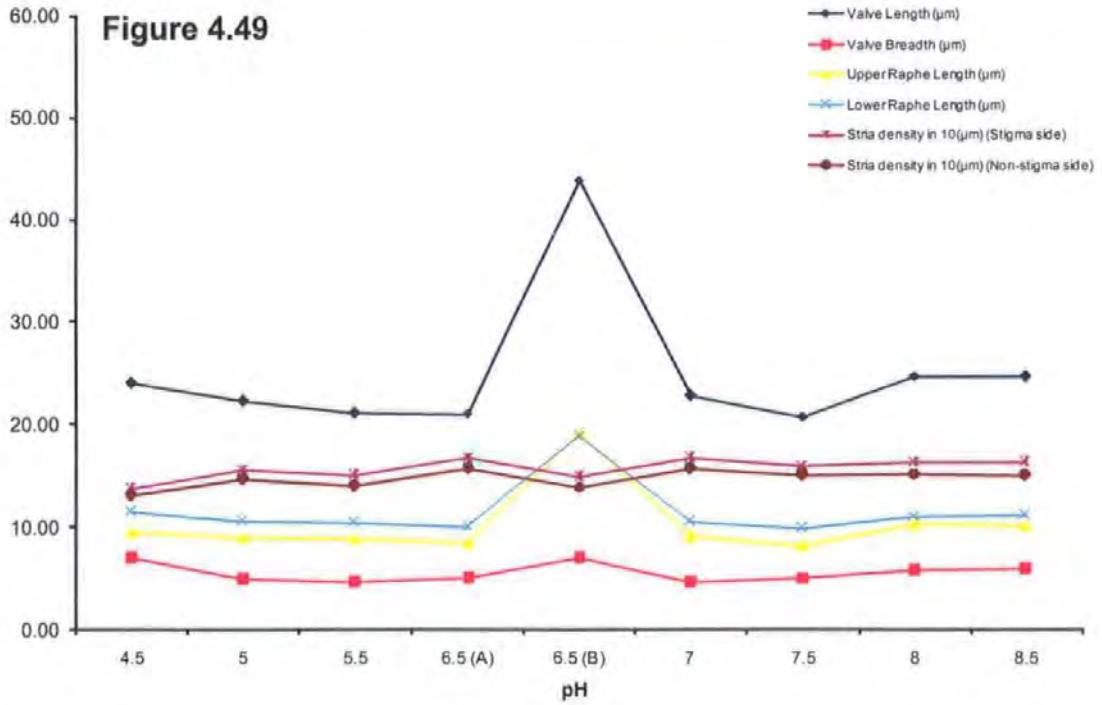
Line graphs for Scion Pond (Copper Regime) and Ham Gate Pond (pH Regime) clones respectively, showing mean length, breadth, raphe length and central striae density in 10µm. **NB:** (A) = Post auxospore cells and (B) = parents that gave rise to post auxospore cells.

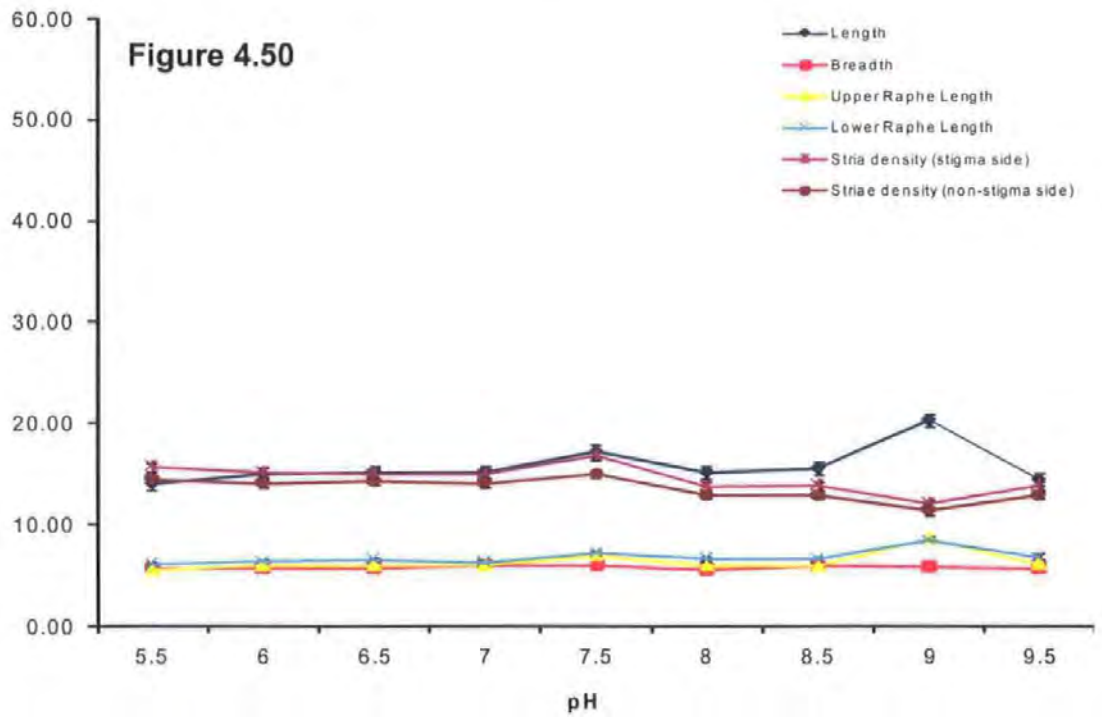




Figures 4.47 and 4.48

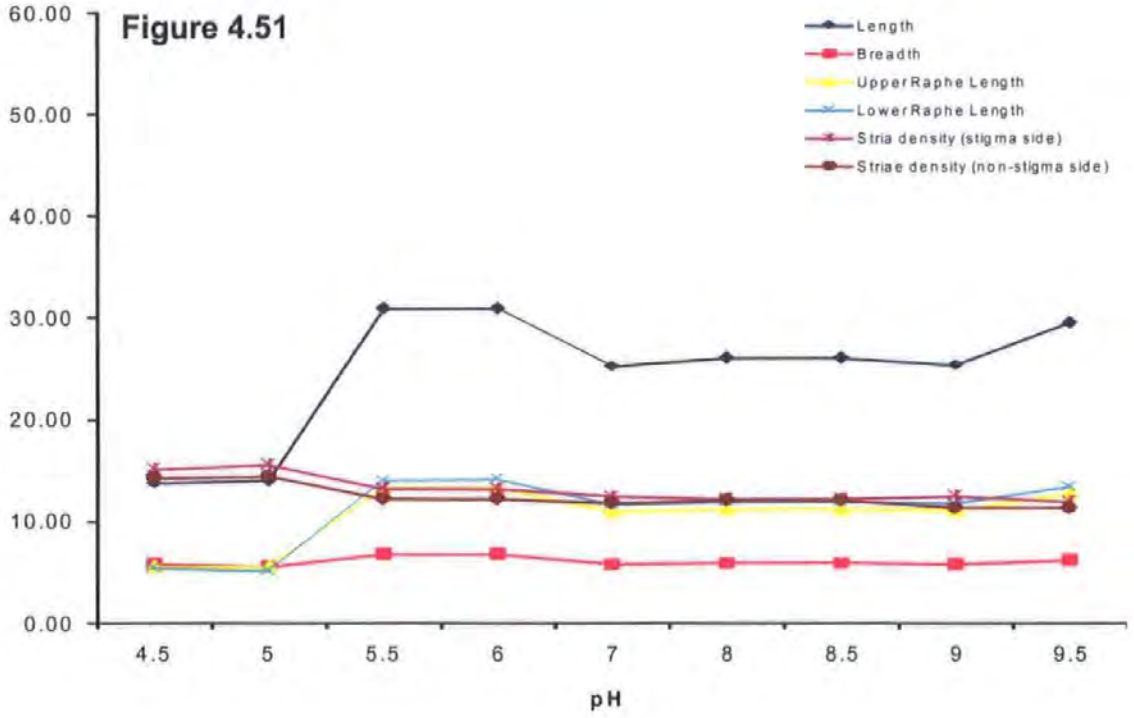
Line graphs for Ham Gate Pond (Sewage Regime) and Llyn Idwal (Zinc Regime) clones respectively, showing mean length, breadth, raphe length and central striae density in 10µm. **NB:** (A) = parents that gave rise to post auxospore cells and (B) = Post auxospore cells.

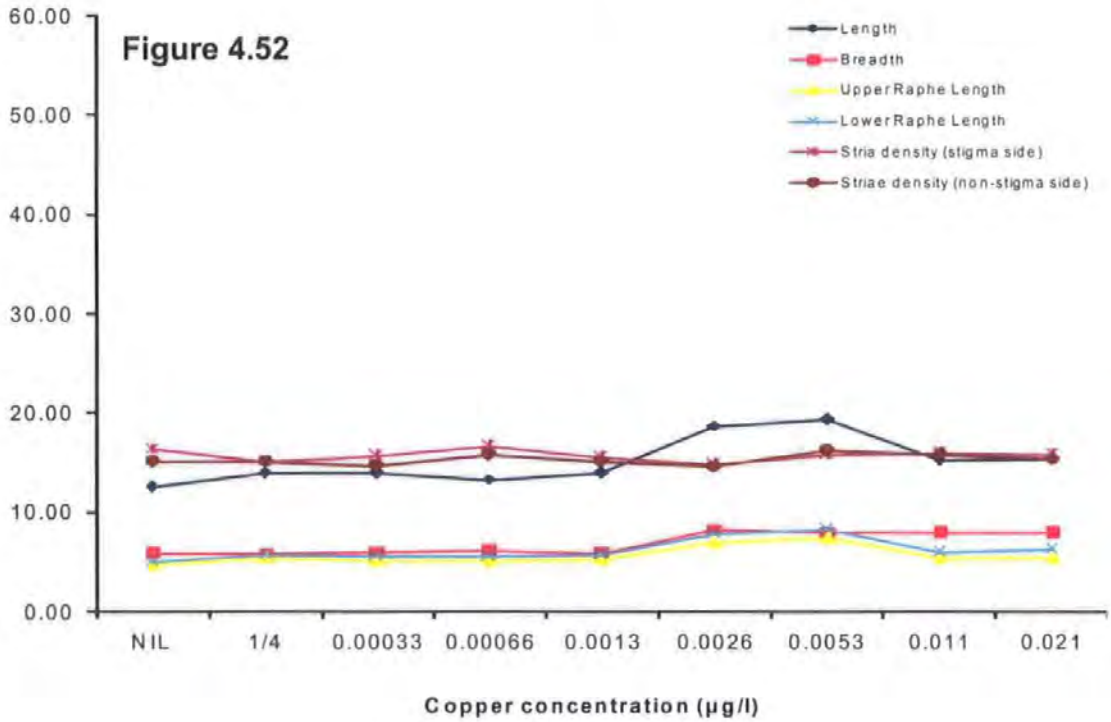




Figures 4.49 and 4.50

Line graphs for Llyn Ogwen (pH Regime) and Scion Pond (pH Regime) clones respectively, showing mean length, breadth, raphe length and central striae density in 10µm. **NB:** (A) = parents that gave rise to post auxospore cells and (B) = Post auxospore cells and.





Figures 4.51 and 4.52

Line graphs for Pen-y-Bryn (pH Regime) and Parys Mountain (G_1) (Copper Regime) clones respectively, showing mean length, breadth, raphe length and central striae density in 10µm.

Qualitative characters included observations for striae, valve shape and apex shape. Striae direction did not depart from radiate in any clone for any assay with the exception of clones from Ham Gate Pond at pH 6.0 (10% convergent) and Llyn Idwal at pH 59/01 5.5, 7.5, 8.0, 8.5 and 10 (10% convergent and 20% convergent at pH 10 respectively). The River Kennet clone produced no central short stria in the pH and copper regimes, however some valves in the cadmium regime were observed to have two short central striae, whilst and 2-3 short striae were observed in the pH, copper and zinc regimes. All had occurrences between 10-30%. This clone also produced valves with 4 central short stria in the copper regime at a concentration of 0.00016mg/l (10% of population). Compared to stock cultures, the occurrence of more than 1 or no short central striae increases with increasing pH, sewage and metal concentration.

The presence of non-central short striae, inserted striae and broken striae, occurred in all assays for nearly all clones. These three characters occurred more often in the metal (particularly copper) and pH regimes, whilst the sewage regime had relatively few occurrences of these characters. Clones from River Kennet and Parys Mountain (G_2) under the sewage regime, and from Scion Pond and Parys Mountain (G_2) under the zinc regime, did not produce any of these three characters. Generally, non-central short striae occurred more often than inserted or broken striae, and inserted striae occurred more often than broken striae. The occurrence of inserted or broken striae never exceeded 60% and 50% of the assay population respectively, though these high occurrences were rare. A 10-30% occurrence was more common. The occurrence of non-central short striae ranged between 0 and 100%, although 0-40% was more common. Clones from Kings Mere, Ham Gate Pond, River Kennet and Parys Mountain (G_2) had the highest occurrence of non-central short striae (all ≥50%) in the pH regime, whereas in the copper regime, clones from Scion Pond, Ham Gate Pond, Llyn Idwal and Parys Mountain (G_1)

had the highest occurrence. Clones from Ham Gate Pond and Parys Mountain had high occurrences of this character in the cadmium regime.

Both valve shape and apical pole shape remained unchanged, regardless of assay concentration, in clones from Scion Pond (pH and sewage regime), Kings Mere and Parys Mountain (G_1 and G_2) (zinc regime), Ham Gate Pond (copper and zinc regimes), Llyn Idwal (cadmium regime), River Kennet (zinc and cadmium regimes) and Pen-y-Bryn (copper and cadmium regimes). In clones from Llyn Ogwen and Parys Mountain (G_1), changes in valve and apical pole shape appear linked to increasing pH concentration (excluding auxosporulation). In the River Kennet clone, valve shape changes if copper is omitted, whilst in the Parys Mountain clone (G_1) head pole shape changes if copper concentration increases.

Abnormalities occurred within 10-20% of an assayed population. Two exceptions were the Parys Mountain clone (G_1) at a copper concentration of 0.011µg/l (40% of the population) and the Ham Gate Pond clone at cadmium concentrations of 5.0 and 12.5µg/l (30 and 40% of the population respectively). The clone from Barnwood Pond was most afflicted with abnormalities (in pH, copper and zinc regimes), followed closely by the Ham Gate Pond (in pH, copper, zinc and cadmium regimes). Clones with the least occurrence of abnormalities were Llyn Idwal (copper regime), Llyn Ogwen (pH regime) and Parys Mountain (G_2) (cadmium regime).

4.5 FRUSTULE MORPHOLOGY AND SEXUAL REPRODUCTION

Observation revealed that differences in shape, size, symmetry and valve morphology, between opposing ends of the size spectrum and intermediaries, equate to different taxonomic identities. **Figures 4.8, 4.9, 4.11 and 4.15** for stock cultures, illustrate the changes in size and morphology for each auxosporulating clone and the taxonomic identity of intermediate entities where different. It can be seen that the larger post auxospore cells are morphologically very different from their parent cells and in turn, the smallest cells are very different from the larger and intermediate sized cells. These differences are particularly exaggerated between parental cells prior to auxosporulation and initial cells post auxosporulation, when differences are so great that they would be identified as different species in any wild population. This brings into question the identity of *G. parvulum* and any associated taxon, i.e. *Gomphonema gracile* Ehrenberg and *G. hebridense* Gregory. In particular, early initial cells **(Figures 4.53a-d)** have apical poles that are barely heteropolar and rounded, whilst transapically the cells are often somewhat expanded, and/or the cell is apically asymmetrical. Striae and raphes are often disrupted in initial cells, and the poles often bowed, giving a cymbelloid-like appearance in valve view and corresponding to the concave/convex hypotheca/epitheca observed in stock culture parental cells. The size ranges for sexualised cells are shown in **Table 4.3**.

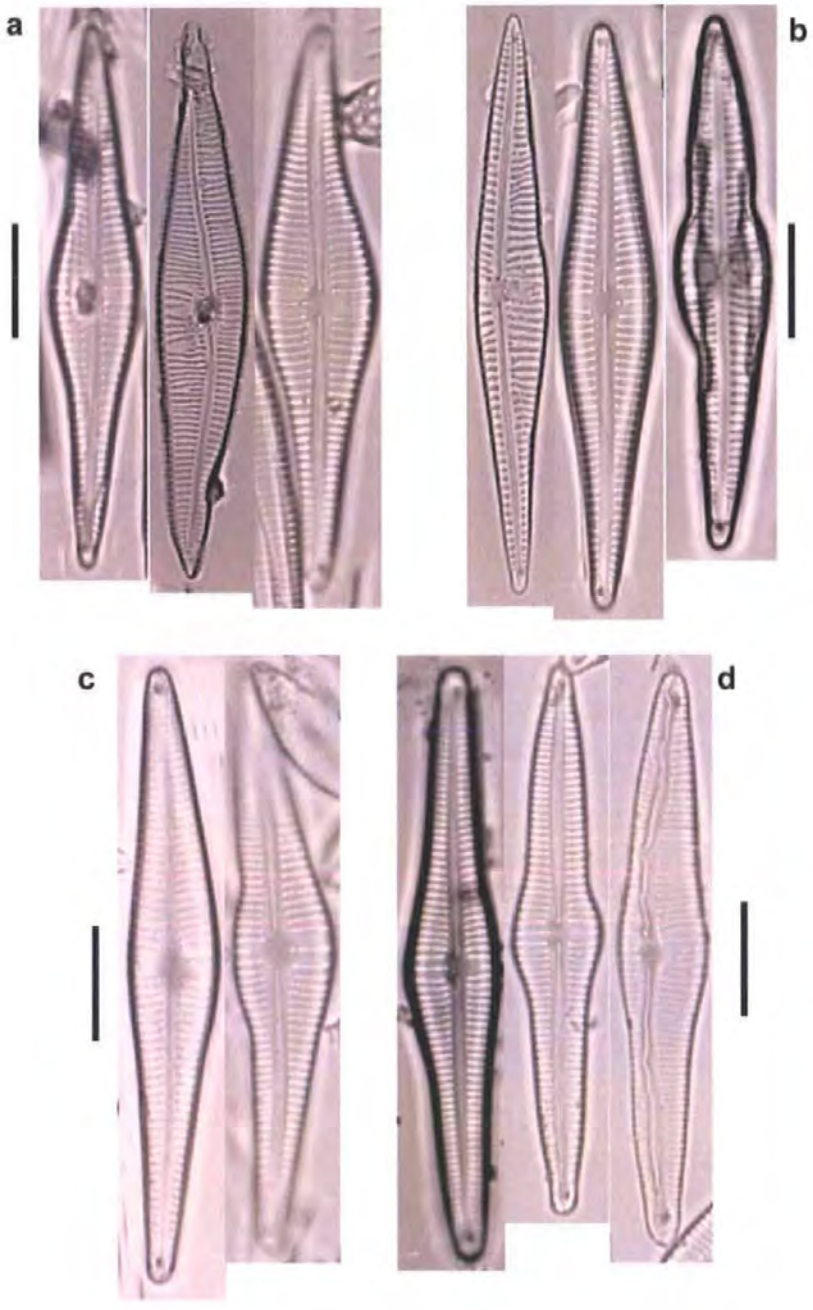


Figure 4.53: a-d

LMs examples of initial cells **(a)** Kings Mere **(b)** Ham Gate Pond (**c**) Llyn Idwal and **(d)** Parys Mountain. All scale bars = 10μm.

Table 4.3

Summary of length and breadth measurements of sexualised clones detailing size for parent and offspring whilst in stock culture.

	Parent Cells				Offspring			
	Length (μm)		Width (μm)		Length (μm)		Width (μm)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Kings Mere	16-19	17.14	6-8	7	56-59	57.4	7-10	9.3
Ham Gate Pond	12-21	15.61	6-10	6.98	47-50.9	49.2	5.6-8.4	7.6
Llyn Idwal	14-17.5	16	6-7	6.5	46-58.5	52.2	4.5-9.5	8.5
Parys Mountain	14.5-18	16	5-7	6.2	48-53	51.1	7-8.5	7.6

4.6 DISCUSSION

The purpose of this study was two fold, (i) to investigate the range of morphologies exhibited by different *G. parvulum* clones by observing how morphology changed with reduction in size and sexual reproduction over several generations, and (ii) what effects certain environmental conditions have on morphology, testing whether morphological variability is a response to changes in those conditions.

4.6.1 Stock Culture Clonal Morphology

Geitler, (1932) has shown that *G. parvulum* is capable of auxosporulation and has a size reduction cycle. It is therefore expected that length, width, striae density and valve and apical pole shape will vary widely. In particular, auxosporulating clones exhibit the most morphological variability. The lack of variability in the River Kennet clone however, is surprising. This clone survived 16 months in culture, therefore one would expect to see a wider size range compared to, e.g. the Llyn Ogwen clone, which survived just 8 months in culture but shows much wider morphology. The River Kennet clone does not exhibit typical diatom size reduction. In fact, it appears to increase slightly in size with each generation.

The data for the River Kennet clone suggests either errors in measurement, vegetative or sexual cell enlargement or another as yet unidentified mechanism. Measurement errors seem unlikely, as the growth is steady and consistent with time. For vegetative enlargement to have occurred one would expect to come across much larger cells on par with post auxospore cells, however no auxospores, initial cells or otherwise enlarged cells were observed. Some other mechanism must therefore be at work. It has been suggested

(pers. comm. Cox, 2006), that there is sufficient flexibility in the mantle of the valve and the girdle bands in some diatoms to allow for some enlargement without auxosporulation or vegetative cell enlargement. Although this type of cell enlargement would manifest primarily as a change to the depth of the frustule, minor changes to length could occur as valve mantle is flexed outwards. The small but progressive increases in valve length in the River Kennet clone could accommodate this model of cell expansion and if so, suggests that the River Kennet clone, and possibly also the Barnwood clone, are asexual.

Although the size ranges for all clones overlap, significant differences between clones (except for clones from Ham Gate Pond and Parys Mountain) largely reflect the differences between auxosporulating and non-auxosporulating clones. Overlaps in the ranges of length and breadth and the lack of clear-cut separation of clones, suggest that the traditional morphological measures of length and breadth may be insufficient in separating *G. parvulum* clones. A similar problem was encountered for two demes of *Sellaphora pupula* (Mann *et al.* 2004). The occurrence of auxosporulation in four clones however, highlights the problem of relying on traditional characters of length, breadth and striae density to separate taxa. If none of these clones had auxosporulated, and the full size range was not known, several clones could easily have been separated into discrete entities. This highlights the need to know the full size range and morphology of a taxon before naming.

The characters broken, inserted and non-central short stria do not necessarily represent voigt discontinuities, as often there may be more than a single occurrence in a single valve. The data shows that striae density is closely related to length, increasing in most

clones as they get smaller, suggesting that despite the variation, mean striae number should be a stable character if related to length. Only the Kings Mere and Parys Mountain clones do not conform to this pattern. Striae direction in this study was uniformly radiate for all clones. The degree of curvature, especially for the central striae, along with angle might however prove better measures of striae direction. Taxonomic characters or descriptors such as "radiate" are a matter of degree, but if quantifiable could refine the usefulness of the character further, e.g. does the degree of striae radiation (or other descriptor of direction) change during the cell cycle and/or is it influenced by environmental factors? Are particular regions of striae more susceptible to change (e.g. central area striae)?

Although the Pen-y-Bryn and Parys Mountain clones have been identified as the same taxon, they differed in their principle valve shape. However as clone 17/02 auxosporulated and 14/02 did not, this accounts for the difference. In most clones, there is a progressive change in valve shape as cells get smaller, e.g. lanceolate to lanceolate-clavate to ovate-clavate to ovate. From the data, it can be concluded that in the clones from River Kennet, Llyn Ogwen and Barnwood Pond, valve shape is a stable character.

Whereas apical pole shape is often similar, it is not necessarily the same at both apices. The head pole is often wider and less attenuated than the foot pole, resulting in loss of apical detail in the head pole faster than in the foot pole. A practical reason for this difference, aside from the clavate morphology of the valve, is that the mucilage pores for attachment are located at the basal pole. Loss of basal pole detail to the extent of loss of mucilage pores would be a distinct disadvantage to this taxon. Post auxospore cells however, are typically narrow lanceolate and almost isopolar, suggesting that

heteropolarity is not fixed, but develops after several vegetative cycles. Transapical expansion or swelling is also common in these valves, reflecting the origin of the expanding auxospore after plasmogamy.

The data also show that abnormalities are a normal part of the population, rarely occurring above 3% and largely confined to the smallest cells in favourable conditions. In the assays however, the types of abnormal morphology are often the same but occur with higher frequency, suggesting that pH, sewage and metals act similarly on the silica deposition process, perhaps interfering with key developmental pathways. It is still unclear how teratological forms arise or why. Not all teratological forms are thought to arise from the same cause, i.e. similar changes in environmental conditions can produce dissimilar teratological forms and possibly vice versa (Barber & Carter, 1981). It has been suggested that the mechanical injury caused by, e.g. overcrowding during vegetative reproduction, may cause some of the abnormalities seen and that these are passed to each clone in an approximation of the original deformity (Drum, 1964a). Heavy metals and low pH however, have been implicated as having a causal effect. It has also been suggested (Barber & Carter, 1981) that deformities may be a protective measure within the natural variability of the genetic make-up of the cell, when normal conditions rapidly change. That proposition could apply in this study, though it is difficult to understand what "protection" the deformity might bestow unless there is some yet undisclosed structural benefit.

4.6.2 Assay Morphology

Although the assays produced similar morphological responses in different clones, each clone exhibited different sensitivity to a particular assay and no two clones exhibited the same tolerance profile. Cadmium however had less of an influence over morphology than

pH, copper or zinc, perhaps reflecting its non-biological nature (Although, Lane & Morel, 2000 have shown a biological function for cadmium in the marine diatom *Thalassiosira weissflogii* under constant conditions of low zinc).

The occurrence of isolated incidences of abrupt size change, or unequal division within a subculture, anomalous to the general trend, is unusual. However, it does not seem sensible to conclude that the relevant assays had a real effect on growth, i.e. if there was an effect at say 0.0013µg/l zinc concentration, one would expect to see a similar and more noticeable effect at adjacent higher or lower concentrations, which was not the case. For the Pen-y-Bryn clone (pH & zinc) it is possible that auxosporulation occurred in the pH regime, but that turnover in the subsequent vegetative cycle at higher pHs is enhanced, such that come harvest, there is a noticeable difference in size. In the zinc regime however, cell size is stable from Nil through to 0.0021µg/l concentration, but length decreases dramatically at 0.042µg/l concentration, suggesting that concentrations at this magnitude are in some way inhibitory. In the subsequent extended run however, an increase in cell size occurred which cannot be explained.

Abrupt cell size reductions are recorded for other taxa (Chepurnov & Mann, 1997; Geitler, 1932; Kling, 1993; Roessler, 1988), possibly in response to limiting nutrients. It has been suggested (Edlund & Stoermer, 1997; Roessler, 1988) that this phenomenon may enable diatoms to shorten the life cycle when conditions are suboptimal. In this study, the inconsistent occurrence of the phenomenon suggests causes such as (i) medium chemistry altered during assay culture causing protoplasm to either progressively shrink away from the cell wall resulting in progressively smaller cells, or (ii) the original inoculum may have consisted of a single aberrant cell that was at the smaller end of the vegetative

reproductive cycle compared to the other assay subcultures, perhaps reproducing at a slower or faster rate than the main population.

The Parys Mountain clone proved to be the least affected by any assay except the sewage regime. This clone originates from a metal contaminated, low pH site and it is therefore not surprising that it should be tolerant of metals, however there are differences between the parent cells and the post auxospore progeny, with the smaller parent cells being less tolerant of copper and zinc and the progeny being less tolerant of pH, suggesting crucial differences in physiology at different stages in the life cycle. Similarly, the Scion Pond clone comes from a nutrient rich site and therefore expected to be tolerant of sewage as was shown. The absence of tolerance to sewage other than in the Scion Pond clone is intriguing, as it goes against the literature. However, bacteria became a significant problem either within days of inoculation in the case of full strength sewage, or over the following week or two of culture at lower strengths, and this may have exacerbated and confounded the results. The decrease in length with increasing assay concentration for some clones is an indication that there is some sensitivity to sewage levels.

4.6.3 Taxonomic Implications

Whilst all clones in the data set have the features typical of *G. parvulum* sensu stricto, the initial separation of clones into variety or form was based primarily on traditional diatom taxonomy using length, breadth, striae density and overall shape according to the literature. Only SEM reveals further detail than described in the literature (Dawson, 1973b, 1974; Geitler, 1932; Krammer & Lange-Bertalot, 1986, 1991a, 1991b) however, there are disagreements. (Dawson, 1973b, 1974) stated that *G. parvulum* has reniform

slits that do not lye in depressions on the valve face whereas, e.g. *G. acuminatum* and *G. intricatum* do. The observations in this study disagree with both the terminology and the observations. The pores on the valve face are not reniform slits. The term slits give the impression of a narrow thin opening, whereas the pores are in fact more rounded and reniform and clearly composed of at least three layers internally. The depressions in *G. acuminatum* are no different from those observed in this study and therefore are not a valid basis for separating *G. acuminatum*. (Dawson, 1973b, 1974) also separates *G. gracile* based on transverse striae, yet the diatom floras of (Krammer & Lange-Bertalot, 1986) and her SEMs show variation from transverse to slightly radial, similar to *G. parvulum*. Similarities in pore structure between *G. parvulum* and *G. gracile* have been noted by Krammer and Lange-Bertalot (1986), but without a series of intermediate forms, as shown here, no firm taxonomic link could be inferred. A link has not been made in the literature between *G. parvulum* and *G. hebridense*, though (Dawson, 1973b, 1974) and Krammer and Lange-Bertalot (1986) note its similarity to *G. gracile*, which in turn is similar to *G. parvulum*. The taxonomy of *G. gracile* and *G. hebridense* will now need to be re-examined in light of these findings. (Dawson, 1973b, 1974) also states that the raphe in *G. parvulum* is straight, whilst observation in this study has shown that it is slightly undulate. The observations in this study therefore disagree with the basis of Dawson's separation using these characters. The comparison also highlights the need for standard taxonomic terminology accompanied by diagrammatic and micrographic representations.

The results call into question the taxonomic status of *G. gracile, G. hebridense*, possibly also *G. lagenula* and G. *innocens;* all clonal morphologies shown to be part of the life cycle of *G. parvulum*. Further examination of the type materials and finer discrimination

between the different morphologies included under *G. parvulum*, is required before a full taxonomic revision can be applied.

5 OBSERVATIONS ON LIVE MATERIAL

5.1 INTRODUCTION

This chapter presents the results of observational notes on live clonal, stock and experimental cultures over a period of approximately two years. Initially, the aim of this chapter was to provide evidence of basic behavioural and protoplastic changes, as observed under the light microscope, in response to different culture regimes. The discovery of sexually reproducing clones and the production of unusual forms of Extracellular Polysaccharide Substances (EPS) however, provided additional interesting material for discussion. Therefore, this chapter aims to identify the environmental factors influencing behaviour and protoplasmic responses of *G. parvulum* in an ecological context, to assess whether clones can be differentiated on one or more of these characters.

5.2 GENERAL OBSERVATIONS

A cell that did not auxosporulate continued vegetative reproduction resulting in cell diminution, to a point where the cell's ability to function was severely affected both in terms of physiology and reproduction, i.e. almost as many cells died as were being produced. Thus, turnover rate was low, and cells eventually reached a finite size at which all cellular functions were at their end limit. At this latter vegetative stage, most organelles would not be able to reduce their size further without also reducing functionality, e.g. chloroplast size diminishes proportionate to frustule size, probably leading to loss in photosynthetic ability and energy production. Smaller cells may require and produce less energy, however a cut-off point must inevitably arise where the energy requirements

versus the energy production is not balanced. Consequently, the cultures eventually die out. All cultures were eventually lost in this manner.

The by-clone and by-regime percentage occurrence of a live observational characters within a clone or within a regime, are summarised in **Tables 5.1** and **5.2**, respectively. The number of Operational Units (OUs) refers to the individual subcultures for each clone and any G_2 offspring, plus each time period for exponential ($K_{0\text{-}6}$), and post exponential (K_{6+}) growth (see Chapter 2). The low number of OUs for Barnwood Pond, Abbey Lakes River, Llyn Idwal (G_1) and Llyn Ogwen clones and the light and temperature regime, are due either to the death of a clone prior to assay, or missing data as highlighted in the preface to this thesis.

Table 5.1

Summary of by-clone % occurrence of live observational character states. † = Number of Operational Units per clone. **A** = total number of clones exhibiting a single character state and **B** = total number of character states exhibited by a single clone.

		Number of Operational Units per Clone (†) and % Occasion Character Occurred										
		Barnwood Pond	Scion Pond	Kings Mere	Ham Gate Pond	Abbey Lakes River	Ilyn Idwal	River Kennet	Pen-y-Bryn	Llyn Ogwen	Parys Mountain	A
CODE	Character Description	7†	72†	93†	126†	10†	133†	121†	63†	7†	186†	
Attachment Mode												
ATT1	Suspended cells		0.82	0.30	0.55	1.00	0.41	0.30	0.46	0.29	0.43	9
ATT2	Attached in valve view	1.00	0.74	0.90	0.77	1.00	0.74	0.93	0.86	1.00	0.79	10
ATT3	Attached in girdle view	0.57	0.69	0.86	0.76	1.00	0.70	0.88	0.84	1.00	0.78	10
ATT4	Attached by mucilage pad at foot pole	0.43	0.07	0.24	0.42		0.47	0.16	0.43		0.25	8
ATT5	Attached by mucilage stalk at foot pole			0.04	0.07						0.05	3
Colony Type												
COL1	Single cells	1.00	0.93	0.95	0.95	1.00	0.89	1.00	0.97	1.00	1.00	10
COL2	Paired cells		0.29	0.44	0.32		0.25	0.26	0.19	0.57	0.22	8
COL3	Short chains (valve face to valve face)		0.04	0.03	0.02	1.00	0.03	0.01	0.03	0.29	0.01	9
COL4	Short chains (girdle to girdle)		0.01	0.01		0.80		0.03	0.03	0.14	0.01	7
COL5	Tufts			0.22	0.41		0.38	0.06	0.38		0.19	6
COL6	Mucilaginous matrix				0.05			0.07		0.14		3
COL7	Dense mass aggregations		0.11		0.05			0.01	0.06		0.03	5
Chloroplast Colour												
CHL1	Dark brown chloroplast	0.14	0.01	0.10	0.02	0.10	0.02	0.03	0.03		0.01	9
CHL2	Golden brown chloroplast	1.00	0.82	0.77	0.74	1.00	0.83	0.72	0.97	0.86	0.88	10
CHL3	Pale golden brown chloroplast		0.18	0.37	0.38		0.29	0.47	0.17		0.16	7
CHL4	Yellow chloroplast		0.01	0.01	0.02			0.07			0.01	5
CHL5	Green chloroplast	0.14	0.01	0.03	0.02		0.05	0.02			0.01	7
CHL6	Colourless chloroplast		0.04	0.01	0.05		0.11	0.04	0.02	0.14	0.01	8
Chloroplast Size												
CHL7	Chloroplast normal	1.00	0.82	0.83	0.89	1.00	0.79	0.64	0.89	1.00	0.87	10
CHL8	Chloroplast lobes extended into apical		0.25	0.23	0.24		0.09	0.17	0.17		0.19	7
CHL9	Chloroplast occupies entire cellular		0.13	0.01	0.04		0.02	0.02		0.14	0.06	7
CHL10	Chloroplast constricted to medial area	0.14	0.04	0.03	0.06	0.10	0.11	0.32	0.35		0.11	9
CHL11	Chloroplast constricted to cell walls	0.14	0.13	0.53	0.24		0.23	0.36	0.38		0.15	8
Pyrenoid		0.14	0.19	0.19	0.22	0.20	0.12	0.06	0.06		0.11	9
PYR1	Pyrenoid triangular	0.14	0.25	0.24	0.25		0.23	0.12	0.08		0.36	8
PYR2	Pyrenoid rounded	1.00	0.79	0.96	0.95	1.00	0.95	0.90	0.95	1.00	0.89	10
Vacuoles												
VAC1	Vacuoles 2			0.01	0.01			0.06	0.08		0.02	5
VAC2	Vacuoles, 1	0.57	0.17	0.29	0.30		0.26	0.74	0.60		0.33	8
Other												
OIL1	Lipids		0.39	0.59	0.52		0.63	0.26	0.40		0.81	7
GRAN1	Granules		0.08	0.11	0.10		0.05	0.02	0.10		0.05	7
OTH1	Dark bodies											
OTH2	Teratology		0.06	0.09	0.04		0.05	0.10	0.02	0.14	0.03	8
OTH3	Auxosporulation		0.01	0.01	0.02		0.02			0.14	0.09	6
	B	14	28	30	31	12	26	30	26	15	31	

Table 5.2

Summary of by-regime % occurrence of live observational character states. † = Number of Operational Units per clone. **A** = number of regimes exhibiting a single character state and **B** = number of character states exhibited within a regime by all clones.

		Number of Operational Units per Clone (†) and % Occasion Character Occurred							
		Stock	L&T	pH	Sewage	Copper	Zinc	Cadmium	
CODE	Character Description	155†	15†	184†	63†	174†	136†	138†	A
Attachment Mode									
ATT1	Suspended cells	0.31		0.64	0.63	0.36	0.38	0.42	6
ATT2	Attached in valve view	0.87		0.81	0.71	0.87	0.87	0.74	6
ATT3	Attached in girdle view	0.81		0.69	0.71	0.87	0.87	0.75	6
ATT4	Attached by mucilage pad at foot pole	0.22	1.00	0.29	0.08	0.39	0.43	0.21	7
ATT5	Attached by mucilage stalk at foot pole	0.05		0.01	0.06		0.07		4
Colony Type									
COL1	Single cells	0.99	1.00	0.96	1.00	0.92	0.93	0.99	7
COL2	Paired cells	0.42		0.38	0.03	0.24	0.43	0.02	6
COL3	Short chains (valve face to valve face)	0.15		0.01		0.05			3
COL4	Short chains (girdle to girdle)	0.10				0.01	0.02		3
COL5	Tufts	0.08	1.00	0.13		0.32	0.46	0.27	6
COL6	Mucilaginous matrix	0.04		0.01	0.06			0.05	4
COL7	Dense mass aggregations	0.03		0.04		0.05	0.02	0.02	5
Chloroplast Colour									
CHL1	Dark brown chloroplast	0.10		0.03		0.01		0.04	4
CHL2	Golden brown chloroplast	0.81	1.00	0.84	0.95	0.76	0.86	0.77	7
CHL3	Pale golden brown chloroplast	0.11		0.26	0.06	0.41	0.38	0.31	6
CHL4	Yellow chloroplast	0.01		0.02		0.05	0.01		4
CHL5	Green chloroplast	0.07		0.03	0.03	0.01		0.04	5
CHL6	Colourless chloroplast	0.03		0.07	0.05		0.05	0.06	5
Chloroplast Size									
CHL7	Chloroplast normal	0.85	1.00	0.76	0.97	0.84	0.80	0.77	7
CHL8	Chloroplast lobes extended into apical pole	0.06		0.33	0.08	0.25	0.16	0.09	6
CHL9	Chloroplast occupies entire cellular space	0.05		0.12	0.02	0.02	0.03		5
CHL10	Chloroplast constricted to medial area	0.13		0.10		0.15	0.29	0.12	5
CHL11	Chloroplast constricted to cell walls	0.10		0.12	0.14	0.36	0.59	0.22	6
Pyrenoid									
PYR1	Pyrenoid triangular	0.12		0.18	0.19	0.20	0.11	0.04	6
PYR2	Pyrenoid rounded	0.23		0.64	0.11	0.18	0.07		5
Vacuoles									
VAC1	Vacuoles 2	0.92		0.94	0.90	0.86	0.90	0.97	6
VAC2	Vacuoles, 1			0.01		0.04	0.07		3
Other									
OIL1	Lipids	0.23		0.56	0.41	0.41	0.26	0.32	6
GRAN1	Granules	0.20		0.65	0.38	0.58	0.57	0.67	6
OTH1	Dark bodies	0.06		0.10	0.02	0.11	0.06	0.01	6
OTH2	Teratology	0.03		0.06		0.13	0.05		4
OTH3	Auxosporulation	0.07		0.02			0.08		3
	B	31	5	31	22	28	28	23	

5.2.1 Clonal Stock Cultures

Nearly 200 subcultures of *G. parvulum* were established between September 2001 and September 2002, representing 10 different clones, and where those clones reproduced sexually, the G_2 offspring. Clonal survival lasted between 3 and 21 months with the Scion Pond clone surviving the longest. Ordering clones by length of survival gave the following ranking, with number of months in brackets:

1. Scion Pond (21)
2. Llyn Idwal (G_2) (18)
3. Abbey Lakes River, Pen-y-Bryn and Parys Mountain (G_1) (14)
4. River Kennet (16)
5. Llyn Idwal (G_1) (15)
6. Kings Mere (G_2) and Ham Gate Pond a(G2) (13)
7. Barnwood Pond (12)
8. Kings Mere (G_1), Ham Gate Pond (G_1) and Parys Mountain (G_2) (10).

Ranking of clones by the total number of character states observed **(Row B, Table 5.1)** indicates that smaller sized cells, such as the Barnwood Pond, Abbey Lakes River and Llyn Ogwen, were less variable in their mode of attachment, colony type and chloroplast morphology. They were also less likely to contain granules or lipids.

A few clones exhibited a consistent mode of attachment throughout their period of culture; clones from Barnwood Pond (attached in valve view), Llyn Ogwen (attached in valve and girdle view), Abbey Lakes River (suspended cells or attached in valve or girdle view) and Ham Gate Pond (G_2) (attached in valve or girdle view or by stalk). All other clones were observed to increase the propensity to attach with increasing age of clone, with the exception of the Abbey Lakes River clone, which remained stable. Small chains of cells (3-10 cells long) which adhered valve face to valve face, occurred in a few subcultures, often in clones at the lower end of the size spectrum. Chains of cells adhering girdle to

girdle, occurred only in those clones that already formed valve face to valve face chains, though not necessarily in the same subculture. Tufted colonies usually 'sprouted' from a shared mucilage pad and because they took a little while to develop, appeared only in older subcultures.

Some clones produced more unusual types of colony. A thin sheet, or irregular shaped amorphous extracellular matrix, containing many single cells was formed by clones from Ham Gate Pond (G_2) and Llyn Ogwen. The matrix was often suspended in the water column, though it could also be tenuously attached to the base of the culture dish by a thread. This occasionally, but not always, occurred in subcultures that were adversely affected by bacteria (Ham Gate Pond (G_2) and Llyn Ogwen). Mass aggregation of cells also formed in some of the latter subcultures for clones from Scion Pond and Parys Mountain (G_1).

Pyrenoids were not always easily seen, but when visible they were large and usually rounded (hemispherical), however the triangular form could occur in the same clones and/or the same subculture.

Occasionally, vacuoles were not visible/observed (as in some of the subcultures for clones from Kings Mere (G_2), Ham Gate Pond (G_2), River Kennet and Parys Mountain (G_1)). Sometimes this was due to cell size (very small cells <10μm) or because the chloroplast appeared to fully occupy the cell. In certain clones and subcultures, the

vacuoles were extremely large, constricting the chloroplast to the cell wall. No clone or subculture from the stock cultures was observed to have a single vacuole.

Lipids were less likely to be observed in the smaller clones, e.g. Kings Mere (G_1), Ham Gate Pond (G_1), Abbey Lakes River, Llyn Idwal (G_1) and Llyn Ogwen. When bacterial presence was high, some clones increased lipid content in response (Ham Gate Pond (G_2) and River Kennet), or produced a gelatinous coating around individual cell. Small granular bodies were occasionally observed to exhibit chaotic Brownian movement. The function of these granular bodies is not known.

Teratologies were not obvious in the stock cultures, but occurred in at least one subculture each for clones from Llyn Idwal (G_2), River Kennet, Parys Mountain (G_2) and Llyn Ogwen.

5.2.2 Experimental Regimes

Ranking of regime by the total number of character states observed **(Row B, Table 5.2)**, gave the following:

1. Stock cultures and pH Regime
2. Copper and Zinc Regimes
3. Cadmium Regime
4. Sewage Regime
5. Light and Temperature Regime.

5.2.2.1 Light and Temperature Regime

Whilst in exponential growth phase, the Barnwood clone appeared to "avoid" the light at 5°C high light, by crowding at the very edge of the culture dish. The Ham Gate Pond (G_1) clone auxosporulated at 20°C low and medium light, 25°C low, medium and high light, and at 30°C high light between days 2 and 8 of exponential growth. The progeny of both produced 'tufted' colonies post exponential growth, and grew less well at 5°C low, medium and high light. The Abbey Lakes River clone grew well at all light and temperature levels, mostly as single attached cells in valve and girdle view. Many clones also began to exhibit paler chloroplast as both temperature and light intensity increased, from 25°C high light to 30°C low, medium and high light. Many of the clones had paler chloroplasts as both temperature and light intensity increased, from approximately 25°C high light to 30°C low, medium and high light.

Cell pairings in opposing polar orientation i.e. head pole to foot pole, were often a pre-curser to sexual reproduction (whether successful or not). The Kings Mere clone (G_1 was seen to pair in this manner on days 2 and 4 of the exponential growth phase in the light and temperature regime, but in most cases did not produce auxospores. The morphologically similar clone from Ham Gate Pond (G_1) auxosporulated at 20°C low and medium light, 25°C low, medium and high light and at 30°C high light between days 2 and 8 of the exponential growth phase, indicating warmer temperatures may be a necessary cue for sexual reproduction.

5.2.2.2 pH Regime

Most clones (except Ham Gate Pond, G_2), exhibited a more stable and consistent mode of attachment during the exponential growth phase than the period thereafter, with

attachment in valve and girdle view dominating. The dominant attachment mode post exponential phase was suspended cells. The dominant colony mode both during and post exponential growth, was as single cells (exception, Kings Mere (G_2) at pH 8.0 and Ham Gate Pond (G_2) at pH 4.0-4.5 and 6.5 and 8.0 to 8.5). Cells forming tufts were half as common during post exponential growth phase than during exponential growth.

Cells forming chains linked valve face to valve face, and cells forming an amorphous mucilaginous matrix, occurred only in one clone and one pH level each. The former in the Llyn Idwal clone (pH 4.0), and the later in the River Kennet (pH 8.5). In acid conditions, the River Kennet clone was observed to form branched stipate colonies (as opposed to individually stalked cells). The branches being mucilaginous stalks at the end of which was a single cell.

Chloroplast colour and size was more variable during the post exponential growth phase than during exponential growth. Pale chloroplasts were often, but not always, associated with chloroplasts that had been restricted to cell walls, usually by an enlarged vacuole. All chloroplast sizes and colours could co-occur in a single subculture. Dark brown, yellow, green and colourless chloroplasts were only observed during post exponential growth, as were chloroplasts that were restricted medially. There was also an increase in the number of subcultures exhibiting chloroplast constricted to the cell wall in post exponential growth.

Teratologies were only observed in the post exponential growth phase, having spent longer in the assay medium. Auxosporulation occurred during the post exponential growth phase in clones from Scion Pond and Ham Gate Pond (G_2), at pH 9.0 and 5.5 to 6.0, respectively. However, initial cells appeared slightly deformed in the Scion Pond clone at pH 9.0, having a medially concave 'dent'. In the Ham Gate clone, auxosporulation was unsuccessful at pH 5.5 and 6.0, and cells tended to die before forming initial cells.

Bacteria and precipitates were a problem in some pH assays. Precipitates tended to form around day 6 onwards, and only at higher pH's in clones from Kings Mere (G_2) (pH 10.5 to 11.0) and Abbey Lakes River and Parys Mountain (G_2) at pH 11.0. Bacteria contaminated clones from Kings Mere (G_2), Abbey Lakes River, Llyn Idwal (G_2), River Kennet, Pen-y-Bryn and Parys Mountain (G_2), at pH's 6.0, 9.0 to 9.5, 9.0, 5.0, 4.0 to 10.0 and 5.0 to 10.5 respectively. There was a tendency for less, or no bacteria in the more acid conditions. Generally, the more alkaline the conditions, the higher the number of dead, empty 'ghost' valves, particularly in clones from River Kennet and Parys Mountain (G_1 & G_2).

In the Ham Gate Pond clone (G_2), at pH 4.0 and 4.5, and the River Kennet clone at pH 4.5 and 5.5, some cells in low pH culture were observed to grow and crowd at the margins of the culture dish, close to the dish walls or on the walls, apparently 'avoiding' the centre of the dish. The reason for this behaviour was not apparent.

5.2.2.3 Sewage Regime

In the Sewage Regime, during the exponential growth phase, the dominant mode of attachment was single cells attached in valve and girdle view, at all sewage strengths for all clones except Ham Gate Pond (G_2), in which single suspended cells dominated all sewage strengths. Mode of attachment and colony type were very similar post exponential growth.

Bacteria were a problem in most subcultures due the nature of the medium, and despite all efforts to the contrary (e.g. aseptic techniques and antibiotic treatment). They were a significant problem in the full strength sewage assays and quickly overran the subculture.

5.2.2.4 Copper Regime

Mode of attachment and colony type were highly variable both in the initial and the extended runs, however single suspended and single cells attached in valve view dominated during the exponential growth phase, followed closely by attached cells in girdle view, and cells attached by a mucilage pad. Single cells attached in valve view and/or girdle view dominated latter cultures, followed closely by suspended cells and cells attached by a mucilage pad.

The branched stipate colony observed in the pH and sewage regimes for the Ham Gate Pond clone, also occurred for the same clone at copper concentrations of 0.0013µg/l and 0.0026 during the post exponential growth phase.

No clones exhibited chloroplasts where the lobes extended into the poles, occupied the entire cellular space or were restricted medially during exponential growth, however all three chloroplast types were observed post exponentially in all clones except Parys Mountain (G_2). The occurrence of triangular and rounded pyrenoids both during and post exponential growth was approximately equal.

Cells were observed to have one large polar vacuole in clones from Kings Mere (G_2) and Ham Gate Pond (G_2) (at 0.0053µg/l), River Kennet (0.0013, 0.0053, 0.05 and 0.25µg/l) and Pen-y-Bryn (0.0026µg/l). Lipids and dark bodies occurred in more subcultures and clones post the exponential growth phase.

5.2.2.5 Zinc Regime

The Zinc Regime was dominated by cells that attach in valve and girdle view, both during and post the exponential growth phase, and in all clones at most zinc concentrations. No clone exhibited attachment by stalk during exponential growth, however it was observed in latter cultures of the Parys Mountain clone (G_1) (Nil to 0.011µg/l). Single cells were the dominant colony type both during and post exponential growth, and observed in all clones and most zinc concentrations.

During post exponential growth, the Kings Mere (G_2) (0.00066µg/l) clone was observed to have a large number of pale chloroplasts, and clones from River Kennet (0.0013, 0.10 to

1.0µg/l), Pen-y-Bryn (0.011µg/l) and Parys Mountain (G_1) (0.042µg/l) all had a high proportion of colourless cells.

There were occasions when only one vacuole was present during post exponential growth in clones from River Kennet (0.053, 0.011 and 0.021µg/l), Pen-y-Bryn (0.00066, 0.0053, 0.011 and 0.042µg/l) and Parys Mountain (G_1) (Nil and 0.00016µg/l).

5.2.2.6 Cadmium Regime

Cells forming mass aggregations or suspended in a matrix were rare, and occurred only during post exponential growth in clones from River Kennet (Nil to 0.0013µg/l) and Pen-y-Bryn (mass aggregation only, 0.00033 to 0.0013µg/l). Branched stipate colonies occurred in clones from Kings Mere and River Kennet for the cadmium regime at 0.02 and 0.10µg/l respectively.

Chloroplast colour and size was more variable during post exponential growth days. Vacuoles on the other hand, were normal in all clones and at all cadmium concentrations during and post exponential growth days, except for clones from River Kennet (0.05 and 0.25µg/l) during exponential growth, and Pen-y-Bryn (0.05µg/l (No EDTA) and 12.5µg/l (No EDTA)) during post exponential growth phase.

5.2.3 EPS production

The types of Extracellular Polysaccharide Substances (EPS) produced by *G. parvulum* clones in this study include stalks, pads and an amorphous matrix. Additional EPS are presumably produced by cells adhering to the culture vessel surface in valve or girdle view, in paired cells, chains of cells, tufts and mass aggregations, the latter of which over time produce floc-like clumps.

In LM, **cells that attached in valve and/or girdle view without a stalk** were common. Often the majority of cells were inactive, occasionally however, the majority of cells were observed to be very active and motile, often in the morning. All cells appeared to be heading directly for a central mass congregation of cells. This behaviour occurred only in the River Kennet clone and was never repeated or observed in any other culture.

In **stalked cells,** the stalk can be seen to consist of a collar and shaft. There are also three fine transverse bands in the upper part of the shaft near the collar, which may indicate growth spurts. The shaft also appears to be differentiated longitudinally, i.e. there is thicker, denser material at the edges of the stalk, with either a central tube or thinner central portion. The stalks were invariably very long, often more than 3 times the valve length. Dichotomously branched stalks were observed in clones from Kings Mere (G_2) for the cadmium regime, Ham Gate Pond (G_2) for the copper and sewage regimes, and River Kennet for the pH and cadmium regimes. These branched colonies were not large, and only a few occurred in each of the cultures mentioned, alongside single stalked cells. The end of each stalked branch bore a single cell. **Cells forming mucilage pads** were distinguished from stalks in being considerably shorter. The structure was difficult to

discern in LM, but in at least one case appeared to consist of a collar and shaft much the same as the stalked cells.

Non-sexual paired cells were often observed in cultures, and on occasion could be seen to constitute over 50% of the population. Due to the clavate nature of *G. parvulum*'s outline shape, pairing was observed to occur along the upper part (near the head pole) of both cells, the lower part (near the foot pole) in larger cells, or nearer the centre in smaller cells. These paired cells were always in the same polar orientation. Mucilage pads at the site of attachment were not observed, but as these pairing were always girdle to girdle, it is probable that either the mucilage coating around individual cells or additional mucilage secreted from the girdle region, facilitates this pairing. Pairings were always observed either pyrenoid to pyrenoid or non-pyrenoid to non-pyrenoid sides, never pyrenoid to non-pyrenoid side.

Although the base of **tufts** were not observed, it is likely that the cells are joined by a shared mucilage pad, though this is not always securely anchored to the base of the culture vessel, and several subcultures were observed to have floating tufts, i.e. a majority of tufts were suspended in the medium. These are possibly precursors to the formation of algal flocs.

This thesis is the first reported case of *G. parvulum* forming a mucilage matrix, and appears to be confined to clones from Ham Gate Pond (G_2), River Kennet and Llyn Ogwen. It was more common however in the River Kennet clone in the cadmium regime.

Cells appear to be embedded in the matrix rather than actively moving through it. The matrix was observed to be tenuously attached by a 'cord' of several threads, to the base of the culture dish, though it was also found floating free in some cases. In each of the cultures in which this behaviour was observed, the matrices occurred as single entities with either small flocs of clumped cells, or cells attached in valve view or girdle view.

When cells formed sufficient densities, some began to mass together to form tight bundles/clumps of cells or mass aggregations. These were not tufts, rather, a muddle of cells very densely packed together and either attached to the base of the culture dish, or floating free in the medium as floc.

Cells did not form **chains** often, and there were rarely more than 6-8 cells in any one chain, though a few chains of 10 cells were observed. There was no consistency in the occurrence and no 'active' cell joining was observed. The majority of chains were formed in older cultures when cells were at their smallest.

Examination of individual cells in SEM, showed that mucilage secretion (EPS) occurred from the valve face, the mantle, the apical pore field and sutures. Looking at the valve face, not all the clones examined under SEM had mucilage secretions, and in some cultures secretions were present, but only in very small amounts **(Figures 5.1: a-f)**. However, in every clone where mucilage secretions were evident, it was clear that secretions were exuded from valve face pores and the raphe **(Figures 5.2, 5.3, 5.4, and 5.6)**. The mucilage is in the form of strands and occasionally, as a smooth deposit on the

valve surface **(Figures 5.4: e and 5.6: a-d)** or a granulised deposit **(Figures 5.6: e-g)**. The mucilage strands also originate from the stigma **(Figures 5.2: d, 5.4: f, 5.5: c, h and l)**. In **Figures 5.6: h-l**, there is evidence of mucilage between two adjacent frustules lying valve face to valve face. In girdle view, mucilage secretions followed much the same pattern od deposition as shown on the valve face, i.e. strands, smooth layers and granulised layers. Mucilage originated from mantle pores, the pores on the girdle band and sutures **(Figures 5.7, 5.8, 5.9)**. Overall, the mucilage appears relatively thick in places (on the raphe, in-between cells adhered valve face to valve face and with mucilage pads), and where it is a smooth deposit, EPS is seen to obscure pore openings. It also appears quite opaque, though this may be an artefact of the drying process.

Figures 5.9: a-b, show a mucilage pad, apparently exuded from the apical pore field on both valves of a single frustule, to form a thick, short, and undifferentiated mucilage pad. Neither the collar nor the substratum pad was visible. **Figures 5.9: c-d**, show fibrous strands of mucilage, also exuded from the apical pore field, linking three single frustules. The morphology is very different from that of the mucilage pad in **Figure 5.9: a-b**, being more fibrous and considerably thinner. Again, there is no clear collar, shaft and pad visible.

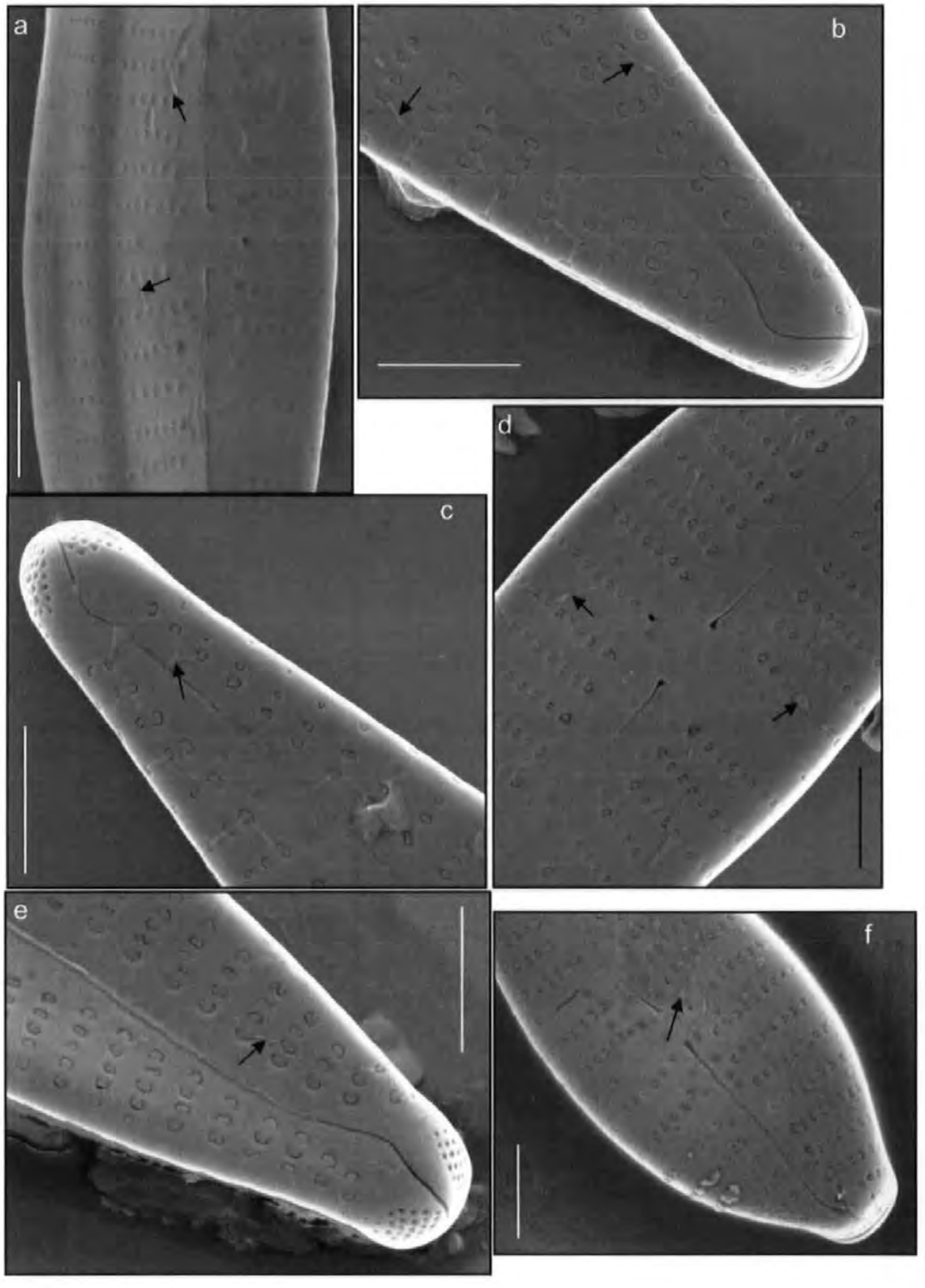


Figure 5.1: a-f

SEMs showing small amounts of EPS present on uncleaned valves, as strands of material originating from valve face pores (arrows) **(a)** Barnwood Pond **(b-c)** Kings Mere (G2) **(d-e)** Parys Mountain (G_1). Scale bars = 2μm.

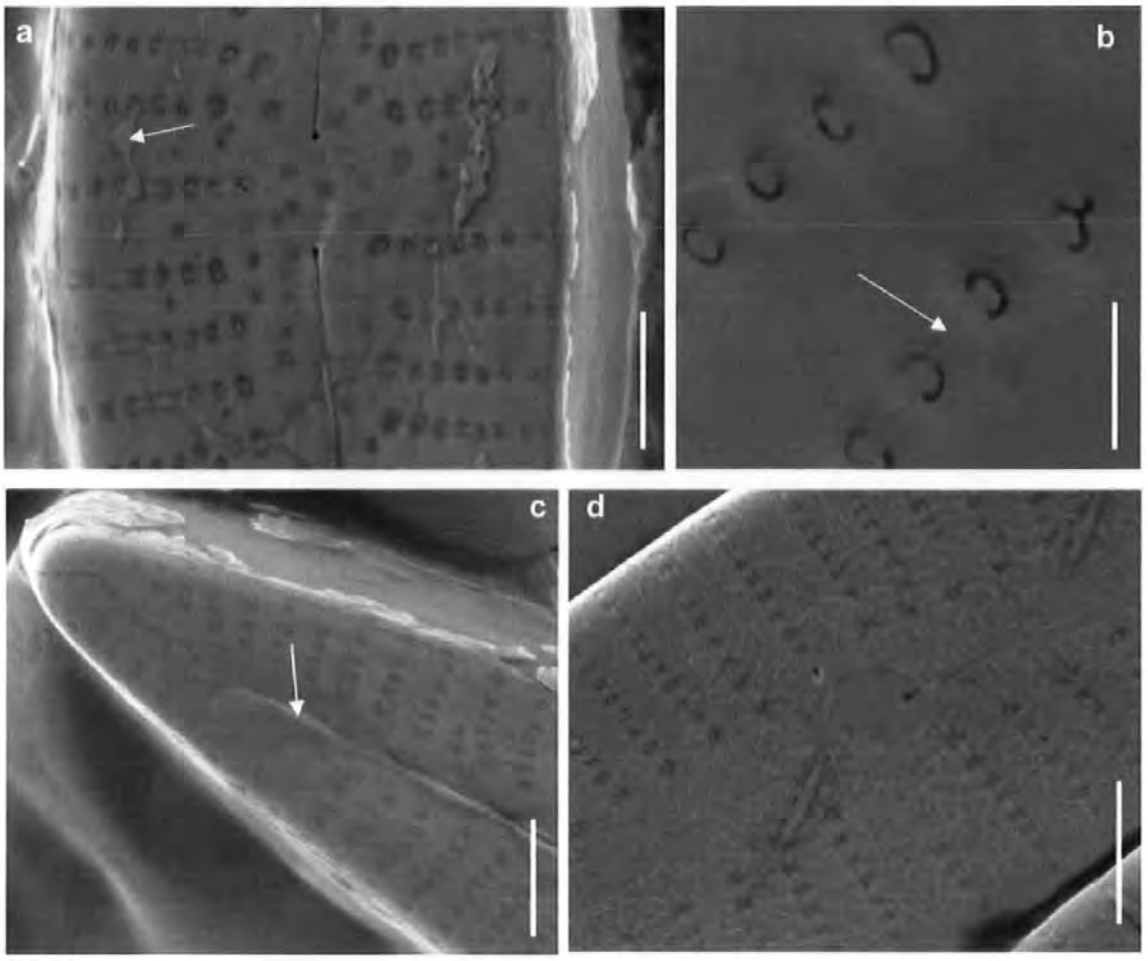


Figure 5.2: a-d

SEMs showing EPS present on uncleaned valves, as strands of material originating from valve face pores (arrows). **(a-b)** Kings Mere (G_2), subculture 08/05/02. Note the mucilage originating from the valve face pores in Fig. b. **(c-d)** Kings Mere (G_2), subculture 01/07/02. Note the mesh of fine interlacing strands in Fig d. Scale bars = 2μm.

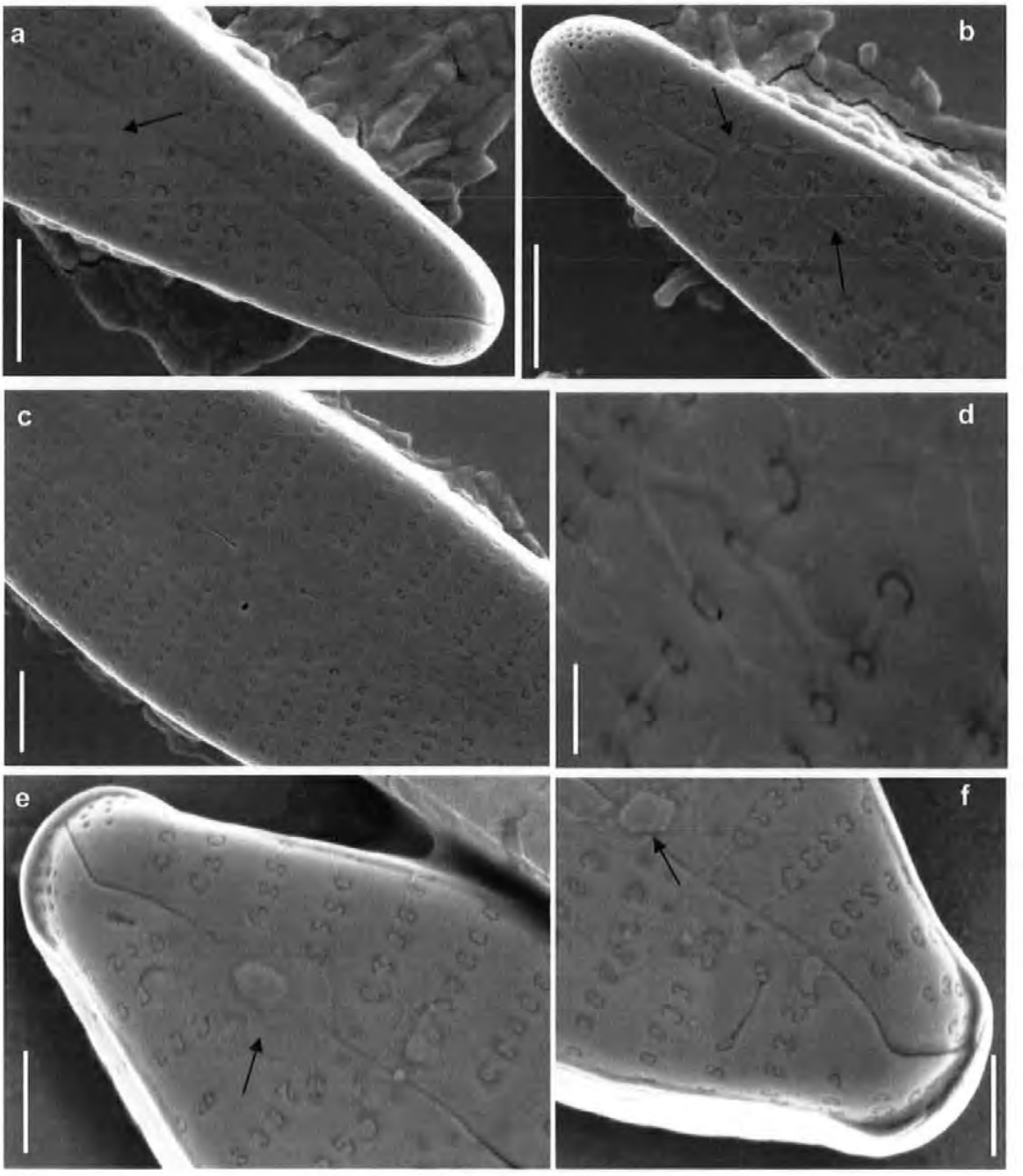


Figure 5.3: a-f

SEMs showing EPS present on uncleaned valves, as strands of material originating from valve face pores (arrows). **(a-d)** Hamgate Pond (G_2), subculture 01/07/02. Note the mucilage originating from the valve face pores and raphe in Fig. b. **(e-f)** Abbey Lakes River, subculture 01/12/01. Note the coalescing of mucilage in Fig d and e. Scale bars = 2μm.

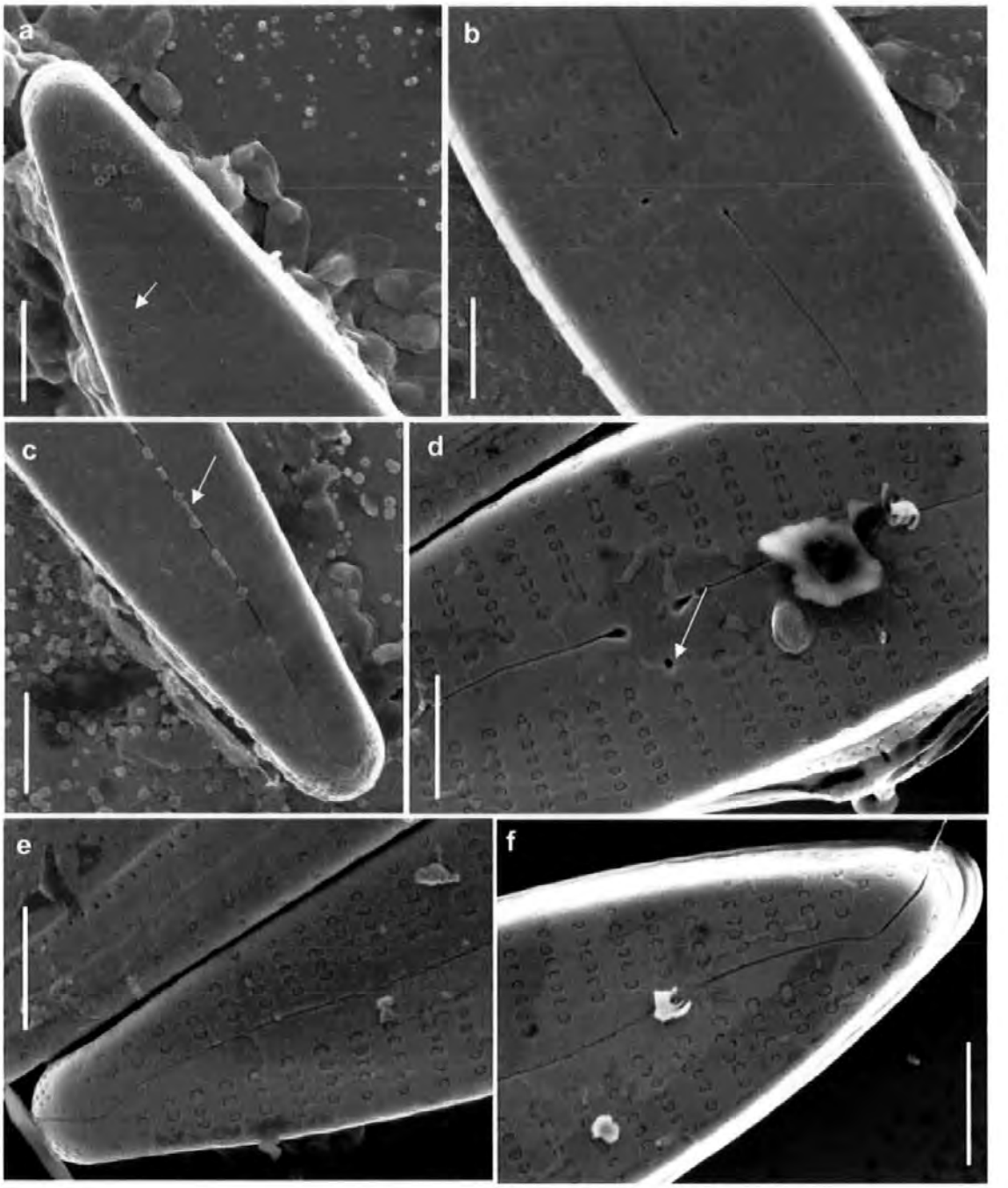


Figure 5.4: a-f

SEMs showing EPS present on uncleaned valves, as strands of material originating from valve face pores (arrows). **(a-c)** Llyn Idwal (G_2), subculture 01/07/02. Note the mucilage originating from the raphe in Fig. c. **(d-f)** Parys Mountain (G_1), subculture 01/07/02. Note the mucilage emanating from the raphe in Fig d. and from the stigma in Fig e. Scale bars = 2μm.

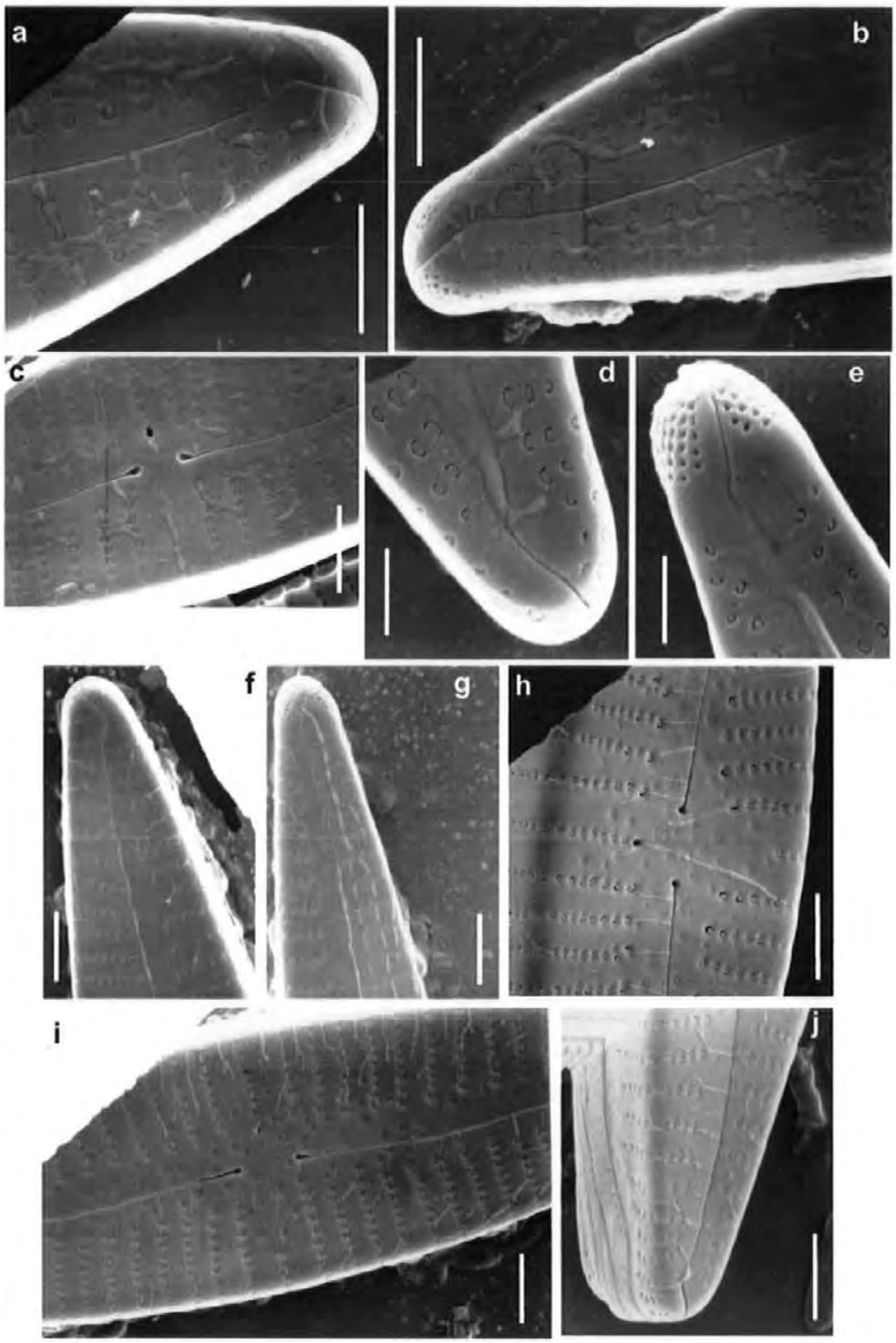


Figure 5.5: a-j

SEMs showing EPS present on uncleaned valves, as strands of material originating from valve face pores (arrows). **(a-c)** Parys Mountain (G_1), subculture 01/07/02. **(d-e)** Kings Mere (G_2), subculture 01/07/02. **(f-j)** Llyn Idwal (G_2), subculture 01/07/02. Scale bars = 2μm.

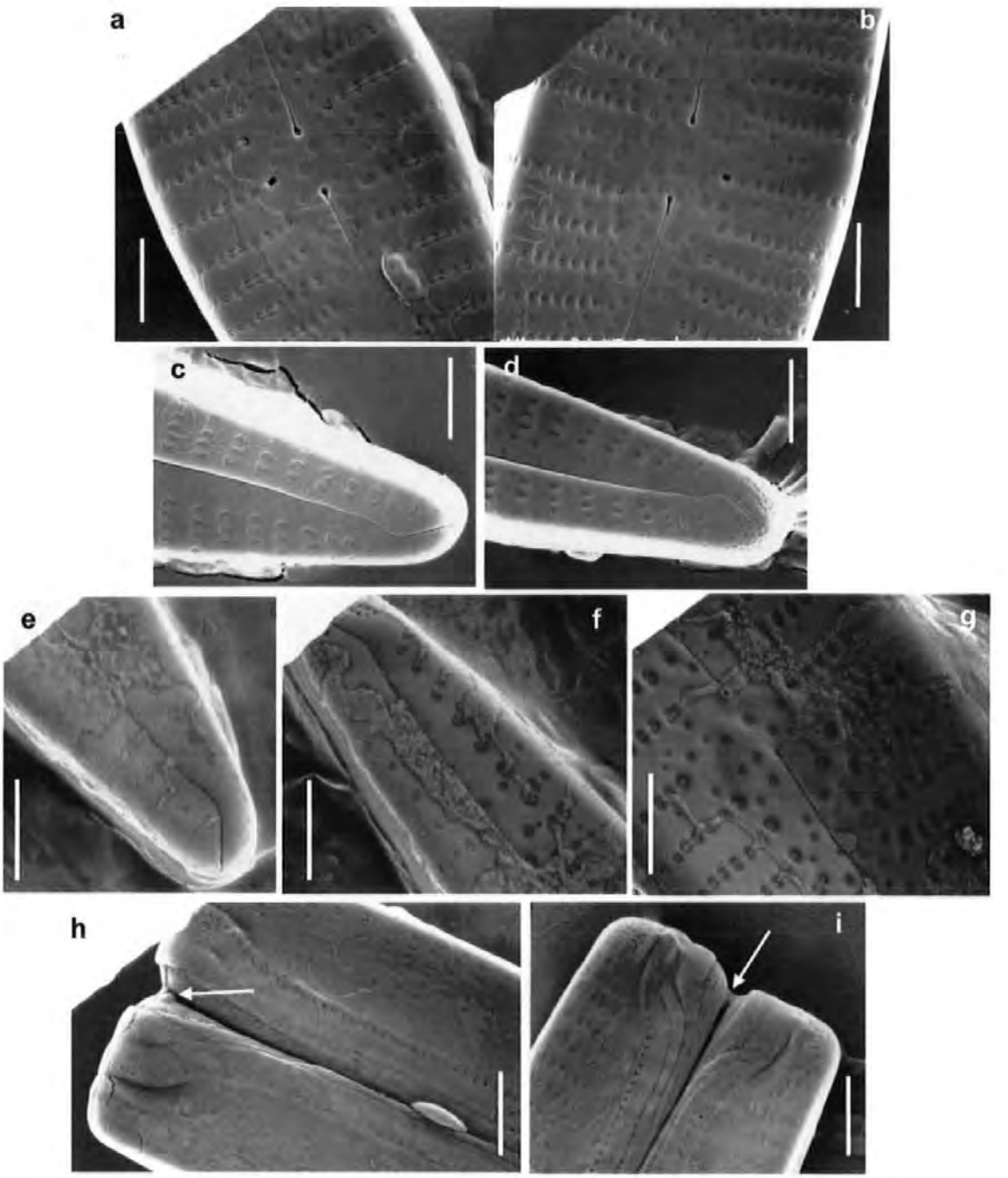


Figure 5.6: a- i

SEMs showing EPS present on uncleaned valves. **(a-d)** Mucilage forms a smooth covering over the valve surface in **(a)** Kings Mere (G_1), subculture 01/07/02 **(b-d)** Ham Gate Pond (G_2), subculture 01/07/02. **(e-g)** Mucilage as a granulized deposit over the valve surface in Kings Mere (G_2), subculture 08/05/02 **(h-i)** Mucilage (arrows) between paired cells in Parys Mountain (G_1), subculture 07/06/02. Scale bars = 2μm.

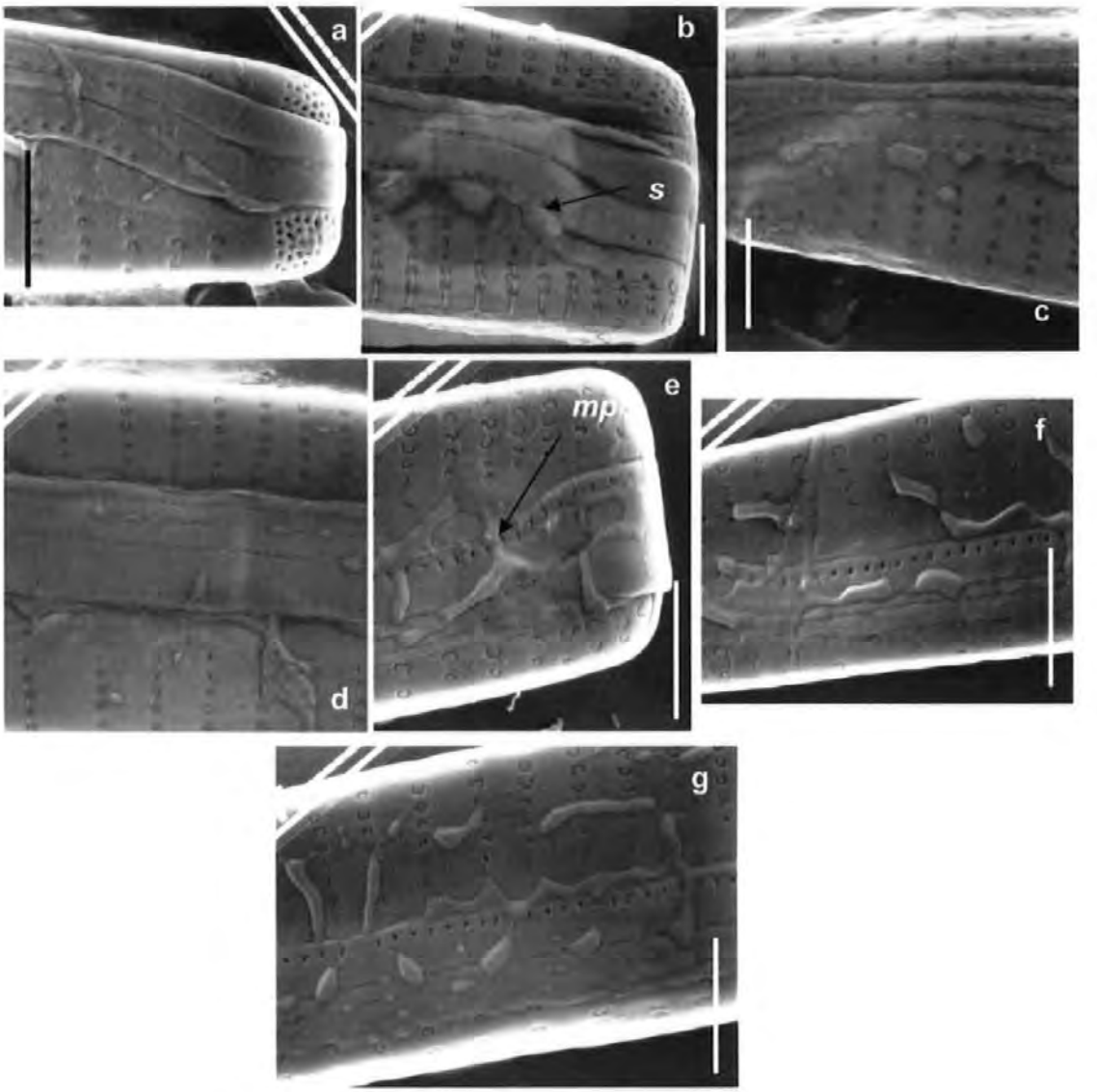


Figure 5.7: a-g

SEMs showing EPS present on uncleaned valves. Mucilage originating from mantle pores (***mp***) and sutures (***s***). **(a)** Kings Mere (G_2), subculture 01/07/02 **(b-d)** Kings Mere (G_2), subculture 08/05/02. **(e-g)** Parys Mountain (G_2), subculture 01/07/02. Scale bars = 2μm.

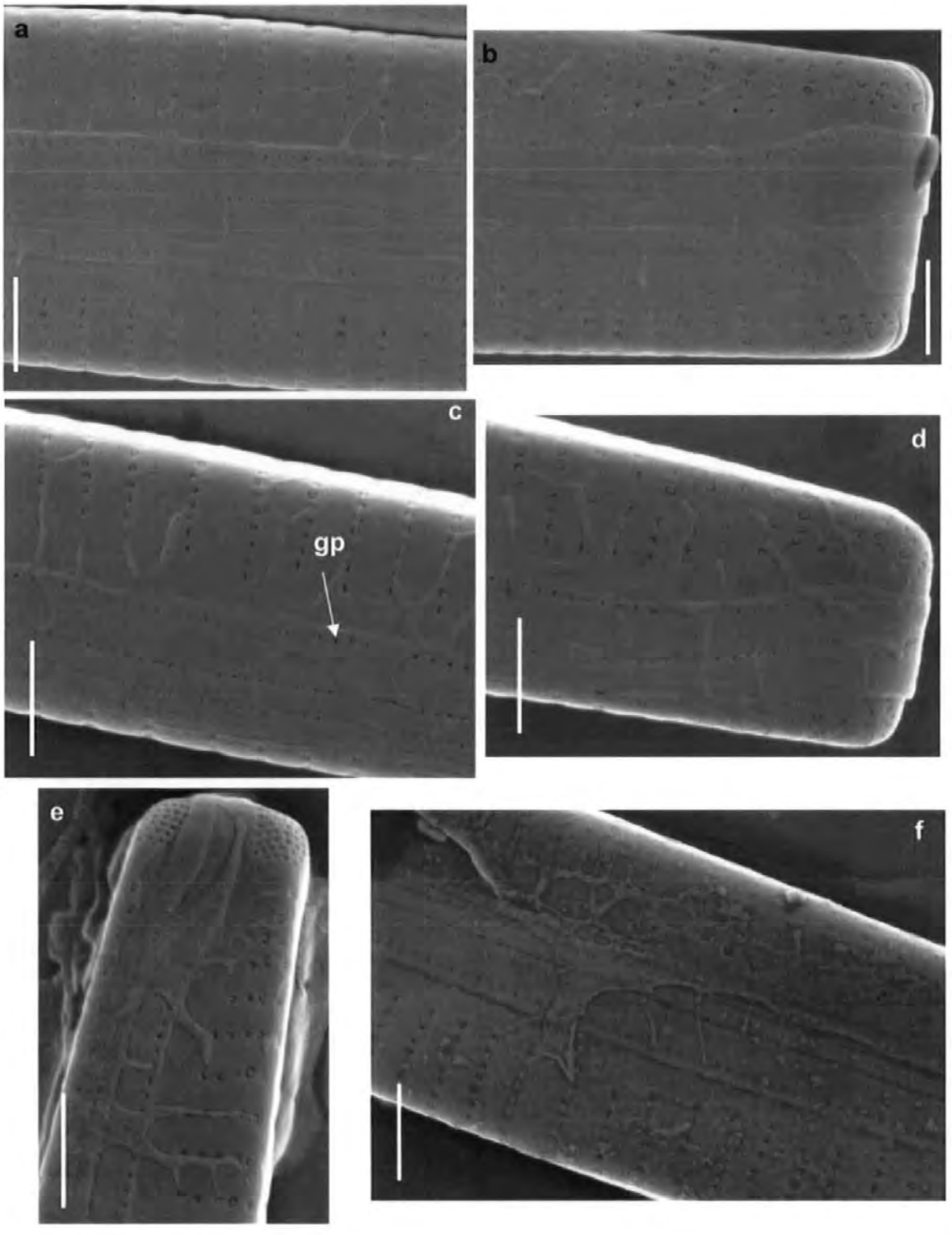


Figure 5.8: a-f

SEMs showing EPS present on uncleaned valves. Mucilage originating from mantle pores, girdle band pores (***gp***) and sutures. **(a-f)** Kings Mere (G_2), subculture 01/07/02. Scale bars = 2μm.

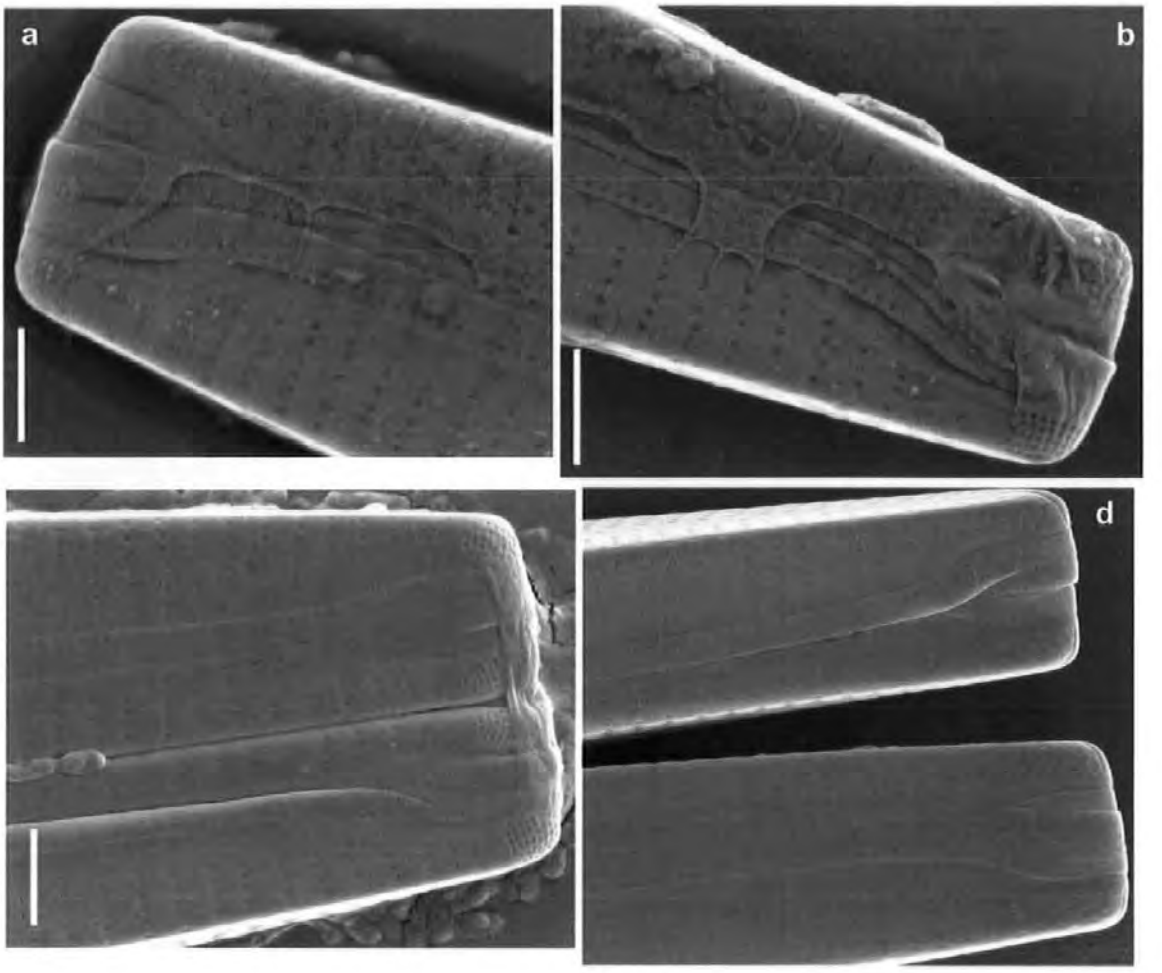


Figure 5.9: a-e

SEMs showing EPS present on uncleaned valves. Mucilage originating from mantle pores and girdle pores. **(a-b)** Kings Mere (G_2), subculture 01/07/02. **(c-d)** Barnwood Pond, subculture 01/07/02. Scale bars **(d)** = 5μm all others = 2μm.

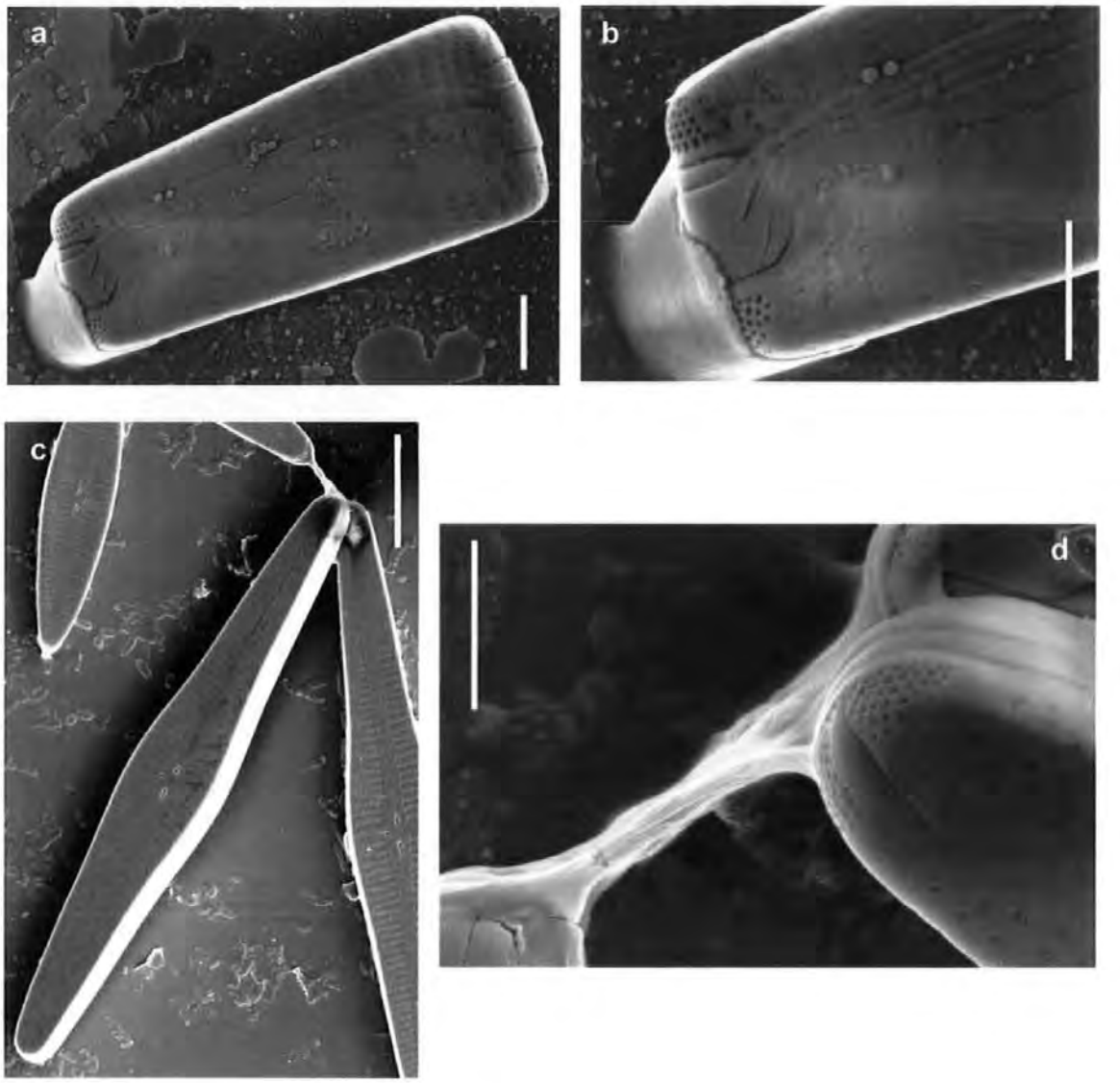


Figure 5.10: a-d

SEMs showing EPS present on uncleaned valves. **(a-b)** Mucilage pad originating from apical pore field of both valves in a single frustule in Abbey Lakes River clone, subculture 01/12/01. **(c-d)** Strands of EPS produced from apical pore fields of three single frustules, joining the three cells together in clone from Parys Mountain (G_2), subculture 20/05/02. Scale bars = 2μm.

5.2.4 Sexual Reproduction

As mentioned, certain clones under particular conditions were able to auxosporulate. However, these sexual stages were often unsuccessful, other than in the clonal stock cultures and the pH regime, suggesting that this stage in the life cycle of *G. parvulum* is especially sensitive to extreme changes in water chemistry. **Figures 5.11-5.12**, are simple diagrams illustrating some of the sexual reproductive stages as observed under LM for the Ham Gate Pond (G_1) clone at pH 5.5, and the Parys Mountain (G_2) clone from subculture dated 20/08/02. The process was the same for all four clones exhibiting auxosporulation, i.e. Kings Mere, Ham Gate Pond, Llyn Idwal and Parys Mountain. Not all sexual stages were observed.

Sexual reproduction was assumed to have begun when two vegetative parent cells pair-up, usually in opposite polar orientation **(Figures 5.11:a and 5.12:a)**, but sometimes lying on top of one another. Meiotic division was assumed to occur in both parent frustules (but not observed), giving rise to two gametes per parent cell **(Figure 5.11:b and 5.12:b)**. One gamete of each parent cell was passive, exhibiting no movement, whilst the other was active and moved across to the opposite partner cell to effect plasmogamy **(Figures 5.11:c and 5.12:b)**. During this process, a semi-protective matrix must have been exuded by both parents to then allow the valves of the frustule to either part or open in some way.

Plasmogamy produces a bi-nucleate cell, with nuclear fusion two zygotes were produced, one in each of the two parental thecae **(Figure 5.11:d)**. The zygotes are assumed to form a protective organic wall around themselves, and exhibit bipolar expansion, which eventually ruptures the parental thecal walls, though this was not observed, **(Figures**

5.11:e and 5.12:c). The nucleus was often obscured, and therefore its position could not be determined.

The zygote, now an auxospore, continued to expand. Perizonal caps at the poles of the expanded auxospores were only observed on one occasion. Within the organic casing of the expanded auxospore, an initial cell formed via mitosis **(Figure 5.12:d)**. The initial cell silicifies and further mitotic divisions occur to produce the initial valve and the first vegetative cell **(Figure 5.11:f)**. These initial cells varied from the basic features of a normal vegetative cell in that they were longer, had more rounded features and often, disrupted striae patterns.

The term auxospore is often used as an encompassing term including all developmental stages from the moment of plasmogamy to just before the laying down of silica to form the initial cell thecae. The stages in auxospore development and the formation of the initial cell are not protected by a silica wall, but by an organic layer and thus are more vulnerable to changes in the environment. This was evident in some of the deformations observed **(Figure 5.12: c)**. The lack of a restrictive cell wall produces initial cells with a modified morphology, notably expanded across the transapical plane with either only a very slight, or no heteropolarity in the apical plane. More often, initial cells appeared either isopolar or somewhat cymbelloid-like in outline. Additionally, initial cells and the first few vegetative cells do not have the typical rostrate or capitate head pole of the species, and the lengths of the polar raphe fissures were equal in size compared to vegetative cells. These "modified" morphologies suggest that the gross taxonomic characters of heteropolarity, symmetry and head pole morphology in *G. parvulum*, may not be determined until after a series of vegetative divisions.

Several clones were also mated in all possible pairwise combinations and left to grow for two weeks. At no time was auxosporulation observed suggesting that either all the clones tested were reproductively isolated or simply that conditions were not favourable.

The mean size ratio of initial cell length to parent cell length, produced by clones from Kings Mere (G_1), Ham Gate Pond (G_1), Llyn Idwal (G_1) and Parys Mountain (G_1), was on average 1: 3 (for Kings Mere and Ham Gate Pond) or 1: 2 (for Llyn Idwal and Parys Mountain). However, this could sometimes be as much as 1: 4 (Parys Mountain) or 1:5 (Kings Mere and Llyn Idwal), especially when particularly small parent cells were involved. The average length of a parent cell undergoing sexual reproduction varied between 9.5 and 17.5μm (Kings Mere =15μm, Ham Gate Pond = 11μm, Llyn Idwal = 9.5μm and Parys Mountain = 13μm).

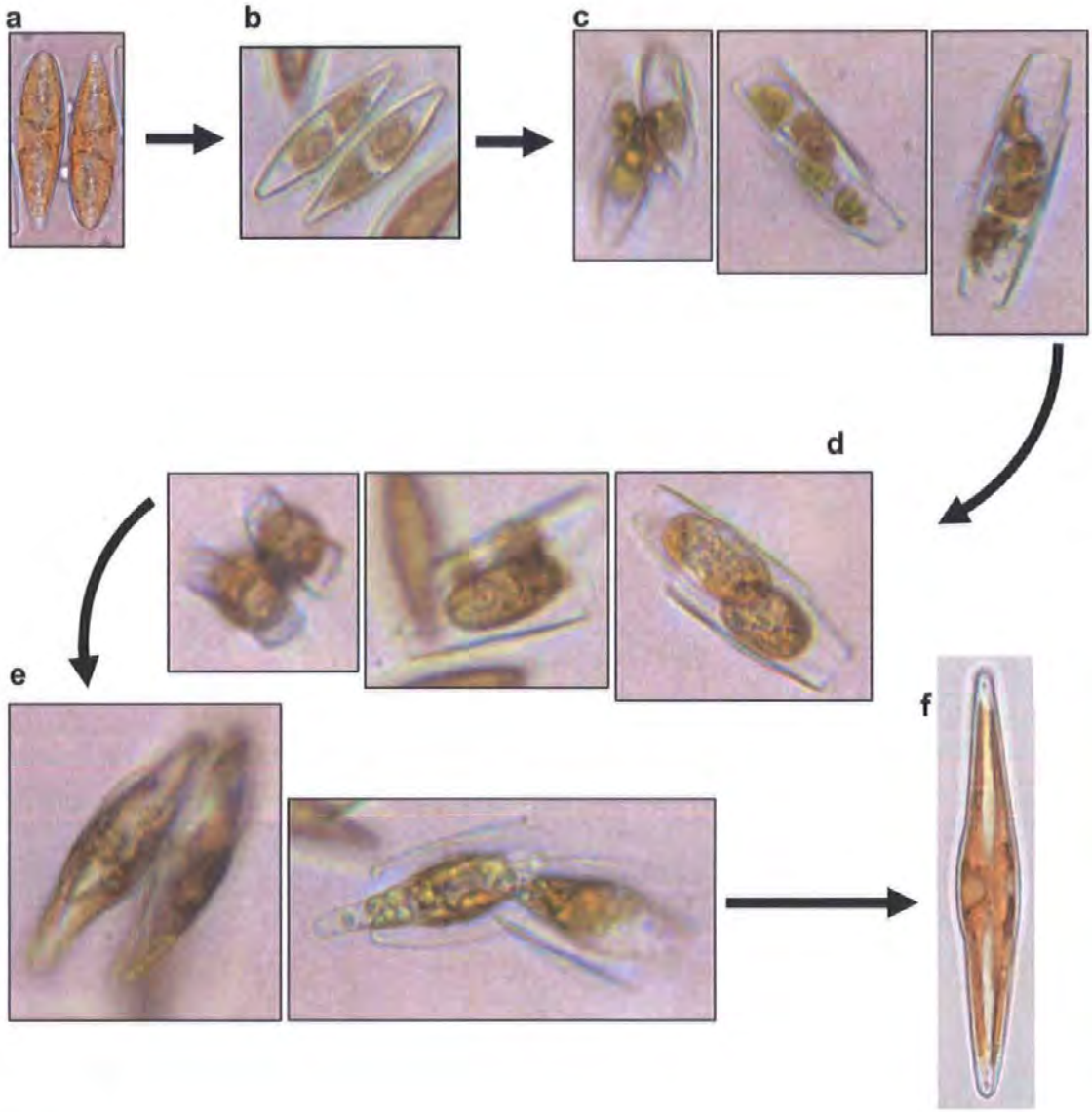


Figure 5.11: a – f

Sexual Reproduction and Auxosporulation in clone from Kings Mere whilst in experimental culture (pH Regime, pH 5.5). Respectively, **(a)** Cells pair up in opposite polar orientation to each other, girdle to girdle or lying with one cell under the other **(b)** Meiosis occurs in both cells, giving rise to two gametes each **(c)** One of the gametes is passive and one active. The active gamete from each cell moves to its partner cell to effect plasmogamy **(d)** Within the organic casing of the expanded auxospore, an initial cell forms via mitosis. The initial cell silicifies and further mitotic divisions occur to produce the initial valve. Further mitotic divisions occur to produce the first vegetative cell **(e)** Zygotes may have an organic casing, but this was not observed. Zygotes expand, eventually rupturing the parental thecae walls **(f)** vegetative cell.

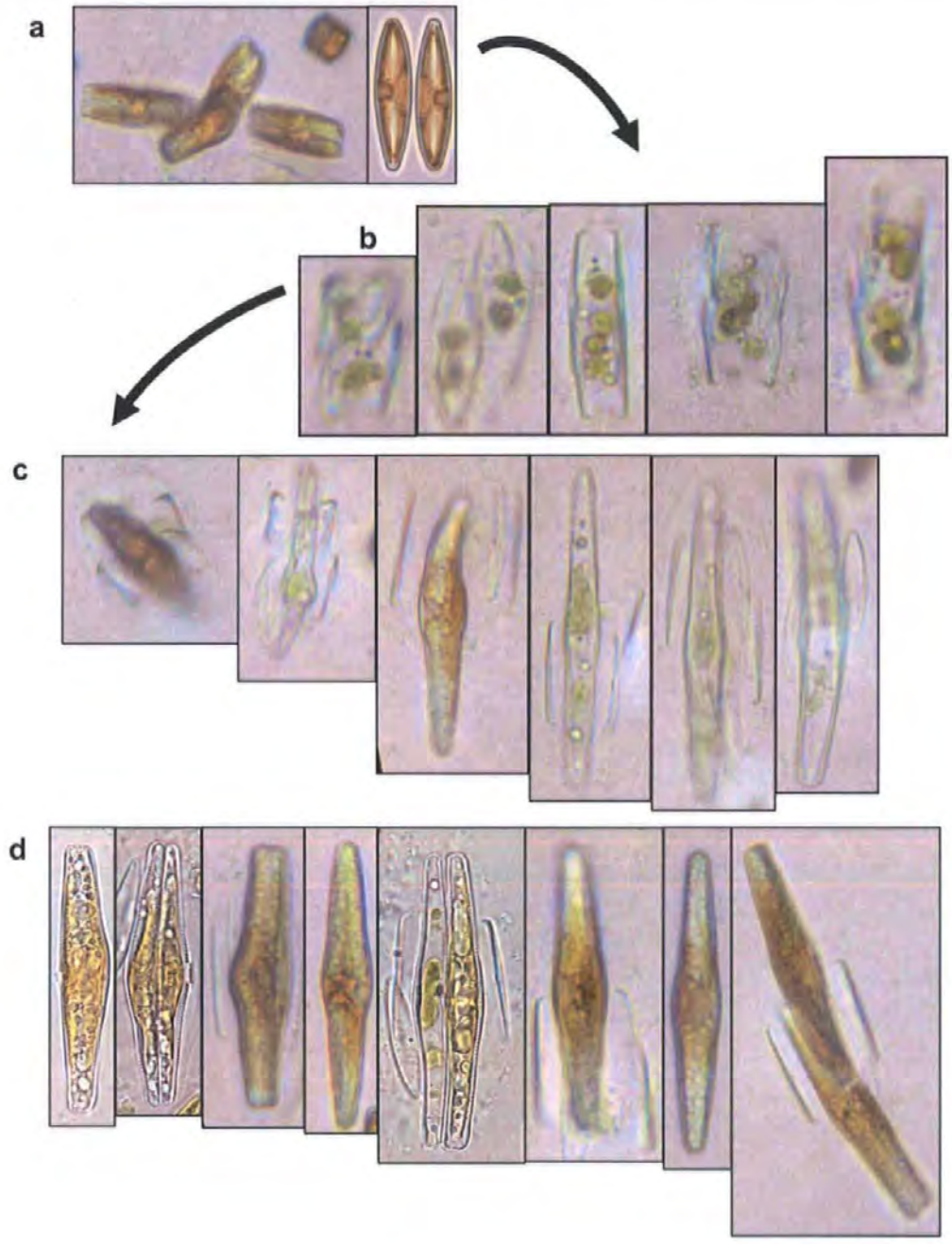


Figure 5.12: a-d

Sexual Reproduction and Auxosporulation in clone from Parys Mountain, subculture 20/08/02. Respectively, **(a)** Cells pair up in opposite polar orientation to each other, girdle to girdle or lying with one cell under the other. **(b)** Meiosis occurs in both cells, giving rise to two gametes each. One of the gametes is passive and one active. The active gamete from each cell moves to its partner cell to effect plasmogamy **(c)** Nuclear fusion produces two zygotes, one in each parent theca. Zygotes expand, eventually rupturing the parental thecae walls. **(d)** Within the organic casing of the expanded auxospore, an initial cell forms via mitosis. The initial cell silicifies and further mitotic divisions occur to produce the initial valve. Further mitotic divisions occur to produce the first vegetative cell.

5.3 CLASSIFICATION OF LIVE OBSERVATIONS

The purpose of the classification was to identify any major differences or similarities in observed characters between clones, and infer the probable causes of those differences. Where characters are stable, they may be used as a tool for differentiating between different eco- or morphotypes of the *G. parvulum* species complex.

Dendrograms are automatically truncated and provide within and between clone comparisons and within and between experimental regime comparisons. Where applicable, divisions in a dendrogram are denoted by the presence (+) or absence (-) of a character state, i.e. a character occurring/not occurring in all OUs for the cluster or group of clusters. Occasionally, a division may not be clear-cut and presence/absence of a character will not occur in all OUs for a cluster. Classification results are summarised in **Tables 5.3 and 5.4.**

Table 5.3

Summary of by-clone cluster analyses where n= number of Operational Units (OUs) in each cluster and

■ = Character state present in all OU's within a cluster

□ = Character state absent from all OU's within a cluster

▯ = Character state present in some, but not all OU's within a cluster

NB: Refer to Chapter 2 for character state code descriptions and examples.

			CHARACTER STATES FOR LIVE OBSERVATIONS																														
Clone	n	Cluster	ATT1	ATT2	ATT3	ATT4	ATT5	COL1	COL2	COL3	COL4	COL5	COL6	COL7	CHL1	CHL2	CHL3	CHL4	CHL5	CHL6	CHL7	CHL8	CHL9	CHL10	CHL11	PYR1	PYR2	VAC1	VAC2	OIL1	GRAN1	OTH1	OTH2	OTH3
Barnwood Pond	2	A																															
	1	B																															
	1	C																															
	3	D																															
Scion Pond	1	A																															
	1	B																															
	12	C																															
	58	D																															
Kings Mere	2	A																															
	1	B																															
	7	C																															
	1	D																															
	82	E																															
Ham Gate Pond	8	A																															
	3	B																															
	4	C																															
	111	D																															
Abbey Lakes River	5	A																															
	1	B																															
	2	C																															
	1	D																															
	1	E																															
Llyn Idwal	10	A																															
	96	B																															
	22	C																															
	5	D																															
River Kennet	11	A																															
	1	B																															
	5	C																															
	104	D																															
Pen-y-Bryn	5	A																															
	2	B																															
	2	C																															
	48	D																															
	6	E																															
Llyn Ogwen	1	A																															
	4	B																															
	1	C																															
	1	D																															
Parys Mountain	2	A																															
	1	B																															
	3	C																															
	180	D																															

Table 5.4

Summary of by-regime cluster analyses where n= number of Operational Units (OUs) in each cluster and

■ = Character state present in all OU's within a cluster

□ = Character state absent from all OU's within a cluster

▯ = Character state present in some, but not all OU's within a cluster

NB: Refer to Chapter 2 for character state code descriptions and examples.

			CHARACTER STATES FOR LIVE OBSERVATIONS																													
Clone	**n**	**Cluster**	**ATT1**	**ATT2**	**ATT3**	**ATT4**	**ATT5**	**COL1**	**COL2**	**COL3**	**COL4**	**COL5**	**COL6**	**COL7**	**CHL1**	**CHL2**	**CHL3**	**CHL4**	**CHL5**	**CHL6**	**CHL7**	**CHL8**	**CHL9**	**CHL10**	**CHL11**	**PYR1**	**PYR2**	**VAC1**	**VAC2**	**OIL1**	**GRAN1**	**OTH1**	**OTH2**	**OTH3**
Stock Cultures	1	A																															
	2	B																															
	3	C																															
	149	D																															
Sewage Regime	2	A																															
	4	B																															
	2	C																															
	55	D																															
pH Regime	1	A																															
	2	B																															
	10	C																															
	44	D																															
Copper Regime	1	A																															
	35	B																															
	53	C																															
	85	D																															
Zinc Regime	3	A																															
	4	B																															
	15	C																															
	6	D																															
	20	E																															
	88	F																															
Cadmium Regime	5	A																															
	4	B																															
	17	C																															
	12	D																															
	100	E																															

5.3.1 Clonal Stock Cultures

A cluster analysis revealed nested clusters for clones from Scion Pond, Kings Mere, Llyn Idwal and River Kennet, whilst the remaining clones showed a clearer separation amongst clusters. Nearly all by-clone analyses produced a 3 level, 4 cluster classification at between 73 and 94% similarity, except clones from Kings Mere, Abbey Lakes River and Pen-y-Bryn which produced 5 clusters and 4 level dendrograms at between 74 and 97% similarity.

Overall, a common factor that emerges from the by-clone cluster analyses is the separation of a number of OUs into a single branch cluster. This single branch, primarily consisted of circumneutral to alkaline OUs with the exception of the clone from Ham Gate Pond, which consisted of acid to circumneutral OUs, and the River Kennet clone, which included acid and alkaline OUs. The results suggest that pH is a major contributing factor to difference between clones. The results also suggest that pH has more of an effect on chloroplast morphology than any other character. Most of the separation also occurred for OUs during post exponential growth, when one might expect differences to occur due to initial shock of a different medium; however, the results may indicate a lag in behavioural and protoplasmic responses to changing environment.

Additionally, the separate analyses of parents (G_1 cells) and offspring (G_2 cells) for the Parys Mountain clone suggest that the initial run of the zinc regime and low levels of copper are contributing factors accounting for differences between parents and offspring and that there is a physiological basis to that difference.

Finer, more reliable clusters among clones can be achieved by including additional observations with more characters where type and equipment allow.

5.3.2 Experimental Regimes

A cluster analysis of clones under each experimental regime revealed typically nested clusters for all regimes except zinc. The stock cultures, sewage regime and copper regime all produced a 3 level, 4-cluster classification at between 73 and 80% similarity, whilst the pH and cadmium regimes produced 4 and 5 level classifications respectively, also between 73 and 80% similarity. The zinc regime produced a 5 level, 6-cluster classification at 77% similarity.

In the by-regime classification, results suggest that culture regime contributes to differences between clones and that some clones may have particular tolerances/sensitivities.

5.4 DISCUSSION

5.4.1 Relationship between Culture Regime & Observed Live Characters

Growth mode and EPS production: Mann (1999) noted in casual observations and in controlled laboratory experiments, that colony characteristics often change in response to environmental conditions as well as seasonally, and may be due to changing abundance of clones with different growth habits, phenotypic plasticity or environmental conditions. Chepurnov & Mann (1997) stated that unisexual and bisexual clones tend to grow dispersed, whereas monoeocious clones form dense tufts. If this were true, one would expect to observe the regular formation of dense tufts in the monoeocious *G. parvulum* clones however, most of the clones in this study grew as dispersed, single suspended or attached cells, whilst some grew on stalks or in fibrous EPS matrices. Growth mode was often not consistent within a clone, changing temporally and spatially with size and experimental regime. If changes in size throughout the life cycle of a diatom are also accompanied by changes in morphology, is it reasonable to suggest that growth mode could also change with size and morphology as well as environment.

EPS production by diatoms and other algae has been linked to changes in environmental conditions and different growth stages. Cultures may develop one or several different forms of EPS, and observations on the EPS produced by different clones show that mucilage is produced by all clones, but not necessarily in the same form or circumstances. The clones in this study agree with the literature that stalks and pads are formed and that stalks may occasionally be dichotomously branched. What is new however, is that some clones can produce tufted colonies and colonies in a matrix, which have not previously been observed in *G. parvulum*.

In this study, pad production was more common than tuft production, which in turn was more common than stalk production. Pad production was mainly associated with copper, zinc and cadmium and to a lesser extent pH, though it also occurred in older stock subcultures. Only the River Kennet clone regularly produced pads in the sewage regime. Stalk development was largely seen as single cells on single stalks, but rarely seen in the stock subcultures. The only clones to exhibit this mode of growth were Ham Gate Pond (G_2) (sewage), Parys Mountain (G_2) (zinc) and Kings Mere (G_2) at pH 8.0 and 8.5. Though not tested, it is entirely possible that stalk production was initiated by density (Lewis *et al.* 2002) as stalk production tended to develop late in the culture when cell numbers were higher. It is also possible that stalk production was a response to resource depletion (Hudon & Bourget, 1981; Staats *et al*, 2000). Although growth media were topped up regularly, it is possible that a fast growing population depleted nutrients faster than they were being replaced. This study has also shown that stalks are differentiated according to the studies of Daniel et al. (1987), Hufford & Collins (1972) and Wang *et al.* (1997).

Mass aggregations were of several types, either tightly bunched single cells, or tightly bunched tufted cells forming "island" populations in a sea of single attached cells, or the cells exhibited "swarming" behaviour. The latter is discussed further on (other behaviours). The other two modes of growth however, tended to occur intermittently in only one or two clones of the pH, copper or zinc regimes. It is unlikely to be a reaction to crowding, as the density of the masses would incur considerable self-shading. It is however, possible that massing of cells is a protective measure, particularly if it also encompasses mucilage. Tufts of cells were rare in stock subcultures, but more frequent

than stalk formation, and tended to occur in older cultures. In the experimental regimes, tufts were primarily associated with clones from Ham Gate Pond (G_2) and Llyn Idwal (G_2) under all metal regimes, with intermittent occurrences in other clones, and regular occurrences in clones from Pen-y-Bryn and Parys Mountain (G_2) for cadmium and zinc respectively.

The amorphous, mucilaginous matrices produced by clones from Ham Gate Pond (G_2), River Kennet and Llyn Ogwen, may also have been instigated by cell density or resource depletion as results suggest that EPS production increases generally, from exponential to stationary phase growth (Smith & Underwood, 2000; Staats et al. 1999, 2000). As EPS production requires energy, a lag phase in the growth period would allow time to gather resources and recover from the initial shock of a different environment (which may also have acted as a cue to start producing EPS as a protective measure).

SEMs show that EPS is largely in the form of strands rather than a smooth uniform deposit, however this could be an artefact of the drying and sputter coating process, e.g. size, shape and position of mucilage strands may change via shrinkage or coalescence. Wang et al. (2000) had particular difficulties in preserving stalks of *Achnanthes longipes* Agardh and *Cymbella cistula* (Ehrenberg) Kirchner for SEM and in TEM, due to the high level of hydration. Instead, they utilised high-pressure freeze substitution and cryo-field emission scanning electron microscopy, which had been applied successfully to plant, fungal and animal cells. The mucilage trails and paths preserved with this technique are more detailed and depict an almost web-like structure of mucilage strands. Clearly, better preservation techniques need to be tried and developed in order to clarify EPS structure and origin in *G. parvulum* and other diatoms. Nonetheless, none of the features observed

and described in this study occur in acid cleaned samples, which would seem to confirm that they are not artefacts. The studies of Rosowski et al. (1983) and Wang et al. (2000) together with Edgar (1983), have shown that mucilage is exuded from sutures, raphe and pores of the valve face and mantle (expanding thereafter), therefore agreeing with the observations in this study.

Chain formation among the *Gomphonema* spp. is not recorded in the literature either in nature or in culture, and its rare occurrence in his study suggests it may be an artefact of culturing. Only the Abbey Lakes River clone formed chains regularly in most stock subcultures, and therefore this may be an adaptive strategy for this clone, which was very small when collected. Its small size is unlikely to be encountered in nature. Paired cells on the other hand were more common, occurring in most OUs for clones from Kings Mere (G_2) (copper, zinc, stock subcultures), Ham Gate Pond (G_2) (copper, pH and stock subcultures), Llyn Idwal (G_2) (pH), Llyn Idwal (G_1) (stock subcultures), River Kennet (stock subcultures, pH and zinc), Parys Mountain (G_1), (pH, copper, zinc) and Parys Mountain (G_2) (pH). The pairing observed differed from that of sexualised pairing, in that paired cells were always in the same polar orientation and paired girdle to girdle. Again, this behaviour is not recorded elsewhere for the *Gomphonema*, and its purpose is unknown, but it usually occurred in a large portion of the population.

The most common feature of the cultures were that all clones at some point and fairly regularly, exhibited single cells attached in valve and or girdle view and thus this can be said to be the main mode of attachment in *G. parvulum*. Cells suspended in the water column (usually most or all of the population), were almost as common and a somewhat unusual occurrence for a benthic taxon. Invariably, suspended cells were not motile, other

than the odd cells here and there. There was also no direct correlation with the occurrence of cells containing enlarged vacuoles or those with plentiful oil droplets, therefore, neither vacuoles nor lipids were changing buoyancy in these cells. It is therefore not clear what mechanism was allowing the cells to maintain their buoyancy.

Chloroplasts: Pale chloroplasts were evident in all clones subject to experiment and in a few cases, chloroplast damage in the form of loss of pigment was evidenced by the appearance of a number of green or yellow chloroplasts. In both cases, the frequency of occurrence increased with time and/or higher concentration of test substance. Diatom chloroplast colour is known to vary owing to the carotenoid pigments contained within, which mask the colour of the chlorophylls that normally give plants their green colouration. Pale and even green or yellow chloroplasts however, have been linked to high irradiances and the effects of pH (acid conditions destroy carotenoids revealing the chlorophylls) (Round *et al.* 1990). Studies in higher plants show that higher light intensities are capable of destroying chloroplasts of shade tolerant ecotypes (Bjorkman & Holmgren, 1963). As all the clones in this study are benthic, and from either turbid or disturbed sites. It seems highly probable that they would be shade adapted and therefore more prone to damage by high light intensities. Neither the lamps nor the highest light level used in this study however, are likely to have provided enough damaging short-wave radiation as the highest light levels used were <50 μmol/cd^{-2}/s^{-1}, therefore photoinhibition is an unlikely cause of the pale chloroplasts, or loss of pigmentation often observed. Loss of pigmentation was not confined to experimental regimes; stock cultures also exhibited pale, green and yellow chloroplasts, mostly in older subcultures. Loss of pigmentation may therefore indicate other stresses, e.g. crowding, depletion of nutrients. Although little is known about the impact of temperature on certain environmental factors, e.g. metal toxicity, temperature is thought unlikely to have been the cause of chloroplast

damage in the majority of cultures in this study, as most were kept at the same temperature. Only within the Light and Temperature Regime were light and temperature varied, and here pale chloroplasts developed between 25°C high light and 30°C low, medium and high light. Pigment loss in the pH regime also occurred, but was erratic. Therefore, whilst a combination of temperature and high irradiance may elicit a pigment loss response, the intermittent occurrence of pigment loss in the pH regime would suggest that there are other factors eliciting this response.

Yellow or green chloroplasts were considerably rarer than pale chloroplasts. The only consistent loss of pigment within a clone subject to assay was for Parys Mountain (G_2) (almost all copper concentrations at K_{6+} days), Kings Mere (G_2) (at higher copper , most zinc concentrations at $K_{0\text{-}6}$ days), Ham Gate Pond (G_2), Llyn Idwal (G_2) and River Kennet at low, low to medium and low to high zinc concentrations (K_{6+} days). Interestingly, the Parys Mountain (G_1) clone rarely lost pigment, unlike its daughter progeny, in which loss of pigment occurred the most frequently for the copper regime. Loss of pigment was not however, a major feature of the cadmium regime, with the Llyn Idwal (G_2) clone being the most affected at most or all concentrations, though the clone from Ham Gate Pond (G_2) was more affected at $K_{0\text{-}6}$ days than at K_{6+} days, and the River Kennet clone affected at K_{6+} days. The results suggest that in some clones at least, loss of pigment may be a sign of metal stress, but that compensatory mechanisms may work to alleviate or repair initial damage.

Accessory pigments are also able to protect against damage at high irradiances by accumulating and absorbing excess photons, and producing additional carotenoids or alternatively, as shown by Gallagher & Alberte (1985), increase photosynthetic capacity

at low irradiances by increasing pigmentation. Whether this translates as darker chloroplasts is not clear, but several clones (no discernable pattern) exhibited darker chloroplasts in both stock and experimental cultures. Occurrence was rare and inconsistent, but primarily within the pH and cadmium regimes (one in the copper regime), it therefore seems unlikely that increases in pigmentation were a direct response to experimental assay, but may be a measure of increased metabolic activity in particular clones in response to an unknown (genetic?) factor. The increase in pigments in low light has however, been attributed to the effect of nutrient limitation in batch cultures (Beardall & Morris, 1976). This may account for the intermittent occurrence in both stock and experimental cultures, and changes in pigment content may optimise the cells ability to harvest light. Only further experimentation will determine if darkened chloroplasts are inducible, and which pigments are involved.

Clonal cultures also exhibited changes to chloroplast size. Ballantine & Forde (1970) were able to show that the number of chloroplasts per 0.01mm^2 in higher plants, differed significantly between temperature regimes, though not with light intensity. With only a single chloroplast and therefore an inability to increase chloroplast numbers, *G. parvulum* must find other ways to maximise photosynthetic capacity, therefore the size of the chloroplast relative to cell size may be important. It may however, be difficult to separate from normal chloroplast changes related to life cycle stages and cell diminution. Nonetheless, reduced size chloroplasts were repeatedly seen in all clones, increasing in frequency of occurrence as time progressed. Size reduction generally took two forms; one caused by enlarged polar vacuoles squeezing the chloroplasts to the cell wall and therefore reducing photosynthetic capacity, the other condensing the chloroplast to the centre of the cell. The latter was not necessarily accompanied by enlarged vacuoles.

Medially condensed chloroplasts may be a response to alkaline conditions, which are known to dissolve silicate, and thus diatom frustules. Alkaline conditions tend to produce diatoms with thinner valves and less prominent features, which may affect the permeability of the cell, resulting in shrinkage of the chloroplast away from the cell wall. This character was observed in alkaline conditions in this study however, it was also observed in circumneutral and acid conditions in the River Kennet clone and additionally, in a number of the metal regimes (particularly copper and zinc), and in some of the sewage and stock regime cultures. In the cadmium regime, condensed chloroplasts occurred more often at the lower concentrations, in only one or two odd cultures. As shrinkage also occurred in a few stock cultures this, like pigment loss, may be a sign of stress rather than pH or metal concentration. In the sewage regime, the stress factor may have been the fast increase in the bacterial population. The link with metals however is less clear. Ivorra et al. (2002) however, have shown that non-viable or dying cells have strongly condensed chloroplasts or are irregularly shaped or fully spread throughout a vacuolised cell. This may account for increased occurrence of highly vacuolated cells with condensed chloroplasts after several weeks in culture as well as a number of cultures with expanded chloroplasts. Canterford (1980) has shown that cells exposed to 300μg/l copper, had protoplasts that withdrew from the cell walls and chloroplasts concentrated in a small group. Whilst no effect on the protoplast was observed, and the copper concentrations used were considerably less, the condensed chloroplasts could indicate physiological stress due to pH in the River Kennet clone, possible crowding or nutrient stress in stock cultures, and metal stress in clones from River Kennet (copper, zinc and cadmium), Pen-y-Bryn (copper, zinc and cadmium) and Parys Mountain (G_1 and G_2) (zinc).

Chloroplasts were also seen to exhibit expansion. The extension of the chloroplast into the poles of the cell may in part, be a response to size diminution, i.e. in order to maximise light intake and photosynthesis, though expansion occurred in larger cells too. Alternatively, slight expansion and contraction may simply be part of the vegetative cycle. Further observation, experimentation and analysis are required.

Vacuoles, oil droplets, granules and dark bodies: Occasionally vacuoles coalesced into a single large vacuole occuping one pole, pushing the cytoplasm and chloroplast to the other pole. There is no reference to this phenomenon in the literature. On the other hand, Sicko-Goad & Stoermer (1979) have demonstrated that the volume of the vacuole could increase considerably after phosphate starvation and remain high even after phosphate uptake in *Diatoma tenue* var. *elongatum*. Whilst none of the cultures in the study were phosphate starved, and media were topped-up on a regular basis, the speed at which phosphate depleted would be dependant upon the clone and its growth rate. There is no clear indication as to why vacuolisation should increase unless benthic diatoms employ similar tactics to that of plankton diatoms, and utilise the vacuole as a buoyancy device to obtain nutrients. It seems more likely however that the enlarged vacuoles are a symptom of metal toxicity and pH tolerance/intolerance possibly affecting permeability.

Granular inclusions consistently occurred in nearly all subcultures of clones from Llyn Idwal (G_2) and Parys Mountain (G_2), all pH levels for clones from Kings Mere (G_2) Ham Gate Pond (G_2), Llyn Idwal (G_2), Parys Mountain (G_1) and Parys Mountain (G_2), most or all copper concentrations for clones from Kings Mere (G_2), River Kennet and Parys Mountain (G_2), most or all zinc concentration for clones from Kings Mere (G_2) and Parys

Mountain (G_2) and all cadmium concentrations for clones from Ham Gate Pond (G_2), Llyn Idwal (G_2), Parys Mountain (G_1) and Parys Mountain (G_2). It is likely that these are polyphosphate granules as indicated by Dawson (1973a). Sicko-Goad & Stoermer, (1979) have shown that these granules are capable of incorporating heavy metals as a means of reducing toxicity, and that some form of exocytosis may occur to rid the cells of the metals. The results would then suggest that most clones have this capability, however none of the smaller cells (Kings Mere (G_1), Ham Gate Pond (G_1), Abbey Lakes River, Llyn Idwal (G_1), Llyn Ogwen) of the stock subcultures nor clones subject to the sewage regime had granules. In the case of the latter, this was presumably because phosphate was plentiful however, for the smaller cells the requirement for phosphate may not have been as high as in larger cells and the culture medium supplied sufficient nutrients. Alternatively, the small size of the cells coupled with large and/or expanding chloroplasts may have obscured observations.

Frustular teratologies: Whilst frustular morphology has shown that teratologies are produced by environmental conditions, they were more difficult to observe consistently in live cells under LM. Only clone Kings Mere (G_2) consistently exhibited some form teratology whilst under the copper regime, all other teratologies were observed in the pH, copper or zinc regime; none were observed for the copper or sewage regimes. The teratologies largely take the form of a bulging theca, i.e. in girdle view one valve would be either considerably convex or considerably concave. Sometimes the convex or concave extremes were polarised and sometimes the convexation was in the form of a medial "dent" in the thecae. This suggests that stresses were placed on the cells either during vegetative reproduction whilst the daughter thecae were being laid down (resulting in one distorted valve) or, whilst an initial cell was being formed inside an auxospore (resulting in

a two distorted valves). As isolated entities, these few teratologies are not necessarily indicative of population stresses and may instead reflect differences in cell fitness.

Other behaviours: There was some unusual behaviour observed for one or two clones that in many ways were akin to faunal responses, e.g. clones from Barnwood Pond in the Light and Temperature Regime, at the lower temperature of 5°C, Ham Gate Pond (G_2) at pH 4.0 and 4.5 and River Kennet at pH 4.5 and 5.5, all appeared to "avoid" the light by crowding at the edges and walls of the culture vessel. This phenomenon is not mentioned anywhere else in the literature. In one culture believed to be the clone from the River Kennet (damaged notes), cells were observed to actively "swarm" toward, as well as in and around, a large mass of vigorously active cells in the culture dish reminding one of slime mould pseudoplasmodia, the streaming together of single cells into one giant organism. One can speculate that the frenzied activity had something to do with reproduction however, no auxospores were ever formed in this clone. It might also have been precursor behaviour to the formation of a mucilage matrix, as exhibited by this clone at pH 8.5.

5.4.2 Sexual Reproduction

Sexual reproduction was seen to initiate in several stock subcultures, pH regime cultures and L&T regime cultures however, only the stock cultures successfully auxosporulated. As most of the population for the successful clones underwent sexualisation, the clones would seem to fit either the first life history strategy of Edlund & Stoermer (1997), i.e. "synchronous sexuality under favourable growth conditions" as stock cultures had been maintained in these conditions for some time prior to sexualisation. Whereas the

unsuccessful sexualisation of some of the clones in the pH and L&T assays, would appear to fit the second life history strategy "synchronous sexuality under unfavourable growth conditions", i.e. the shock of placement into an alternative environment. Having said that, most of the light and temperature conditions at which sexualisation occurred should have been favourable. Additionally, the clone from Ham Gate Pond (G_1) sexualised at $K_{0\text{-}6}$ days, whereas clones from Scion Pond and Ham Gate Pond (G_2) in the pH regime sexualised at K_{6+} days (Scion Pond also had deformities). Sexualisation however occurred in only a small part of the population and so perhaps is better described as asynchronous sexualisation. Alternatively, sexualisation in a small part of the population suggests rapid decay of environmental cue (Armbrust et al. 1990) which in turn may have caused failure to reproduce. Without further experimentation, it is not possible to say which life strategy these clones employing.

There have been few comprehensive studies on the use of live material in diatom taxonomy and yet it was an important part of early taxonomist's toolbox. Without the use of SEM or TEM, good preservation techniques and often with lower resolution microscopes, early diatomists would always describe the live diatom cell or colony prior to any analysis of the cleaned frustule. Indeed these early descriptions often relied on the live features to inform taxonomic circumscription. Whilst these descriptions were largely basic compared to today, the habit of looking at live material appears to have fallen out of use, perhaps due to the ease with which numerous samples can now be cleaned and examined. Additionally, photographic film is expensive and camera lucida drawings time consuming. However, the advent of high specification digital cameras and digital video has made examination of live material easier, more accessible and considerably more cost effective in the long term.

By not including descriptions on live material and considering how those features in addition to cell wall morphology, change over time and in different environments, diatomists may be missing vital information relating to the ecology and ontogeny of diatom taxa. Additionally, many floristic descriptions that do mention live characters and habit, do so only briefly and there is the possibility that some of these, in the absence of evidence to the contrary, are simply descriptions that originate from those early taxonomists that have been repeatedly recycled without context, or are based on the authors own, but unpublished observations. Such descriptions need to be verified and substantiated if they are to be held up as taxonomic truths. One way to do this is to conduct culture experiments, another to observe cells in situ in either cultured or natural environments, and diatomist need to be encouraged to publish such observations. Just as diatomists have built up extensive databases of taxonomic information based on cell wall morphology, diatomists can equally build complimentary databases of information based on live material.

5.5 CONCLUSION AND SUMMARY

The results of the present investigation show that the adaptive strategy of *G. parvulum* clones primarily involves variation in the morphology of the chloroplasts and mode of attachment, and that pH is a dominant factor or predictor in separating clones, suggesting there are discrete ecotypes. Culture regime can account for differences in live morphology in clones from Scion Pond, Parys Mountain (G_1 and G_2) and Llyn Idwal (G_2) showing that these clones are either particularly sensitive or adapted to pH, copper, zinc and cadmium respectively. The universal mode of attachment for all clones is as single cells attached in valve or girdle view to the substratum however, most clones were also able to develop pads, stalks and mass aggregations involving EPS production though not in a consistent manner that would allow separation of clones based on mode of

attachment. There are suggestions that crowding, bacteria and possibly nutrient depletion may act as cues to EPS production.

Stalk and pad morphology has been established as consisting of a collar and differentiated shaft, with stalk length exceeding 3x length of the cell. EPS production in most clones and regimes developed in the stationary growth phase (K_{6+} days) and EPS originates from areolae on the valve face, the raphe, mantle areolae, girdle band pores and sutures and forms stands of polysaccharide over the surface of the cell.

Clones subject to pH assay were more variable in live character morphology than any other assay, whilst copper and zinc regimes elicited similar morphological profiles and sewage and cadmium regimes were the least variable, though loss of pigment may indicate physiological stress.

Sexual reproduction is physiologically and behaviourally anisogamous, however size differences occur between clones entering the sexual phase with the parent: initial cell length ratio varying between 1: 2 and 1: 5 μm. Non-sexualised populations continue asexual division until cell size reaches a point where a cells ability to function is compromised and the population dies. Whislt clonal survival is highly variable, lasting between 3 and 21 months depending of age and size, smaller cells are more prone to producing chains of cells and dense aggregations than larger cells, but overall are less variable in terms of the live characters.

This study has shown that even the simplest of studies can yield valuable information and aid in the understanding of diatom ecology and life cycle.

6 GENERAL DISCUSSION

The aims of this thesis were to document variation in the distribution and morphology of *G. parvulum* and establish whether different morphological races have particular autecologies, or whether morphology is a response to environment, prompting a taxonomic revision of the species and the recognition of ecotypes or ecomorphs. The combination of an historical perspective and diatom culture observation and experimentation, enabled these aims to be explored.

6.1 CIRCUMSCRIPTION, NOMENCLATURE, TYPIFICATION AND SPECIES CONCEPTS

Whilst contemporary botanical science and the ICBN recognises genotypic and ecophenotypic variation in morphology, the recognition of new species occurs because the material differed in some small way from the designated type (Rines, 1994), and regardless of intent, such workers are following a typological species concept (Rines, 1994). This is an outmoded concept no longer accepted by the ICBN. Today, the type serves a nomenclatural rather than taxonomic role (Longino, 1993; Williams, 1993), but in doing so fixes the morphology of a specific specimen, inadvertently promoting its comparison with similar and related entities. However, regardless of shifts in species concepts, changing circumscriptions and the inclusion/exclusion of individuals from the taxonomic set, the name remains with the type specimen.

With the ICBN rules in mind, it therefore falls to a defined species concept to delimit taxa within the *G. parvulum* complex. However, defining a group of taxa based on a single species concept (Biological, Ecological, Evolutionary, Phylogenetic) intrinsically requires

the taxonomist to place a larger emphasis and importance on a particular set of criteria, sometimes to the exclusion of all others.

There are many different species concepts (typological, nominalist, biological, phenetic, phylogenetic, ontogenetic and evolutionary) and no single concept has been identified or accepted as the taxonomic standard. Each has a number of problems, and lack correspondence with each other. For example, the Biological Species Concept (BSC) rests on the idea that interbreeding (or the ability to interbreed) is of prime importance in evolution. Yet breeding compatibility is only one of several evolutionary forces acting on a species. Mann & Kociolek (1990) reviewed species concepts in diatoms with particular reference to the raphid diatoms, using it as a basis for comparison between different groups of organism. This is despite the large number of problems with this concept in the literature (e.g. it excludes asexual reproduction and extinct species) (Gallagher, 1980, 1982; Ghiselin, 1987; McCourt & Hoshaw, 1990; Templeton, 1989). Yet in diatoms, taxonomic species are still largely defined by the morphology of the silica cell wall (phenetic concept) as evidenced by the taxonomic monographs routinely used for identification purposes. The extent to which morphology is reflected in the genotype however, is largely unknown. Whether a phenetic, ecological or biological concept is used, they all inherently suffer non-correspondence and consequently are difficult to compare or apply uniformly.

As a unifying species concept does not exist, the general approach is different classifications are utilised depending on the requirements of the researcher and the questions asked, or where one tries to accommodate and explain different aspects of each species concept (a pluralistic approach). Mishler & Donaghue (1982) state that

single, optimal, general purpose classifications are possible for particular situations, but that the criteria in each case are likely to be different. Choice of criterion is of course largely down the questions asked and the preferences of the researcher. As long as the reasoning is expressed clearly however, reciprocal comparisons should be possible. However, data should be sought from all areas of research , where feasible to build up a complete picture. Yet in diatoms, cleaned valve morphology is consistently used to both group and rank taxa, but does not necessarily reflect biological or evolutionary trajectories.

6.2 EVIDENCE FOR DISCRETE TAXA

6.2.1 Morphology

This study agrees with floristic literature that considerable morphological variation is inherent in the cell wall of *G. parvulum*, as evidence by each of the clones studied, with the exception of clone River Kennet, which was altogether different. A large proportion of the morphological variability observed is a direct result of cell size reduction and auxosporulation, accounting for much of the statistically significant differences encountered. There were however morphological and behavioural differences attributable to assay concentration. Therefore, there is a direct link between physiology/biology and morphology and ecology however, morphological variability confounds efforts to separate the clones into discrete entities. Whilst the clones can be separated qualitatively into morphologically distinct entities based on floristic micrographs and descriptions, and more simply by eye, most characters chosen in this study have proven to be insufficiently stable to allow quantitative separation. On the other hand, the ratio between upper and lower raphe slit, and the ratio between stigma-side and non-stigma side central stria density remained constant regardless of size. Both Geitler (1932) and Geissler (1970a) have shown that stria density remains relatively constant with decreasing valve size. If

different *Gomphonema* spp. can be shown to have the same intraspecific, but different interspecific ratios these two characters may serve to discriminate at the species level. Whilst the data shows that a predominant valve shape occurs in each clone, it is not consistently associated with a particular size class.

Qualitatively however, the Scion Pond clone can be separated based on stria density (14-17/10μm) on the stigma side of the valve. In all other clones, both the stigma and non-stigma sides have the same stria density, 10-15/10μm). Additionally, clones from River Kennet and Llyn Ogwen can be separated based on an unchanging valve shape. This is particularly marked in the River Kennet clone, in which there was little variance in any character except the number of central short striae.

Morphological data also indicate that certain clones are more tolerant of certain environmental conditions than others, though no one culture treatment induced changes, that were consistent with *G. parvulum* varieties established in published diatom floras. However ecophysiologically, the Scion Pond clone can be separated from on the basis of its tolerance to organic pollution, the Llyn Idwal, River Kennet and Parys Mountain (G_1) clones on their pH tolerance, Pen-y-Bryn and Parys Mountain (G_2) clones on their copper tolerance, Scion Pond, River Kennet and Parys Mountain ($G_1$and G_2) clones are tolerant of zinc, and Ham Gate Pond, Llyn Idwal, River Kennet, Pen-y-Bryn and Parys Mountain (G_2) clones are tolerant of cadmium.

These data highlight that traditional morphological measures are insufficient to separate *G. parvulum* clones at the species level (also shown by Trobajo & Cox, 2006). It is recommended that future studies on *G. parvulum* take a keener interest in SEM level characters such as the distance between striae, or areola density, the latter has been shown to be a reliable character in other taxa (e.g. *Amphora*). Additionally, a quantitative measure of shape such as contour analysis, rectangularity, Legendre or Fourier descriptors (Loke *et al.* 2004; Mou & Stoermer, 1992; Stoermer & Ladewski, 1982; Rhode *et al.* 2001) may yield further information.

Despite the lack of discriminating characters delimiting the taxa, this study has highlighted new and important morphological features not previously recorded for *G. parvulum*.

- **The *G. parvulum* complex encompasses other previously described taxa:** The *G. parvulum* species complex includes two previously described species, *G. gracile* and *G. hebridense*, as evidenced by four clones that underwent auxosporulation. Clones from Kings Mere, Ham Gate Pond and Llyn Idwal, were initially identified as 3 morphs of *G. parvulum* var. *parvulum* fo. *saprophilum* (the clone from Abbey Lakes River was also identified as this taxon), and the clone from Parys Mountain identified as a morph of *G. parvulum* var. *exilissimum* (clone from Pen-y-Bryn was also identified as this taxon) according to modern floras. After auxosporulation and subsequent size reduction, it was clear that clones from Kings Mere, Ham Gate Pond and Llyn Idwal consisted of *G. gracile* valves and Parys Mountain of *G. hebridense* valves. The data also show that other clones consist of previously described taxa, i.e. the clone from Barnwood Pond consists of *G.* cf. *lagenula*, the clones from Scion Pond and River Kennet, *G.* cf. *innocens*

and the Abbey Lakes River clone a third unidentified *G. parvulum* morph (denoted *G. parvulum* #3).

- **The heteropolarity assumption:** Auxospores and initial cells are almost isopolar, indicating that marked heteropolarity in this taxon requires several vegetative divisions before "typical" gomphonemoid heteropolarity is formed.

- **The absence of size reduction:** Clone River Kennet does not undergo size reduction, a rarely recorded phenomenon (e.g. Geitler, 1932). Mann *et al.* (1999) Round *et al.* (1990) suggest this is possible if the girdle is sufficiently arched. This clone however has also shown a small degree of non-sexual cell enlargement. Cox (pers. comm.2006) has suggested there may be sufficient flexibility in the valve mantle to allow for some cell enlargement. This phenomenon is rarely recorded (see Geissler, 1970a, 1970b). Measurement error has been discounted.

- **Non sexualised abrupt changes in cell size:** A number of anomalous (not linked to assay level) abrupt cell size changes occurred in culture. Mann *et al.* (1999) stated that occasional disturbances in cell division can cause abrupt changes in cell dimensions. This would seem to suggest that assay level effected disturbance to cell division. If this were the case however, one would expect to see similar effects at neighbouring assay levels. This was not the case. Therefore, it seems likely that the culture medium was in some way compromised.

- **Production of other forms of EPS:** The clone sin this study were seen to produce not only stalks and pads typical of the taxon but also, on occasion, a mucilage matrix, tufts and flocs, non-sexualised pairings and when very small in

size, chain of cells. This is the first time these modes of growth have been observed in this taxon and may be taxon specific and/or related to a change in environment and/or size.

These observations strongly argue against reliance on diatom valve morphology as the sole criterion on which to delimit taxa, and provide a compelling argument for the benefits of algal culturing and observation of live material. There are serious issues for the sampling of waters and the use of diatom indices of water quality. Diatom (or any biological) sampling, by its very nature, provides a snapshot of the community at a particular point in time. Even if a particular site is sampled repeatedly every week or month, it becomes meaningless unless something is known about the ecology and life cycle of that taxon and the time span over which the life cycle occurs. The reason why actively sexualised cells are not encountered in samples may simply be that we are not looking in the right places at the right time, that sexualisation occurs only in part of the population, or that like cells at their lower size limits, sexualised cells are diluted out of the population (Round *et al.* (1990), especially if auxosporulation is relatively rapid. If one cannot accurately identify the different stages in the life cycle (as a zoologist would identify the juvenile tadpole and adult frog), community analyses, water quality indices and taxonomic studies will be invalid, especially if different parts of the life cycle are shown to be ecologically as well as morphological variable.

6.2.2 Experimental Assays

Overall, all the clones were remarkably tolerant, with widely varying tolerance ranges for light, temperature, pH, sewage, copper, zinc and cadmium, only failing to grow at the higher metal assay levels. Growth rate generally decreased with increasing metal assay

and temperatures above 25°C. Overall, there is little difference between the clones in their light, temperature and metal tolerances and ranges. Apparent differences in pH optima should however, be treated with caution as growth rates were very variable and very erratic, both between replicate cultures and between assay levels.

There were marked differences between parent cells and progeny for clones from Kings Mere and Llyn Idwal in the light and temperature regime, and the clone from Parys Mountain in the pH regime. The parent cells grew better under alkaline whilst the progeny grew better under slightly acidic conditions. Generally the data agree with the literature that *G. parvulum* is a broadly pollution tolerant taxa. It is therefore problematic to delimit *G. parvulum* clones based on growth rates.

Some constituents of algal culture media such as Ethylene Diamine Tetra-acetic Acid (EDTA = disodium salt) as well as amino acids, organic matter, humic acids, and fulvic acids can detoxify metals. On the other hand, it could be argued that substances such as EDTA are representative of a natural process whereby portions of the metal are sequestered and therefore a valid addition. It may also be important in not over-stating accumulation rates and tolerances. The separation of the factors involved in diatom responses is however difficult, and this is where laboratory experiments can play an important role. Each environmental variable can be controlled and quantified, aiding recognition of taxa with defined ecological ranges and improving the predictive power of diatoms in water quality monitoring and paleaoecological and climate change studies.

6.2.3 Live Material

The data do not link a particular form of attachment or colony formation to assay level. Observations on live material show *G. parvulum* is as variable cytologically and in colony formation as it is morphologically. The data suggest the adaptive strategy of *G. parvulum* clones involves variation in chloroplast morphology and that pH is a dominant factor or predictor in separating clones, suggesting there are discrete morphotypes. Clones subject to pH assay were more variable in live character morphology than any other assay, whilst copper and zinc regimes elicited similar morphological profiles and sewage and cadmium regimes were the least variable. Culture regime can account for differences in live morphology in clones from Scion Pond, Parys Mountain (G_1 and G_2) and Llyn Idwal (G_2), showing that these clones are either particularly sensitive or adapted to pH, copper, zinc and cadmium respectively. The universal mode of attachment for all clones is as single cells attached in valve or girdle view to the substratum; however, most clones were also able to develop other modes of attachment, though not in a consistent manner that would allow separation of clones. Observations on the light and temperature, and sewage regimes were confounded by the absence of data and the presence of bacteria respectively. Despite the lack of separation, this study has shown that even the simplest of studies can yield valuable information and aid in the understanding of diatom ecology and life cycle.

6.3 SUMMARY AND CONCLUSIONS

The reliability of diatom indices of water quality depends on accurate identification, preferably to species level (Cox, 1991). However, diatom classification still largely rests on the features of the diatom cell wall (Round *et al.* 1990) and assumes that wall morphology is constant and species specific. This study shows that this is not the case and that the diatom can express considerable morphological variability. However, the

development of the cell wall and the ways in which structure and shape are determined remain largely unknown. The outline of a developing hypovalve is probably determined by its parent wall, however major shape changes can be introduced, as shown in this study (clone Llyn Idwal), at the auxospore/initial valve stage when there are fewer constraints on size and shape, and these changes can be propagated over several generations. The initial cells rarely conform to the "typical" morphology of the taxon (clones from Kings Mere, Ham Gate Pond, Llyn Idwal, Parys Mountain) and can take several divisions before "normal" valve pattern and shape is established. This begs several questions. How is the polarity of heteropolar species set? How does the cell know what shape to form? How is the cell able to control its dimensions of length and width, which are species specific?

Considerable further work is required before a revised taxonomy can be considered for *G. parvulum*. It is recommended that similar ecophysiological and morphological studies be carried out in combination with mating experiments and biochemistry to clarify the status of *G. gracile*, *G. hebridense*, *G. innocent, G lagenula* and other *G. parvulum* varieties and forms forming the complex, but not encountered in this study. Samples of the clone from the River Kennet also need to be examined further to establish whether this is a separate taxon requiring upgrade to species. It could be argued that these species contain stages that would have been identified as *G. parvulum*, but the definitions of *G. gracile* and *G. hebridense* need revising. The evidence points to several distinct entities subsumed under *G. parvulum* strongly suggesting that *G. parvulum* should be reappraised and divided into separate species or varieties.

Finally, it is recommended that diatom indices of water quality bear in mind (in the absence of a revised taxonomy) that the taxonomic status of *G. gracile* and *G.*

hebridense are in doubt and that any hypothesis or recommendation made on the basis of indices using *G. parvulum* sensu lato, *G. gracile* or *G. hebridense* are clear in this respect.

APPENDIX I

MEDIA PROTOCOLS AND RECIPES

Standard Woods Hole MBL (Stein, 1973), pH 7.2

Macronutrients	Stock Solution Concentration mg/L	ml of stock solution per 1 Litre of de-ionised water
$CaCl_2.\ 2H_2O$	36.76	1.0
$MgSO_4.7H_2O$	36.97	1.0
$NaHCO_3$	12.60	1.0
K_2HPO_4	8.71	1.0
$NaNO_3$	85.01	1.0
$Na_2SiO_3.\ 9H_2O$	28.42	1.0
Micronutrients		
Na_2 EDTA	4.36	0.25
$FeCl_3.\ 6H_2O$	3.15	0.25
$CuSO_4.5H_2O$	0.01	0.25
$ZnSO_4.7H_2O$	0.022	0.25
$CoCl_2.\ 6H_2O$	0.18	0.25
$MnCl_2.\ 4H_2O$	0.006	0.25
$Na_2MoO_4.\ 2H_2O$	1.0	0.25
Other Ingredients		
IM Tris	121	2.0

- pH adjusted to 7.2 with HCl.
- Provides elemental Cu and Zn concentrations of 2.63 and 5µg/l respectively in the stock solution.
- 0.25 ml of each of Cu and Zn stock solutions in1 Litre of MBL provides elemental Cu and Zn concentrations of 0.0006575 and 0.00125µg respectively.

Artificial Sewage Protocol (adapted from OECD, 1981)

Three solutions made as follows:

A 27.5g of peptone into 250 ml water

B 27.5 g of meat extract into 250 ml water

C 1.875 g of Urea

437.5 mg NaCl

250 mg $CaCl_2.2H_2O$

125 mg $MgSO_4.7H_2O$

} dissolved in 250 ml water

- Mix the three solutions in a 1: 1: 1 ratio. This is the stock sewage solution.
- Full strength artificial sewage solution was made by adding 3.13mls of mixture to 1 litre of water. This gives a BOD of 160 mg/l and a mean concentration of ammonia of 45 mg/l.

Concentration Series for Artificial Sewage Experimental Regime

Using standard MBL as the base for the artificial sewage medium, the following concentration series was made by addition of varying quantities of stock sewage solution.

Sewage Concentration	ml of Stock Sewage Solution per Litre of MBL
Full strength	3.13 ml
1/2 strength	1.565 ml
1/5 strength	0.626 ml
1/10 strength	0.313 ml
1/25 strength	0.1252ml

Copper and Zinc adjusted Woods Hole MBL for Experimental Regimes

Where the required elemental Copper and Zinc concentrations were > 0.05µg/l, the micronutrients $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$ found in standard MBL were suitably adjusted.

Where the required elemental Copper and Zinc concentration were ≥0.05 µg/l, stronger stock solutions were produced as follows:

Micronutrients	Stock solution concentration mg/L
$CuSO_4.5H_2O$	1.9
$ZnSO_4.7H_2O$	2.1988

This provides elemental Cu and Zn concentrations of 499.7 and 500µg/l respectively, in the stock solution.

1ml of this stock solution in 1 Litre of MBL provides elemental Cu and Zn concentrations of 0.5μg each.

Concentration series for Experimental Regimes

A. Copper Regime – Initial Run

ml of Stock $CuSO_4.5H_2O$ per Litre of MBL	µg of elemental Cu in 1 Litre of MBL
16	0.042
8	0.021
4	0.011
2	0.0053
1	0.0026
0.5	0.0013
0.25	0.00066
0.125	0.00033
0.062	0.00016
0.00	0.00

A. Copper Regime – Extended Run

ml of Stock $CuSO_4.5H_2O$ per Litre of MBL	µg of elemental Cu in 1 Litre of MBL
25	12.5
10	5.0
5	2.5
2	1.0
1	0.5
0.5	0.25
0.2	0.10
0.1	0.05

B. Zinc Regime – Initial Run

ml of Stock $ZnSO_4.7H_2O$ per 1 Litre of MBL	µg of elemental Zn in 1 Litre of MBL
16	0.08
8	0.04
4	0.02
2	0.01
1	0.005
0.5	0.0025
0.25	0.0013
0.125	0.00063
0.062	0.00031
0.00	0.00

B. Zinc Regime – Extended Run

ml of Stock $ZnSO_4.7H_2O$ per 1 Litre of MBL	µg of elemental Zn in 1 Litre of MBL
25	12.5
10	5.0
5	2.5
2	1.0
1	0.5
0.5	0.25
0.2	0.10
0.1	0.05

Cadmium Regime

ml/µl of Stock $CdCl_2.2.5H_2O$ in 1 Litre of MBL	µg of elemental Cd in 1 Litre of MBL
2.5ml	12.5
1.0ml	5.0
500µl	2.5
200µl	1.0
100µl	0.5
50µl	0.25
20µl	0.10
10µl	0.05
4µl	0.02
0.00	0.00

As Cd is not biologically required, the concentration series used a stock solution of $CdCl_2.2.5H_2O$ containing 10.158 mg of compound per 1 litre of water, providing an elemental Cd concentration of 5 mg/l.

0.1ml (100 µl) of $CdCl_2.2.5H_2O$ stock solution in 1 Litre of Standard MBL provides an elemental Cd concentration of 0.5µg.

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