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POPULATION ECOLOGY
OF THE DINOFLAGELLATE SPECIES
LINGULODINIUM POLYEDRUM
IN SOUTHERN CALIFORNIA
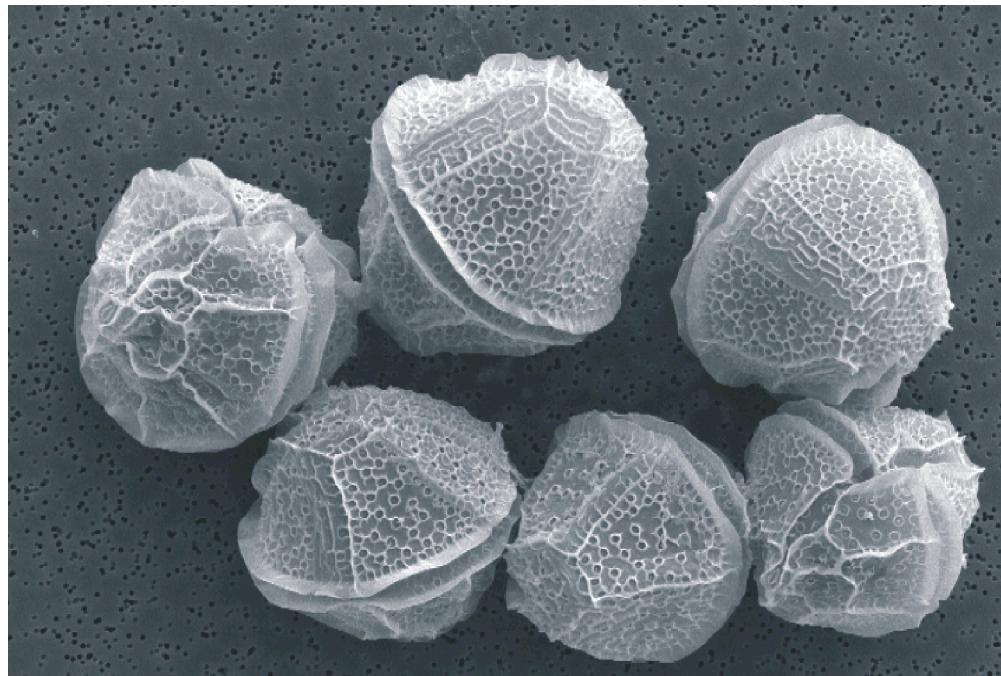
By

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POPULATION ECOLOGY OF THE DINOFLAGELLATE SPECIES *LINGULODINIUM POLYEDRUM* IN SOUTHERN CALIFORNIA

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<u>ABSTRACT</u>

Marine dinoflagellates are an ecologically important phytoplanktonic group that accounts for two thirds of all known harmful algal bloom (HAB) species. This study explores the population ecology of *Lingulodinium polyedrum* (F. Stein) J.D. Dodge, a common bloom-forming dinoflagellate species in Southern California. *Lingulodinium polyedrum* is not considered a HAB species, but functions as one of the main model organisms for dinoflagellate biology. As such, knowledge about this species also contributes significantly to the understanding of dinoflagellate population dynamics at a more general level. In an attempt to understand some of the complex interactions that govern *L. polyedrum* population ecology, laboratory experiments of life cycle control and intraspecific phenotypic diversity were linked with an *in situ* study of the population dynamics and the intraspecific genetic diversity of this species in coastal waters of Southern California.

The life cycle experiments showed that processes such as gametogenesis and ecdysis of *L. polyedrum* are influenced by photon flux density (PFD) and gave a first indication for an involvement of the photosynthetic apparatus in the induction of gametogenesis in dinoflagellates. The light acclimation experiments revealed, for the first time, intraspecific phenotypic diversity in *L. polyedrum*. The two studied strains differed distinctly in their light requirements and light acclimation 'strategies'. For the study of intraspecific genetic diversity in *L. polyedrum* a novel method was developed that allowed the genotyping of individual cells. The application of this novel approach to natural populations showed that population genetic exchange of *L. polyedrum* in the Southern California Bight is tied to water circulation patterns and that both habitat structure and environmental change leave their signatures in the population genetic composition of *L. polyedrum*.

This thesis represents one of the most comprehensive studies of dinoflagellate population ecology and builds the basis for the development of a holistic concept of the population ecology of *L. polyedrum* and other dinoflagellate species.

Acknowledgements

Declaration of authorship

I, Jörg C. Frommlet, declare that the thesis entitled 'Population ecology of the dinoflagellate species *Lingulodinium polyedrum* in Southern California' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

• this work was done wholly or mainly while in candidature for a research degree at this University;

• where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

• where I have consulted the published work of others, this is always clearly attributed;

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• I have acknowledged all main sources of help;

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List of Abbreviations

ACD	Average cell diameter
bp	Base pair
BSA	Bovine serum albumin
CCS	California Current System
DNA	Deoxyribonucleic acid
DSP	Diarrhetic shellfish poisoning
dNTP	Deoxyribonucleotide triphosphate
DVM	Diurnal vertical migration
FRRF	Fast Repetition Rate Fluorometer
Fv/Fm	Quantum efficiency of photochemistry (dimensionless)
HAB	harmful algal bloom
HPLC	High performance liquid chromatography
ITS	Internal transcribed spacer
LHC	Light harvesting complex
PAR	Photosynthetically Active Radiation (400-700 nm)
PCR	Polymerase chain reaction
PFD	Photon flux density
PS I	Photosystem I
PS II	Photosystem II
PSP	Paralytic shellfish poisoning
PSU	Photosynthetic unit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
σ_{PSII}	Functional absorption cross section of PS II ($\times 10^{-20}$ m^2 quanta^{-1})
τ_{Qa}	Rate of electron transport (s^{-1})

Chapter 1

INTRODUCTION

1.1 General introduction

Marine phytoplankton are responsible for 40 to 50% of the global annual primary production, forming the vast amount of approximately 45 gigatons of organic matter per year (Falkowski and Woodhead 1992, Falkowski 1994, Behrenfeld and Falkowski 1997, Falkowski et al. 1998, Field et al. 1998). These numbers underline the immense ecological importance of phytoplankton on a global scale and explain the traditionally strong focus of phytoplankton research on primary production (Smayda and Reynolds 2003, El-Sayed 2005). Diatoms (Bacillariophyceae) are estimated to contribute about half to the total of global primary production but also groups like the dinoflagellates (Dinophyceae) and haptophytes (Prymnesiophyceae) add substantially to this figure (Taylor and Pollingher 1987, Taylor 1990, Green et al. 1990).

The term phytoplankton is derived from the greek terms 'phyton' or plant and 'planktos' meaning 'wanderer' or 'drifter'. Despite this terminology, which implies a rather passive lifestyle, and their seemingly simplicity compared to higher plants, phytoplanktonic organisms are highly adapted to their environment. They have evolved effective mechanisms to influence their exposure to environmental factors, to acclimate to changing environmental conditions as well as manipulate the environment in their favour (e.g. Smayda and Reynolds 2003, Granéli and Turner 2006). Phytoplankton population dynamics are the result of complex interactions of each individual with its biotic and abiotic environment. Consequently, for a better understanding of phytoplankton ecology at the species and population level, these interactions have to be considered in their entireness (Donaghay and Osborn 1997, Roelke and Buyukates 2001). Such a holistic approach to phytoplankton ecology at the species and population level is of particular importance for the understanding of harmful algal bloom (HAB) dynamics (Smayda and Reynolds 2003). The increase of HABs on a global scale (Hallegraff 1993) has driven this research and resulted in HAB species being among the

best studied phytoplanktonic organisms. However, despite major advances in understanding HAB ecology, the sheer complexity of biological, ecological and environmental interactions frequently impedes a full comprehension of how the different ecological traits and adaptive strategies take effect in the natural environment, how they influence phytoplankton population dynamics and how they lead to blooms (Smayda 1997b, Smayda and Reynolds 2001, 2003).

Lingulodinium polyedrum (F. Stein) J.D. Dodge is a typical bloom-forming dinoflagellate species in coastal upwelling regions but is not considered a typical HAB species since adverse effects of blooms on marine life have not been attributed to toxins but to the oxygen depletion during bloom senecense (Allen 1946, Lewis and Hallett 1997, Smayda and Reynolds 2003). Over the last few decades *L. polyedrum* has become a model organism for dinoflagellate physiology and ecology (Lewis and Hallett 1997). Building on this wealth of knowledge, the aim of the present study was to gain new insights into the population ecology of this important bloom-forming dinoflagellate species by linking laboratory experiments of life cycle control and intraspecific phenotypic diversity with *in situ* studies of intraspecific genetic diversity over different temporal and spatial scales in Southern California and in relation to the development of natural populations.

Chapters 2 to 5 are structured in the format of research articles and only include relatively concise introductions to the different aspects of the work presented here. Chapter 1 is therefore intended to give a more general and in depth introduction to HABs, the current challenges of studying phytoplankton population ecology, the dinoflagellates in general and the studied species *L. polyedrum* in particular, and the molecular genetic markers employed. Some of the aims and objectives of this project will be introduced in the course of this general introduction but are also summarized at the end this chapter.

1.2 Harmful algal blooms

Approximately three hundred phytoplankton species from different taxonomic groups are known to form blooms (Sournia 1995) of which sixty to eighty species are considered harmful algae (Hallegraeff 1993). The adverse effects that harmful algal

blooms (HABs) exert on other organisms are a result of toxins that these species produce and/or other effects such as anoxia during bloom senescence (Smayda 1997a). Accumulating evidence suggests that since the 1970s-80s the frequency, magnitude and duration of benign and also toxic, noxious, or otherwise harmful algal blooms increased on a global scale (Smayda 1990, Hallegraeff 1993). Some of this apparent increase may be a result of increased scientific awareness rather than an actual increase in bloom occurrence but in some well documented cases coastal eutrophication and the translocation of non-indigenous species via ship's ballast water could be linked to the occurrence of blooms (Hallegraeff and Bolch 1991). The dinoflagellates are a particularly noxious taxonomic group that accounts for 75% of all known HAB species (Smayda 1997a). Dinoflagellate blooms can cause extensive fish and shellfish kills, resulting in substantial economic losses and represent a risk to human health since shellfish that is contaminated with dinoflagellate toxins can cause illnesses such as diarrhetic (DSP) and paralytic shellfish poisoning (PSP) (Brett 2003, Osek et al. 2006). Exploring the complex interactions that govern outbreaks of harmful algal blooms is a particular focus of HAB research (Donaghay and Osborn 1997, Roelke and Buyukates 2001). However, as pointed out by Smayda (1997b), the study of HAB dynamics is not only essential for a better prediction and management of these events, but also holds the chance for a general improvement of understanding phytoplankton dynamics.

1.3 Challenges in the study of phytoplankton population ecology

Despite major advances in understanding the environmental factors that influence the dynamics of HABs and phytoplankton populations in general, significant gaps in the knowledge remain. The complexity of interactions at different levels of biological organization (e.g., physiological, life cycle, population, trophic) makes the study of population ecology a difficult task in any species. However, in phytoplankton ecology even the concepts of 'species' and 'population' are not easily defined.

Species and population concepts

The different species concepts, e.g. morphological, biological or genetic species concepts follow their own definitions of a 'species', hence the identification of a species

depends on the employed species concept(s) (see review by Manhart and McCourt 1992). Solely morphological criteria, for example, are not always sufficient to distinguish between different phytoplankton species and sometimes only molecular genetic tools have the discriminative power to distinguish between otherwise cryptic or pseudo-cryptic species (Montresor et al. 2003, Sáez et al. 2003, Sarno et al. 2005, Amato et al. 2007). The term 'population' is also difficult to define and there is an ongoing debate as to whether phytoplankton species form panmictic populations (e.g. Finlay and Clark 1999, Finlay 2002) or whether they are divided into different populations (e.g. Medlin 2007).

Life cycle and population dynamics

Another challenge in the study of phytoplankton population ecology is to link the life cycle of a species with its population dynamics (Wyatt and Jenkinson 1997, Garcés et al. 2001). For example, ecdysal cysts can be less prone to viral infections, grazing and parasitic attack, they contribute to the dispersal of species and play key roles in the initiation, maintenance, and termination of blooms (Anderson and Morel 1979, Anderson et al. 1983, Dale 1983, Walker 1984, Ishikawa and Taniguchi 1996, Kremp and Heiskanen 1999, Montresor 2001, Zingone et al. 2001, Anderson and Rengefors 2006). Knowledge of how a species' sexual life cycle is controlled by the environment and how it is interlinked with its population dynamics is however still in its infancy (Wyatt and Jenkinson 1997, Garcés et al. 2001). The way a species reproduces has also a direct effect on its population genetic diversity, such that genetic diversity in mainly asexually reproducing species is generally lower than in sexually reproducing species (Kondrashov 1997). This leads to another challenging subject in the study of phytoplankton ecology: intraspecific genetic and phenotypic diversity.

Intraspecific genetic and phenotypic diversity

In the first population genetic study of a microalgal species Jane Gallagher showed that winter bloom populations of the diatom *Skeletonema costatum* were genetically different from the prevalent summer bloom populations of the same species (Gallagher 1980). Without this knowledge about the population dynamics at the genetic level, the occurrence of summer and winter blooms of the same species would have been difficult to interpret. The study by Gallagher is therefore a good example of how the integration

of population genetic data can help in the understanding of phytoplankton population dynamics. The occurrence of extensive intraspecific diversity in phytoplankton has since been demonstrated at the phenotypic level e.g. in growth rates (Gallagher 1982), tolerance to temperature and salinity (Krawiec 1982, Brand 1984) and toxin profiles (Bomber et al. 1989), to name just a few, as well as at the genetic level (e.g. Cembella and Taylor 1986, Scholin and Anderson 1994, Scholin et al. 1994a, b, Rynearson and Armbrust 2000). Intraspecific diversity has however only been assessed in a relatively small number of species and one of the main challenges is to relate intraspecific diversity to the dynamics of phytoplankton populations.

1.4 The dinoflagellates (Dinophyceae)

One of the three dominant eukaryotic phytoplankton groups in modern oceans is that of the dinoflagellates (Dinophyceae) (Falkowski et al. 2004). Geologically, dinoflagellates are an old group with a fossil record dating back to the Upper Silurian (Walker 1984). Today, approximately 1500-2000 dinoflagellate species are recognised, of which 90% are found in marine, and the other 10% in fresh water environments (Taylor 1990, Sournia 1995, Gómez 2005). Accompanied by the development of numerous forms of nutrition, dinoflagellates have evolved to colonize a broad range of habitats. About half of the dinoflagellate species are heterotrophs, the other half are either photoautotrophs or more often photoheterotrophs (Elbrächter 1991). The boundaries between phototrophy and heterotrophy are however diffuse in this group and an increasing number of species (including *L. polyedrum*) are found to be obligate or facultative mixotrophic (Jeong et al. 2005a, 2005b). Furthermore, a number of heterotrophic species are parasitic, causing severe illnesses on their hosts, and some phototrophic species have developed into symbiotic zooxanthellae in different hosts, such as corals and anemones (Taylor 1987).

Dinoflagellate morphology and taxonomy

Dinoflagellates are mainly unicellular organisms with cell sizes ranging from approximately 5 µm to more than 2 mm. The group is defined by a number of shared characters such as: a unique nucleus, the dinokaryon, which contains permanently

condensed chromosomes and the occurrence of two dissimilar flagella (a longitudinal and the transversal flagellum) at some stage during the life cycle. The two flagella protrude through the cell wall via the flagellar pore(s). Typically, the transverse flagellum runs around the cell in a furrow called cingulum while the longitudinal flagellum runs down the sulcus, a depression on the ventral side at the point of flagellar insertion, and extends tangentially to the cell and perpendicular to the plane of the transverse flagellum. The beating of the longitudinal flagellum and the transverse flagellum result in a spiralling swimming motion, which defines the anterior and posterior of the cell (Fig. 1.1).
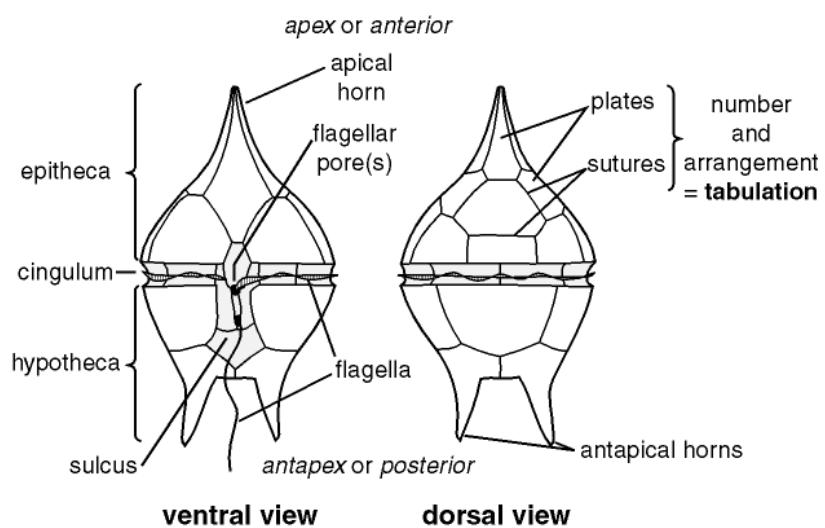
FIG.1.1 Basic anatomy of a thecate dinoflagellate (modified after Evitt 1985).

The main taxonomic features to delineate the different dinoflagellates within the class Dinophyceae are cell shape, features of cell covering, and the orientation of flagella. The cell cover consists of an outer membrane, underlying flattened amphiesmal vesicles, followed by microtubules, sometimes other membranes, and a pellicular layer (Lewis and Hallett 1997). The presence or absence of a visible cell cover allows a distinction between naked (non-armored) and thecate (armored) dinoflagellates. The difference between naked and armoured species lies in the extent of thecal plate formation in the amphiesmal vesicles and the patterns the plates build.

Due to the diversity of nutritional strategies and other plant- and animal-like features, the motile life stages of dinoflagellates have been described by both botanists and

zoologists, while dinoflagellate cysts from the fossil record have been described independently by geologists (e.g. Deflandre and Cookson 1955). According to the International Code of Zoological Nomenclature (ICZN), dinoflagellates belong to the order Dinoflagellida or Dinoflagellata, whereas the International Code of Botanical Nomenclature (ICBN), which today is the more widely accepted nomenclature, puts dinoflagellates into the phylum (division) Pyrrophyta or Dinophyta (Fensome et al. 1993, Greuter et al. 2000). Based on improved methods for the characterization of fine structural morphological differences and a more widespread use of molecular genetic tools, today eleven orders of free living dinoflagellates are recognized (Fensome et al. 1993, Greuter et al. 2000, Gómez 2005). The two largest orders are the naked Gymnodiniales (Lemmermann 1910) and the armored Peridiniales (Haeckel 1894). The naked Gymnodiniales have thecal vesicles containing no plates or, at most, granular material. Typical genera within this order are *Gymnodinium*, and *Gyrodinium*. In the armored Peridiniales the thecal vesicles contain thick, often patterned plates. Typical genera of this order are *Gonyaulax*, *Alexandrium* and *Lingulodinium* (Fensome et al. 1993, Greuter et al. 2000, Gómez 2005).

Lingulodinium polyedrum, the subject of study in the present work, is a good example of an attempt to unite the different taxonomic keys. The motile stage, originally named *Gonyaulax polyedra* (Stein 1883) and the corresponding cyst stage, originally named *Hystrichosphaeridium machaerophorum* (Deflandre and Cooksen 1955) today are merged under the name, *Lingulodinium polyedrum* (F. Stein) J.D. Dodge.

1.5 The bloom-forming dinoflagellate species *Lingulodinium polyedrum*

Lingulodinium polyedrum is a planktonic, single celled, marine, photoheterotrophic dinoflagellate species. Together with *Lingulodinium milneri* (Murray *et* Whitting 1899) Dodge 1989 it represents the whole genus *Lingulodinium*, which, alongside the closely related genera *Gonyaulax* and *Alexandrium*, belongs to the family Gonyaulacaceae (Lindemann 1928) and the order Peridiniales (Haeckel 1894) (Greuter et al. 2000, Gómez 2005). The distinctive morphology and the relative ease of culturing *L. polyedrum* have lead to numerous investigations both in the laboratory and in the field. As a result, *L. polyedrum* has become one of the best-studied dinoflagellate species in terms of its cell chemistry, life cycle, physiology, as well as behaviour, ecology and its

fossil record (reviewed by Lewis and Hallett 1997). The toxicity of *L. polyedrum* has long been argued but recent results confirmed that *L. polyedrum* is capable of producing yessotoxin (Paz et al. 2004, Armstrong and Kudela 2006). The detailed knowledge about this species and the ecological importance of *L. polyedrum* blooms in coastal environments makes it an excellent model organism for a population ecological study of a typical bloom-forming dinoflagellate species.

Morphology and physiology of L. polyedrum

Lingulodinium polyedrum possesses a benthic cyst stage and a planktonic motile stage. The benthic cyst stage is a spherical body of 31- 54 μm in size with numerous spines of variable length and shape (Reid 1974). The motile, vegetative life stage is a thecate single cell of 25-54 μm in diameter with well defined cellulose plates (Lebour 1925). Its plate formula (tabulation) is Po, 3', 3a, 6'', 6c, 7s, 6''', 1p, 1'''', giving the cell an angular or polyhedral appearance in ventral and dorsal view, and appearing circular in apical view (Dodge 1989) (Fig. 1.2).
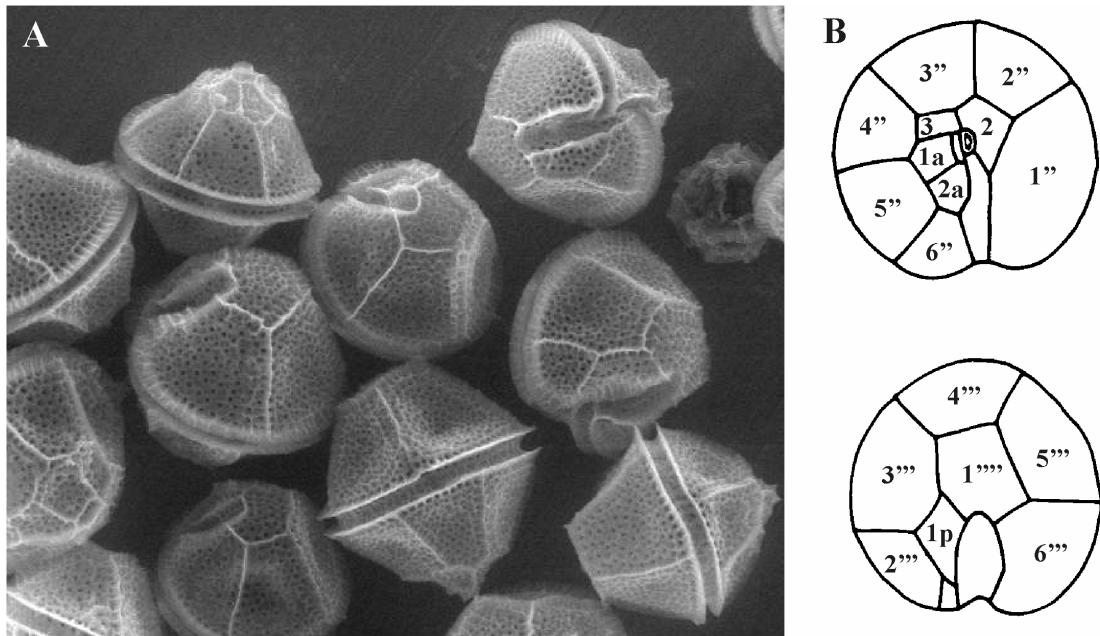
FIG. 1.2 Morphology of *Lingulodinium polyedrum*. A) SEM image of a natural sample of *L. polyedrum* collected in La Jolla Bay showing cells from different angles. B) Tabulation (plate pattern) of *L. polyedrum* from apical (top) and posterior view (bottom). Plates are labelled using conventional Kofoid symbols: apical plates ('), precingular plates ("), postcingular plates ('"), antapical plates (""), anterior intercalary plates (a), posterior intercalary plates (p) (redrawn after Lewis and Hallett 1997).

Lingulodinium polyedrum has been the subject to a wide range of physiological studies covering aspects such as nutrient requirements, its tolerance to salinity, temperature and turbulence as well as its bioluminescence capabilities and the circadian control of its physiology (reviewed by Lewis and Hallett 1997). Since the present study included work on two light related processes: intraspecific variability of light acclimation and the influence of irradiance on the life cycle of *L. polyedrum*, a brief summary of photophysiology related work is given here (see chapter 4 for further details).

Lingulodinium polyedrum possesses a chlorophyll (chl) a-chl c carotenoid pigment system with chl c_2 as accessory chlorophyll and peridinin as major accessory carotenoid (Boczar and Prézelin 1987). Additional carotenoid pigments in *L. polyedrum* include diadinoxanthin, diatoxanthin, dinoxanthin, and *ß*–carotene (Jeffrey et al. 1975, Jeffrey 1976). Light acclimation of *L. polyedrum* involves changes of the photosynthetic pigments chl a/c and peridinin (Prézelin and Sweeney 1978, Boczar and Prézelin 1987), a common type of light acclimation in phytoplankton (Richardson et al. 1983, Raven and Geider 2003). The study of *L. polyedrum* has substantially contributed to the understanding of photosynthesis and light acclimation in dinoflagellates. However, most photophysiological studies in *L. polyedrum* were based on one single strain (e.g. Prézelin and Sweeney 1978), meaning that intraspecific diversity had not been considered. One objective of the present study was to reduce this gap in the knowledge by comparing the light acclimation of different strains of *L. polyedrum*. The aim was to assess whether, and if so, how photophysiology may vary intraspecifically in *L. polyedrum* and to start exploring how intraspecific photophysiological diversity may affect the population ecology of *L. polyedrum*.

Life cycle of L. polyedrum

The life cycle of *L. polyedrum* includes motile and non-motile cells, sexual and asexual stages. Figure 1.3 shows the three main processes that constitute the life cycle of *L. polyedrum*: vegetative (asexual) reproduction, formation of ecdysal stages, and sexual reproduction summarized after Lewis and Hallett (1997) and Figueroa and Bravo (2005). Vegetative reproduction is assumed to be the dominant form of reproduction in *L. polyedrum* and other dinoflagellates. The cells are haploid during this stage and reproduction occurs via binary fission either by sharing of thecal plates (desmoschisis) or by division after the shedding of the theca (eleutheroschisis). Asexual ecdysal cysts

(sometimes referred to as temporary cysts) are formed when conditions become unfavourable. They lack a mandatory dormancy period, allowing a quick return to the water column once conditions improve (reviewed by Lewis and Hallett 1997). The sexual life cycle starts with the formation of gametes, which are morphologically very similar to vegetative cells but appear lighter in colour and are slightly smaller (Kokinos and Anderson 1995). In a recent study, Figueroa and Bravo (2005) showed, that *L. polyedrum* exhibits simple heterothallism (two sexual types, +/-). During sexual reproduction, gametes of opposite sexual types fuse to form a diploid planozygote. Depending on nutrient availability, this planozygote either forms a resting cyst, or a sexual ecdysal cyst.
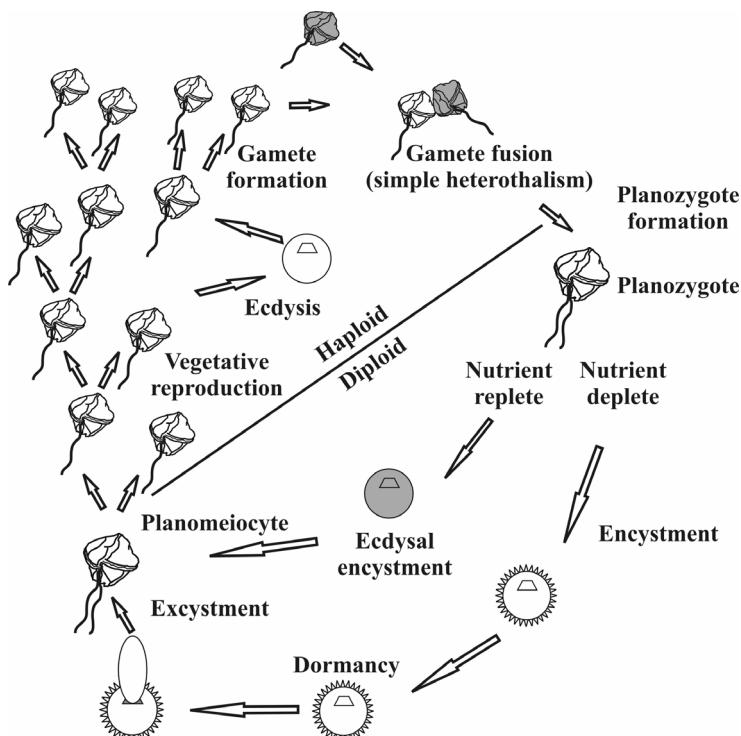
FIG. 1.3 The life cycle of *Lingulodinium polyedrum* (modified after Lewis and Hallett 1997 and Figueroa and Bravo 2005).

Resting cysts sink to the benthos where they have a mandatory dormancy period of typically 2 to 4 months (Figueroa and Bravo 2005). Triggers for germination vary between species but are typically linked to light levels as well as nutrient and oxygen concentrations (Anderson et al. 1987). Both the ecdysal sexual and the resting cysts germinate into a planomeiocyte that returns to the vegetative state by dividing into two haploid cells (Lewis and Hallett 1997, Figueroa and Bravo 2005). In cultures, nutrient

stress has been shown to induce the sexual life cycle in *L. polyedrum* (Blanco 1995, Figueroa and Bravo 2005). However, it is not known whether sexual reproduction in natural populations is triggered by nutrient stress and the role of other environmental factors than nutrient stress in controlling the life cycle of *L. polyedrum* has also not been explored. With the aim to provide the basis for a better integration of life cycle processes into the study of population dynamics, one objective of this study was to test whether life cycle control in *L. polyedrum* is influenced by irradiance.

Biogeography and physiological ecology of L. polyedrum in Southern California

Lingulodinium polyedrum is recorded in many parts of the world (Dodge 1982, Steidinger and Tangen 1997) and has been described as a cosmopolitan estuarine species of cool temperate to tropical regions (Wall et al. 1977). The distribution of *L. polyedrum* over a wide range of biogeographical zones has led to the suggestion that *L. polyedrum* may have several different ecotypes that occupy different ecological niches (Harland 1983). The occurrence of a thin-walled cyst type in Gulf of California and in subtropical assemblages from Australia may support this hypothesis (see Lewis and Hallett 1997).

The physical and chemical characteristics of Pacific coastal waters are influenced by large-scale ocean circulation patterns (Horner et al. 1997). The North Pacific Current approaches the west coast of the United States at approximately 45º N, where a large portion of the water mass turns south to form the California Current System (CCS). The CCS is an eastern boundary current affected by seasonal coastal upwelling during spring and summer in response to strong and persistent northwest winds (Reid et al. 1958, Hickey 1989). One of the sharpest hydrographical and biological boundaries in this region occurs at Point Conception due to a counterclockwise circulation of water masses in the Southern California Bight (U.S. GLOBEC 1994). Surface waters north of Point Conception are typically high in nutrients during the upwelling season (Garrison 1979) whereas surface waters in the Southern California Bight generally have lower nutrient concentrations except during periods of strong upwelling (Eppley et al. 1978). The geographic distribution of *L. polyedrum* along the Californian Coast extends to approximately 35º N, just north of Point Conception. To the South, the distribution is less clear, extending to the Pacific Coast of Mexico (Hernández-Becerril 1988) and further with a recent report of the first *L. polyedrum* bloom in Costa Rican waters

(Morales-Ramirez et al. 2001). From a population genetic point of view, the occurrence of *L. polyedrum* beyond Point Conception to the North is very interesting, as it raises the question whether populations north and south of this point are genetically different. One of the aims of the present study was to test the hypothesis of whether the hydrographical boundary at Point Conception represents an effective barrier for genetic exchange.

In the Southern California Bight blooms of *L. polyedrum* are common from April to November and are associated with calm, warm weather conditions (Allen 1946, Holmes et al. 1967, Eppley and Harrison 1975). Physical oceanographic features, which seem important for the development of *L. polyedrum* blooms in this area, are the formation of a shallow (< 10m) nutrient-deplete mixed surface layer, a steep thermocline and nutrient-rich deeper water masses (Eppley and Harrison 1975). *Lingulodinium polyedrum* is able to exploit these conditions by diurnal vertical migration (DVM) to the nutricline, taking up nutrients at night and photosynthesising in surface waters during the day (Eppley and Harrison 1975, Harrison 1976, Heaney and Eppley 1981). Figure 1.4 summarizes the physical conditions and the physiological ecology of *L. polyedrum* in Southern California according to Lewis and Hallett (1997).
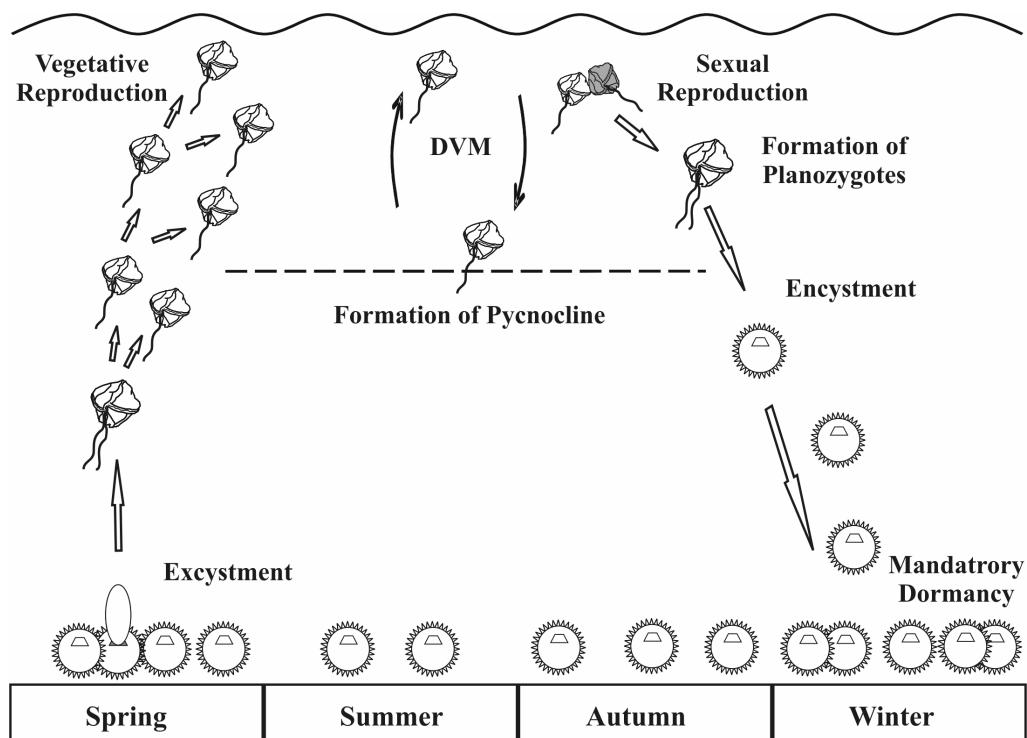
FIG. 1.4 Physiological ecology of *Lingulodinium polyedrum* (modified after Lewis and Hallett 1997).

Although the diagram of Lewis and Hallett implies that sexual reproduction occurs at the end of the growth period, the extent of asexual *versus* sexual reproduction in *L. polyedrum* and whether sexual reproduction occurs continuously or as a concerted event in time and space is actually untested. As mentioned above, in cultures of *L. polyedrum*, gametogenesis can be induced by nutrient limitation (Blanco 1995, Figueroa and Bravo 2005). Since one of the characteristics of surface waters during *L. polyedrum* blooms along the Californian Coast is nutrient depletion, there may be a link between phases of sexual reproduction and blooms. One of the objectives in the present study was to monitor the development of natural populations of *L. polyedrum* over time and space and to identify phases of sexual reproduction in order to test whether sexual reproduction is associated with bloom formation. A main biological advantage for sexual reproduction to occur during periods of dense blooms could lie in an increased encounter rates with mating partners (Wyatt and Jenkinson 1997), and in the frequently observed onshore transport of blooms, which may help in the establishment of cyst beds for future growth periods (Donaghay and Osborn 1997).

1.6 Molecular genetic markers in phytoplankton ecology

In the last two decades, a variety of molecular genetic tools have become available for high-resolution genetic studies at the population level (Parker et al. 1998, de Bruin et al. 2003, Nybom 2004, Agarwal et al. 2008). The choice of a particular molecular marker depends on the extent of genetic polymorphism required to best answer the specific scientific question. It is not the scope of this section to review existing molecular genetic markers and the following section focuses only on microsatellites, the markers used in the present study to assess genetic diversity within *L. polyedrum*. Since the 1980's microsatellites have become one of the most popular molecular markers used in population genetic studies. Their main advantages over other markers are: high levels of polymorphism, high heterozygosity, codominant single-locus inheritance, abundance in the genome and selective neutrality (Estoup and Angers 1998).

Microsatellites: A group of tandemly repeated DNA

Only a small fraction of genomic DNA encodes functional genes that are expressed as proteins. In human DNA, for example, gene-related but un-translated sequences, including introns, promoter regions, and pseudogenes exceed the abundance of functional genes nine times (Bennett 2000). Very common in the genomic DNA of all organisms studied so far are repetitive sequences (Britten and Kohne 1968, Hamada et al. 1982, Tautz and Renz 1984). Dinoflagellates possess large genomes compared to any other phytoplanktonic group (Spector 1984, Rizzo 2003) and it has been suggested that a substantial fraction of their genomes consists of repetitive sequences (Rizzo 1987). Repetitive sequences can occur as single units (interspersed repetitive sequences) or can be arranged in tandemly repeated clusters. Tandemly repeated DNA is divided into three groups based on the length of the core repeat unit and the number of repeats.

1. Satellite DNA consists of repeat units of five to several hundred base pairs (bp) stretching over several hundred kilo base pairs (kb). It was the first of the three groups of tandemly repeated DNAs to be discovered and was named 'satellite' DNA because it separates from the remaining DNA after buoyant density gradient centrifugation as a minor or "satellite" band (Britten and Kohne 1968).

2. Minisatellites are comprised of shorter repeat units (6-50 bp) and expand between 100 bp and 20 kb. Based on their localization in the genome, telomeric and hypervariable minisatellites are distinguished. Telomeric minisatellites protect chromosome ends and possibly play a role in chromosome pairing and cell ageing, whereas hypervariable minisatellites are found throughout the genome (Jeffreys et al. 1985).

3. Microsatellites, also called 'short tandem repeats' (STRs), 'simple sequence repeats' (SSRs) or 'variable number tandem repeats' (VNTRs), consist of tandemly repeated mono- to penta-nucleotide motifs e. g. ACACAC or AGCAGCAGC of short length (usually ≤ 100 bp) and are the most common group of tandemly repeated DNA (reviewed in Bennett 2000). The mechanism by which microsatellite sequences arise is through a process called 'strand slippage', a stepwise mutation process during DNA replication in which repeat units of a core repetitive sequence are added or deleted (Tautz 1989, Schlötterer and Tautz 1992).

For a long time microsatellite sequences were seen as "junk DNA" without any function. However, this view has changed since more and more evidence suggests that microsatellites can have regulatory functions and can also serve as functional coding regions in both prokaryotic and eukaryotic organisms. For example, microsatellite containing genes, so called 'contingency genes', can be switched on and off reversibly via a shift of the open reading frame. This reversible shift, also called 'phase variation' is often caused by a change in the length of unstable penta-nucleotide repeats and enables certain bacterial pathogens and parasitic protists to escape the immune system of their host by rapid changes in epitope composition (Deitsch et al. 1997).

Microsatellites: highly polymorphic markers

As described above, a process during DNA replication of microsatellite regions called 'strand slippage' can result in the addition or deletion of repeat units. This process gives rise to a length polymorphism of a given locus in a natural population (Tautz 1989, Schlötterer and Tautz 1992). Unequal crossing-over and gene conversions during recombination are other possible phenomena that can cause mutations in microsatellites (Hancock 1999). Amplification of microsatellite loci via polymerase chain reaction (PCR) allows the analysis of microsatellite length polymorphisms in natural populations, making them very powerful markers for the study of genetic diversity at the inter- and intra-population level (Parker et al. 1998, de Bruin et al. 2003, Nybom 2004, Agarwal et al. 2008). Microsatellites are inherited in a simple Mendelian fashion and are often selectively neutral (Estoup and Angers 1998). Microsatellite analysis can be applied to numerous ecological and evolutionary studies as population analysis (genetic structure and effective population sizes), assessment of gene flow and hybridization zones, construction of pedigrees, analysis of mating systems and migration, inference of colonization patterns, forensic studies, linkage or genome mapping, and paternity testing (Goldstein and Schlötterer 1999).

The application of microsatellite markers in population genetic studies of marine phytoplankton species has been introduced fairly recently by Rynearson and Armbrust (2000). Since then, microsatellites have become the marker of choice for an increasing number of population genetic studies in phytoplankton and have been successfully employed in phytoplankton groups such as the Bacillariophyceae (Rynearson and Armbrust 2000, 2004, 2005, Evans and Hayes 2004, Evans et al. 2004, 2005),

Haptophyceae (Iglesias-Rodríguez et al. 2002, 2006), Dinophyceae (Nagai et al. 2004, Santos et al. 2004, Alpermann et al. 2006, Nagai et al. 2006a,b, Nagai et al. 2007), and Raphidophyceae (Nagai et al. 2006c), and have given unique information about the genetic structure of populations, gene flow, dispersal mechanisms, and the biogeography of these mainly unicellular organisms. One of the objectives of the present study was to develop microsatellite markers for *L. polyedrum* and employ these markers in a population genetic diversity study of this species.

1.7 Aims and Objectives

The principal aim of research presented in this thesis is to contribute to a better understanding of the population ecology of the dinoflagellate species *L. polyedrum*. In many respects *L. polyedrum* is considered a typical model organism for bloom-forming dinoflagellates, thus, this project may also contribute to the understanding of population dynamics of bloom-forming dinoflagellates at a more general level.

Only a holistic approach can capture the complexity of interactions that control phytoplankton population ecology. In an attempt to start developing such an approach for *L. polyedrum*, the present study was designed to not only assess how the environment may affect the population dynamics of *L. polyedrum* but also to investigate environmental control over the life cycle of this species, how genetic diversity relates to population dynamics and how physiological diversity may relate to the ecophysiology of the species. Since none of these aspects have previously been studied in any detail in *L. polyedrum* the first three objectives of this thesis are:

1. To develop suitable molecular genetic tools for the assessment of population genetic diversity in *L. polyedrum* (Chapter 2).

2. To investigate whether irradiance, a central variable for phototrophic organisms, exerts any control on the life cycle of *L. polyedrum* (Chapter 3).

3. To assess whether there are intraspecific differences in the photophysiology of *L. polyedrum* (Chapter 4).

These first three objectives build the basis for a study of *L. polyedrum* population ecology in Southern California coastal waters that aims to integrate aspects of life cycle control, and phenotypic and genotypic diversity (Chapter 5). The specific objectives of this second part of the thesis are:

4) To employ the developed molecular genetic tools to assess the population genetic distance/differentiation between geographically and hydrographically separated 'populations' in Southern California.

5) To monitor natural populations of *L. polyedrum* in La Jolla Bay over time and with vertical resolution in the water column in order to: a) study the development of natural populations in relation to environmental conditions; b) to identify phases of sexual reproduction in relation to population development; and c) to investigate how population genetic parameters and the abundance of specific genotypes changes over time and space and in relation to population development and phases of sexual reproduction.

A concluding summary of the main findings, limitations and future work is given in Chapter 6.

Chapter 2

MICROSATELLITE GENOTYPING OF SINGLE CELLS OF THE DINOFLAGELLATE SPECIES *LINGULODINIUM POLYEDRUM* (DINOPHYCEAE): A NOVEL APPROACH FOR MARINE MICROBIAL POPULATION GENETIC STUDIES

Abstract

In recent years two new approaches have been introduced in genetic studies of phytoplankton species. One is the application of highly polymorphic microsatellite markers, which allow detailed population genetic studies; the other is the development of methods that enable the direct genetic characterization of single cells as an alternative to clonal cultures. The aim of this present study was to combine these two approaches in a method that would allow microsatellite genotyping of single phytoplankton cells, providing a novel tool for high resolution population genetic studies. The dinoflagellate species *Lingulodinium polyedrum* (F. Stein) J.D. Dodge was selected as a model organism to develop this novel approach. The method described here is based on several key developments: (1) a simple and efficient DNA extraction method for single cells, (2) the characterization of microsatellite markers for *L. polyedrum* (3) a protocol for the species identification of single cells through the analysis of partial rRNA gene sequences, and (4) a two-step multiplex PCR protocol for the simultaneous amplification of microsatellite markers and partial rRNA gene sequences from single cells. This protocol allowed the amplification of up to six microsatellite loci together with either the complete ITS1-5.8S-ITS2 region or a partial 18S region of the ribosomal gene of *L. polyedrum* from single motile cells and resting cysts. This paper describes and evaluates the developed approach and discusses its significance for population genetic studies of *L. polyedrum* and other phytoplankton species.

2.1 Introduction

Recent studies on the genetic diversity in phytoplankton species have given unique information about the genetic structure of populations, gene flow, dispersal mechanisms, and the biogeography of these mainly unicellular organisms (e.g. Iglesias-Rodríguez et al. 2006, Rynearson et al. 2006). A prerequisite for these studies has been that high-resolution population genetic tools, such as microsatellites, have become more widely available (reviewed in Parker et al. 1998, de Bruin et al. 2003, Nybom 2004). Microsatellites consist of tandemly repeated mono- to penta-nucleotide motifs of usually ≤ 100 bp (reviewed in Bennett 2000). A process during the replication of microsatellite regions called 'strand slippage' can result in the addition or deletion of repeat units, which leads to a length polymorphism of a given locus in a natural population (Tautz 1989, Schlötterer and Tautz 1992). The amplification of microsatellite loci via PCR allows the analysis of these length polymorphisms making them powerful markers for the study of genetic diversity at the inter- and intra-population level (Parker et al. 1998, Nybom 2004). Microsatellites are therefore the marker of choice for an increasing number of population genetic studies in phytoplankton and have been successfully employed in phytoplankton groups such as the Bacillariophyceae (Rynearson and Armbrust 2000, 2004, 2005, Evans and Hayes 2004, Evans et al. 2004, 2005), Haptophyceae (Iglesias-Rodríguez et al. 2002, 2006), Dinophyceae (Nagai et al. 2004, Santos et al. 2004, Alpermann et al. 2006, Nagai et al. 2006a,b, Nagai et al. 2007), and Raphidophyceae (Nagai et al. 2006c).

One major challenge in population genetic studies of phytoplankton and other microorganisms is that each single individual only provides minute amounts of DNA. A common approach to overcome this limitation is to establish clonal cultures from natural samples prior to genetic analysis. While this approach solves the difficulty of limiting amounts of genetic material it comes at a price as the isolation and maintenance of cultures is labour intense and time consuming. Low survival rates of isolates pose an additional limitation since a selective bias towards culturable strains might be a source of error in the assessment of naturally occurring population genetic diversity.

The most direct alternative to the use of clonal cultures would be the direct microsatellite genotyping of single individual cells. One area of research in which the amplification of microsatellite loci from single cells has been previously described is the

field of prenatal diagnosis where microsatellites are used for the genetic identification of fetal cells (Von Eggeling et al. 1996, Garvin et al. 1998, Griffin and Ferguson-Smith 1999). Although several previous studies on phytoplankton have introduced the genetic characterization of single cells (Bolch 2001, Marin et al. 2001, Ruiz Sebastián and O'Ryan 2001, Edvardsen et al. 2003, Ki et al. 2005, Richlen and Barber 2005, Takano and Horiguchi 2005, Kai et al. 2006), non of these studies have combined single cell PCR with high-resolution microsatellite markers.

The aim of this study was to develop a method that would allow a high resolution population genetic study of a bloom-forming phytoplankton species using multiplex microsatellite analysis of single cells. The dinoflagellate species *Lingulodinium polyedrum* (F. Stein) J.D. Dodge was selected as a model organism to test this novel approach. The species is an ecologically important primary producer in cool temperate to tropical regions around the globe (Wall et al. 1977, Dodge 1982) and frequently causes red tides in coastal upwelling regions such as the Southern Californian Coast (Allen 1946, Eppley and Harrison 1975). The distinctive morphology and relative ease of culturing *L. polyedrum* have lead to numerous studies and the species has become one of the best described dinoflagellates in terms of its cell chemistry, life cycle, physiology, behaviour, ecology as well as its fossil record (reviewed in Lewis and Hallett 1997). Contrastingly, only one study by Rollo et al. (1995) described a genetic marker for the molecular typing of *L. polyedrum* and, to date, no detailed population genetic study has been conducted on this species. The protocol of multiplex microsatellite genotyping is applicable to both single motile cells and resting cysts of *L. polyedrum*. This approach allows the population genetic analysis of large numbers of individuals without the need to establish clonal cultures. The simultaneous analysis of the ITS1-5.8S-ITS2 region or a partial region of the18S rRNA gene sequences allows the verification of species identity of the cells used for microsatellite analysis. The power of this novel approach of combining single cell PCR with high-resolution microsatellite markers in studies of population genetic diversity is discussed.

2.2 Material and Methods

Origin of strains and DNA preparation

DNA for the development of microsatellite markers was obtained from nine clonal cultures of *L. polyedrum* isolated from Scripps Pier, La Jolla, California, USA (32° 52.024 N, 117° 15.438 W). Seven of the isolates originated from a bloom event in 2003 of which two (CCAP 1121/3 and CCAP 1121/5) are available from the Culture Collection of Algae and Protozoa (CCAP). The other two strains were isolated in 1998 by Amy Shankle of which one strain (CCMP 1932) is available from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (CCMP). Cultures were grown in F/2 medium (Guillard 1975) at 19ºC, and 130 µmol photons $\cdot$ m^{-2} $\cdot$ sec^{-1}, under a 12h light/dark cycle. DNA extractions were prepared with a DNeasy^{TM} Plant Maxi Kit (QIAGEN, Crawley, UK). The concentrations and the quality of DNA extracts were assessed by gel electrophoresis and spectophotometrically using a ND-1000 spectrophotometer (NanoDrop^{TM}, Wilmington, USA).

Development of microsatellite markers

A partial genomic library enriched for microsatellite regions by selective hybridization was constructed. The employed methods were derived from Armour et al. (1994), Kandpal et al. (1994), Toonen (1997) and Hammond et al. (1998). A brief outline of the method follows. The DNA of clonal cultures of *L. polyedrum* was pooled, digested with the restriction endonuclease Sau3A1 (Sigma-Aldrich, Gillingham, UK), and separated on an agarose gel. Fragments of 400-900 bp were cut out from the gel followed by gel extraction using the MinElute^{TM} gel extraction kit (QIAGEN). The recovered DNA fragments were ligated to the linker molecule SAULA/SAULB, which was constructed by annealing equimolar amounts of the oligonucleotides SAULA (5'- GCGGTACCCG GGAAGCTTGG-3') and SAULB (5'-GATCCCAAGCTTCCCGGGTACCGC-3') (Armour et al. 1994). Following the ligation, the constructs were purified using the QIAquick^{TM} PCR purification kit (QIAGEN) and amplified in a single primer PCR using SAULA as a primer according to Hammond et al. (1998). PCR products were purified using the QIAquick^{TM} PCR purification kit (QIAGEN) prior to a joint enrichment of microsatellite sequences by selective hybridization of thirteen 3'-

endlabeled biotinylated probes and capturing of microsatellite containing fragments with Vectrex Avidin D (Vector Laboratories, Peterborough, UK) based on Kandpal et al. (1994) and Hammond et al. (1998). The used probes were: $C(CT)_{12}$, $(TG)_{11}$, $C(AAC)_7$, $G(AAG)_8$, $(AAT)_{10}$, $(AGG)_6$, $(ACC)_7$, $(AGC)_5GC$, $AT(AAAT)_7$, $G(AAAG)_6$, $CT(ATCT)_7$, $(TGTA)_7TG$, and $(AAAAT)_6$. The probes were designed to have similar melting temperatures and to avoid self-hybridization, hair pins, and the formation of cross dimers between probes using the software Netprimer (www.premierbiosoft.com/ netprimer/netprimer.html). Labelling of probes was performed using the 3' EndTag^{TM} nucleic acid labelling system and a biotin (long arm) maleimide, according to the manufacturers' instructions (Vector Laboratories). Recovered microsatellite enriched fragments were reamplified using the SAULA primer, followed by linker removal with the restriction endonuclease Nde II (Sigma-Aldrich). The recovered fragments were ligated into a pUC19 plasmid vector, pre-cut with Bam H I (MP Biomedicals Europe, Illkirch, France), and transformed into SURE® 2 Supercompetent Cells (Stratagene Europe, Amsterdam, The Netherlands). Insert containing colonies were replated on grid petri dishes, followed by a colony lift onto nylon membranes for colony and plaque hybridization (Roche Diagnostics, Burgess Hill, UK). Screening for microsatellite containing colonies was performed by hybridization with digoxigenin (DIG) labelled probes (Toonen 1997) of the same motif as used for the enrichment of microsatellites. Labelling of probes was performed using a DIG oligonucleotide tailing kit (Roche Diagnostics). Chemiluminescent detection was performed according to the 'DIG Application Manual for Filter Hybridization' (Roche Diagnostics) using a VersaDoc Imaging System, Model 3000 (BioRAD, Hemel Hempstead, UK). Positive clones were screened for insert length using M13 universal primer and M13 reverse primer before plasmid preparation using a QIAprep Spin Miniprep Kit (QIAGEN). The inserts were sequenced in forward and reverse direction (GRI, Rayne, UK) and aligned using the software ChromasPro version 1.3 (Technelysium, Eden Prairie, USA). The screening of the partial genomic library resulted in the identification of 17 microsatellites, containing 6 of the 13 repeat motifs screened for. Primers were designed for 14 of the loci using the software Primer 3 (Rozen and Skaletsky 2000). After optimization of PCR components and PCR conditions, 12 loci could be successfully amplified from their respective microsatellite containing plasmids and from DNA extracts of *L. polyedrum*. Six of these microsatellite loci (Table 2.1) could be successfully amplified in multiplex PCR and were chosen for the development of the single cell genotyping protocol.

Table 2.1 Microsatellite loci and respective primers used in multiplex genotyping

Locus (Accession no.)	Microsatellite sequence [a, b]	Product size		Primer sequence 5' to 3'
LP2 (EU164409)	$(GT)_5(AT)_2(GT)_6(AT)_4$ $(GT)_{16}GG(GT)_8GGAT$ $(GT)_7AT(GT)_6GGAT(GT)_{10}$	245 bp	F R	TCTGTCATTGTCTCTACTGTCTCTGTG TCAATCAATCGTCCAGTCAATCA
LP26 (EU164410)	$(ATGGGCCC)A(MTGGCCMY)_3A(MTGGCCMY)_3$ $(CAG)_2(CRK)_4AG(CAG)_5$ $CAT(CAG)_3$	144 bp	F R	GATCCAGCGAACTTCAGACTTC GATCGGTGCTGCTGCTGAT
LP31 (EU164411)	$(ACC)_6TGCGACCGCCGTT$ $GCCCC(TGM)_5CACGGT$ $(TGY)_5CAT(TGC)_2CGTT$ $(ACC)_6TGC(SAC)_2(TGY)_2$ $CGC(TGC)_6$	193 bp	F R	GATCGCTGCCATTACCAC AGTGAGTCTTCCCACCGTGT
LP41 (EU164412)	$GCT)_{13}ACT(GCT)_4GTT$ $(GCT)_6GCCTTC(GCT)_4$	212 bp	F R	CTGCCGCTGCCGTTGG CTCAGTTTCGCTTTGCTTCCATT
LP46 (EU164413)	$(GGC)_4(GDT)_3GGC(GGT)_5$ $GACAGTGAT(GGT)_2$ $(GDT)_4(GGT)_3$	298 bp	F R	CTGCATTCTGCGATGGTGAT GCGGCTAGGGGGTTAGTTT
LP65 (EU164414)	$(RTGCBC)_7(ACCATC)_2AC$ $CGCCC(AGGCCC)_4(ATGV$ $YC)_3GCC(ACC)_4GCC(ACC)$ $_4AACC(AKC)_2(ACC)_2$	237 bp [c] 183 bp [d]	F Fb R	CTGTTGGGGTCTCCAAAATGAAC CTGCCATCCATCACCCTCAT GCTTCCCGTTTATGCTGGTG

[a] Imperfect repeat motifs were pooled by applying the following abbreviations: R = (A,G); M = (A,C); S = (C,G); K =(G,T); Y = (C,T); D = (A,G,T); B = (C,G,T); V = (A,C,G). [b] subscripted numbers following parentheses indicate the number of repeats. [c] Product length using primers LP65 F/R. [d] Product length after nested PCR using LP65 Fb/R.

Primers for the ribosomal gene of L. polyedrum

Based on the *L. polyedrum* rRNA gene sequence AF377944 primers were designed for two regions (Table 2.2). For the amplification of the ITS1-5.8S-ITS2 region (ITS) the primers were designed to bind in the SSU and LSU of the ribosomal gene allowing the amplification of a 681 bp product containing the complete ITS region. The 18S primers were designed to amplify a 732 bp product from the SSU.

Table 2.2 Primers for the partial amplification of the ribosomal gene operon of *L. polyedrum*

Target	Primer sequence 5' to 3' [a]	Amplicon [b]
ITS1-5.8S-ITS2	F GGAAGGAGAAGTCGTAACAAGG R TCGCCGTTACTGAAGGAATC	1718 bp – 2398 bp (681 bp)
18S rRNA gene	F AAGTTGCTGCGGTTAAAAAG R CAGGTTAAGGTCTCGTTCGT	583 bp – 1314 bp (732 bp)

[a] Primer design was based on the sequence AF377944. [b] Positions are based on the sequence AF377944.

Origin of samples and preparation of single cells for genetic analysis

Natural samples of motile cells of *L. polyedrum* were collected from surface waters in La Jolla Bay, California, USA in 2003, 2004, 2005 and off the coast of Plymouth, UK in 2006. The samples were concentrated by inverse filtration followed by gentle pelleting at 700g in a bench top centrifuge. After the supernatant was removed, the samples were preserved in 90% molecular grade ethanol (Sigma-Aldrich) and stored at -20ºC for later picking of single cells. Resting cysts of *L. polyedrum* originated from a sediment core that had been taken in Koljö Fjord north of Gothenburg at 58° 13.602 N, 11° 84.245 E on the 06.04.2006. A sediment sample of 0.5-1 g was homogenized by sonication for 2 min and washed through a 100 μm and a 25 μm sieve with filtered seawater. The cysts in the filtrate were concentrated using sodium polytungstate according to Bolch (1997).

The following steps were done on ice whenever possible. In preparation for the picking of single motile cells a small aliquot (< 50 μL) of the concentrated sample was washed twice in 1 mL of 90% molecular grade ethanol (Sigma-Aldrich) and twice in 1 mL TE-buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) by repeated suspending and gentle pelleting. Concentrated cyst samples were used directly. Single motile cells and resting cysts were isolated under a stereomicroscope using a micro pipette or capillary pipette. The picked cells were once again washed in a new aliquot of TE buffer and then transferred in a volume of 1 μL to a PCR reaction tube that contained a single acid washed and sterile glass bead with a diameter of 0.7-1 mm (Sigma-Aldrich). The transfer of single cells was confirmed by examining the cells in the tubes under the microscope. After the picking of a batch of single cells the TE buffer of the last washing step was kept as PCR negative control. Cells that were not used immediately for PCR were kept at -80ºC. Prior to PCR the cells were broken by vortexing the tubes for 30 s at high speed. During vortexing the tubes were held in a way that caused the glass bead to rotate in the bottom of the tube, facilitating the grinding of the single cell by the glass bead. Tests were also conducted with more than one glass bead, but no improvement of cell breakage was observed. Any droplets created during vortexing were spun down in a brief centrifugation step. PCR components were then added directly to the tubes. The steps following the picking of single cells are also illustrated in figure 2.1 A and 2.1 B.
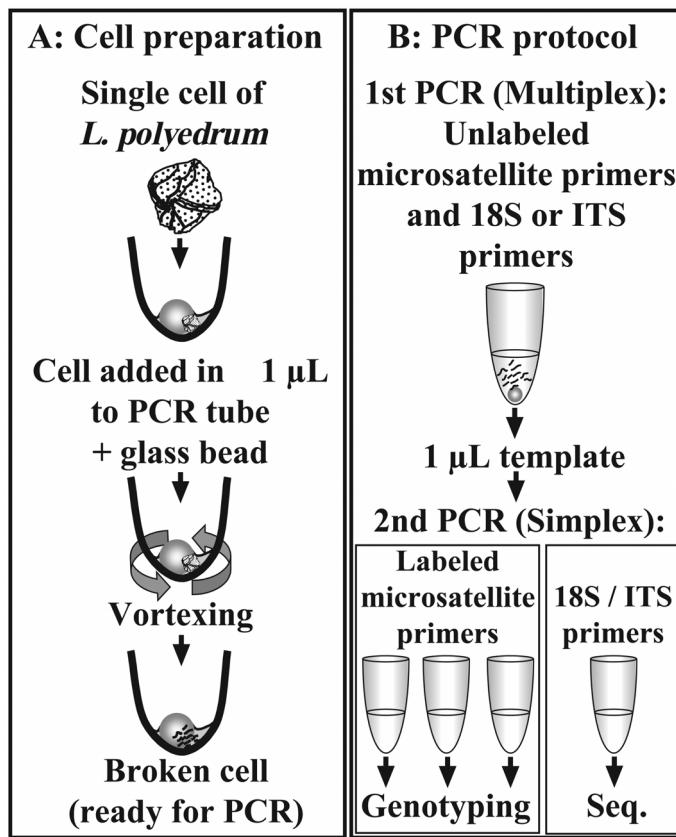
FIG. 2.1 Summary of the method developed for multiplex genotyping of single cells. (A) Preparation of single cells for genetic analysis. (B) Two-step PCR protocol for the amplification of up to six microsatellite loci together with either the ITS or partial 18S region of the ribosomal gene and downstream data analysis. Seq. = Sequencing.

Multiplex PCR protocol

All PCRs were performed in 15 µL reactions on a PTC-200 gradient thermocycler (GRI). The PCR reagents were: Amplitaq Gold (5 U/µL), 10 x Gold buffer, MgCl$_2$ (25 mM) (Applied Biosystems, Warrington, UK); dNTPs (10 mM each) (New England Biolabs, Hitchin, UK); BSA (1 µg/µL) (Promega, Southampton, UK). Unlabeled primers were obtained from MWG (Ebersberg, Germany). 'WellRED' fluorescently labelled forward primers for the amplification of microsatellite loci were obtained from Sigma-Proligo (Paris, France). The protocol for the amplification of up to six microsatellite loci and either the ITS or partial 18S rRNA gene from single cell templates consisted of two rounds of PCR (Fig. 2.1 B). All reactions contained a final concentration of 1x Gold buffer and 0.2 mM of dNTPs (each). The concentrations of other PCR components varied and are listed in Table 2.3.

Table 2.3 PCR components

	1st PCR (Multiplex)		2nd PCR (Simplex)				
	MS [a] + (18S or ITS)	LP65 + (18S or ITS)	LP2	LP26	LP31, LP41, LP46	LP65	18S, ITS
MgCl$_2$	2 mM	2 mM	2 mM	3 mM	1.5 mM	1.5 mM	1.5 mM
Taq polymerase	0.625 units	0.625 units	0.625 units	0.625 units	0.625 units	0.625 units	0.625 units
Primers (each)	0.16 μM	0.16 μM	0.5 μM	0.5 μM	0.5 μM	0.5 μM	0.5 μM
BSA	0	0.2 μg/μL	0	0.2 μg/μL	0	0.2 μg/μL	0
Template	1 Single cell		1 μL of 1st PCR				

All PCRs were performed in 15 μL reactions. [a] MS = All microsatellite loci (LP2, LP26, LP31, LP41, LP46, LP65). All reactions contained a final concentration of 1x Gold buffer and 0.2 mM of dNTPs (each).

All PCR programs started with an initial denaturing step at 94ºC for 8 min followed by 35 cycles of denaturing at 94ºC for 30 s, annealing at variable temperature (Table 2.4), elongation at 65ºC for 60 s and a final elongation step at 65ºC for 2 min. In the first PCR the microsatellite loci and either the ITS or the 18S region were amplified together in a multiplex reaction. In this reaction unlabeled microsatellite primers were used. In the second PCR the different loci were reamplified separately using 1 μL of the first PCR as template for each of the reactions in which fluorescently-labelled forward primers were used for each of the microsatellite loci.

Table 2.4 PCR conditions

	Primers	Annealing
1st PCR (Multiplex)	MS [a] + (18S or ITS)	(57ºC → 50.2ºC) [b] 1min
	LP65 + (18S or ITS)	58ºC 1 min
2nd PCR (Simplex)	LP2, LP31, LP41, LP46	64ºC 1 min
	LP26, LP65, 18S, ITS	58ºC 1 min

[a] MS = All microsatellite loci (LP2, LP26, LP31, LP41, LP46, LP65). [b] Touchdown PCR with a temperature decrease of 0.2ºC per cycle.

Data analysis

Microsatellite PCR products were analyzed on a CEQ 8800 capillary sequencer (Beckman-Coulter, High Wycombe, UK) using a fluorescently-labelled 400 bp internal size standard (Beckman-Coulter). Genetic diversity was calculated according to Nei (1987). PCR products of the ribosomal gene were gel purified (QIAGEN) and sequenced in forward and reverse direction by Geneservice (Cambridge, UK) with the respective primers listed in Table 2.2.

2.3 Results

2.3.1 Amplification of six microsatellite loci plus the ITS or partial 18S region of the ribosomal gene

A typical example of the PCR products obtained from single cells is shown in Figure 2.2. In this case the six microsatellite loci were amplified in conjunction with the partial 18S region. Amplification of all seven loci was possible from cells 1 and 3. Cell 2 resulted in products for 5 of the microsatellite loci and the 18S region of the ribosomal gene. The six microsatellite loci could also be amplified together with the ITS region and resulted in a single product for the ITS region (results not shown). Although the amplification of the six microsatellite loci from single cells was routinely possible, five of the microsatellite loci had various locus-specific limitations. LP2 and LP26 showed moderate levels of polymorphisms (e.g. the PCR products for LP26 from cells 1-3 in Figure 2.2 were 129bp, 147bp, and 105bp respectively) but the success rate of amplifying both loci from single cells was usually only 40 to 50%. Success rates in amplifying LP31, LP41, and LP46 were usually high (90 to 95%). However, LP46 did not show any polymorphism. LP31 and LP41 were both polymorphic yet their exact allele lengths could not always be determined due to stutter bands and banding patterns, which indicated that multiple repetitive regions were amplified. For these reasons the above loci were excluded from further analysis. LP65 showed initially similar patterns of multiple products but nested PCR using the two forward primers listed in Table 2.1 allowed the amplification of a single product. Success rates in amplifying LP65 were consistently high (90 to 95%) and the products showed a clear length polymorphism (Fig. 2.2).
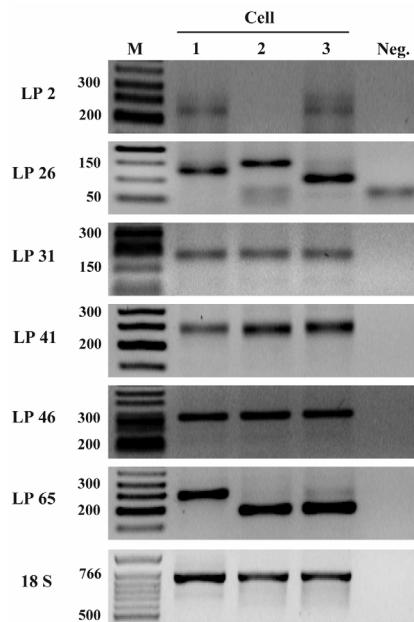
FIG. 2.2 Gel photographs showing the PCR products obtained from three single cells (lanes 1, 2, and 3) following the multiplex amplification of six microsatellite loci (LP2, LP26, LP31, LP41, LP46, and LP65) and a 732 bp product of the 18S rRNA gene. The cells were isolated from a single preserved water sample collected in La Jolla Bay, California in 2004. The left lane (M) contained a 50 bp size standard. Neg. = Negative Control. The low molecular weight band in the negative control of LP26 was due to primer interactions and not contamination of the negative control.

2.3.2 Evaluation of LP65 as a population genetic marker on single motile cells and resting cysts

The PCR products for the locus LP65 and the partial 18S region obtained from 20 single motile cells is shown in figure 2.3 A and 2.3 B, respectively. LP65 could be amplified from 18 cells; the 18S region from 19 out of the 20 cells. In some cases, e.g. cells 5, 7, 10, 14, and 16 in figure 2.3 B, the amplification of the 18S product of 732 bp also resulted in an additional product of approx. 150-200 bp, which was not further characterized. The PCR products for LP65 were mainly distinct single bands of polymorphic length, which could be genotyped directly without any further clean up or processing (e.g. cell 6 in Fig. 2.4). Additionally to the main band cells 15 and 19 showed an additional faint band of higher molecular weight. These bands originated from the outer, unlabeled forward primer during the first of the two amplification steps in the nested PCR protocol and did not affect the analysis as these additional products were not detected during genotyping on a capillary sequencer (e.g. cell 15 in Fig. 2.4). The amplification of LP65 from cells 7, 13, and 20 resulted in two PCR products of similar intensity. The length difference of products from cells 7 and 13 was large

enough to be resolved on the agarose gel while only genotyping revealed that the strong band of cell 20 consisted of two products of similar length. The fact that both products of cells 7, 13, and 20 were fluorescently labelled means that these additional products were not artefacts of the first amplification step (cell 7 in Fig. 2.4).
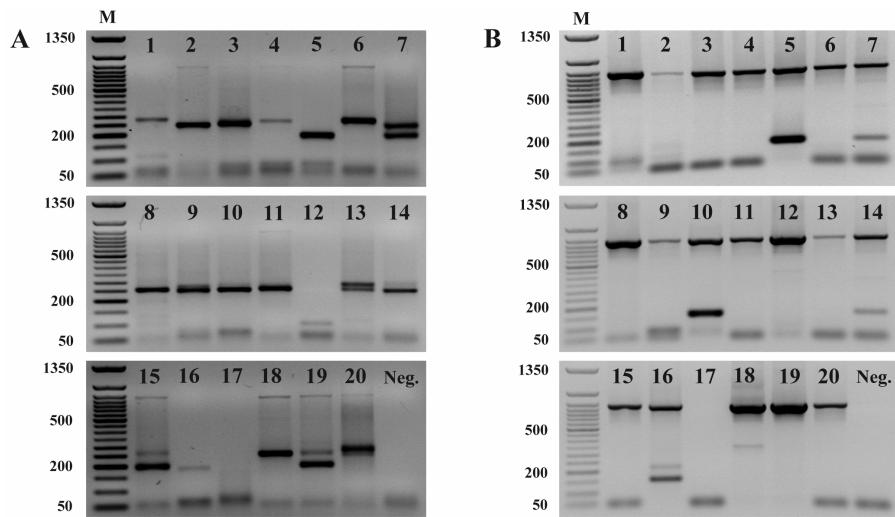
FIG. 2.3 Gel photograph showing PCR products of 20 single cells after the multiplex amplification of the microsatellite locus LP65 (A) and a 732 bp product of the 18S rRNA gene (B). The 20 cells were isolated from a single preserved water sample collected in La Jolla Bay, California in 2004. The left lane (M) contained a 50 bp size standard. Neg. = Negative Control.
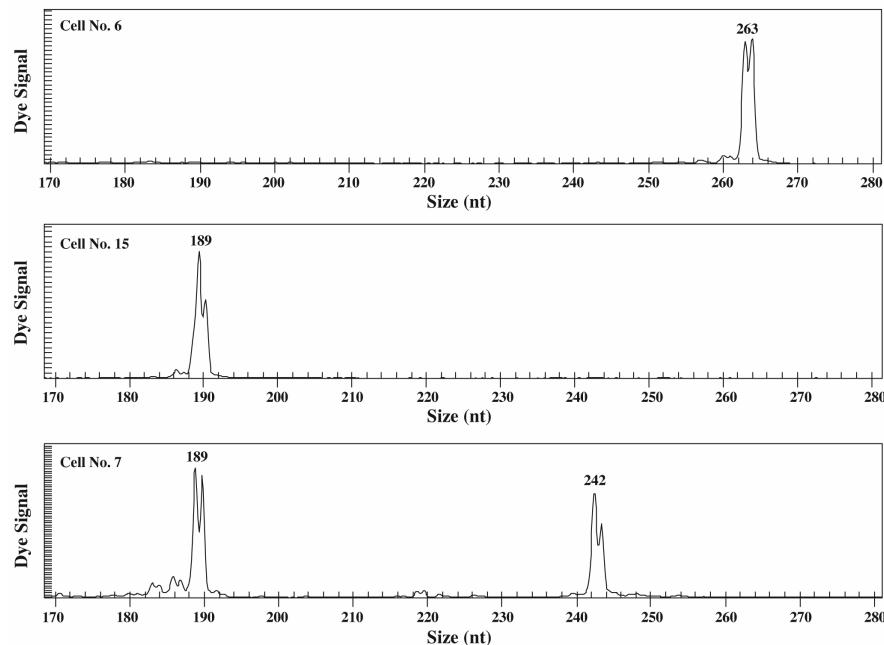
Fig. 2.4 Genotyping results for LP65 from three single cells. The cell numbers corresponds to the numbering of cells in figure 2.3. The lengths of alleles are shown in nucleotides (nt). The additional peaks of slightly longer length are additions of adenine by the *Taq* polymerase during PCR and did not affect the analysis.

LP65 and the 18S region were also amplified from 26 single cysts isolated from a sediment sample. LP65 could be amplified from 24 of the cysts, and resulted in the identification of 12 heterozygous and 12 homozygous cysts. The allelic frequencies of both samples (the 18 single motile cells and 24 cysts) are shown in figure 2.5 A and 2.5 B respectively. Most of the observed alleles were in line with the stepwise addition or deletion of the tri- and/or hexa-nucleotide repeat motifs expected for LP65 (see microsatellite motifs of LP65 in Table 2.1). Within the sample of single motile cells, 10 different alleles were identified and, within the sample of cysts, 14 different alleles could be distinguished. The only shared allele between the samples was the one of 183 bp. The gene diversities were 0.866 for the sample of single motile cells and 0.787 for the cyst sample.
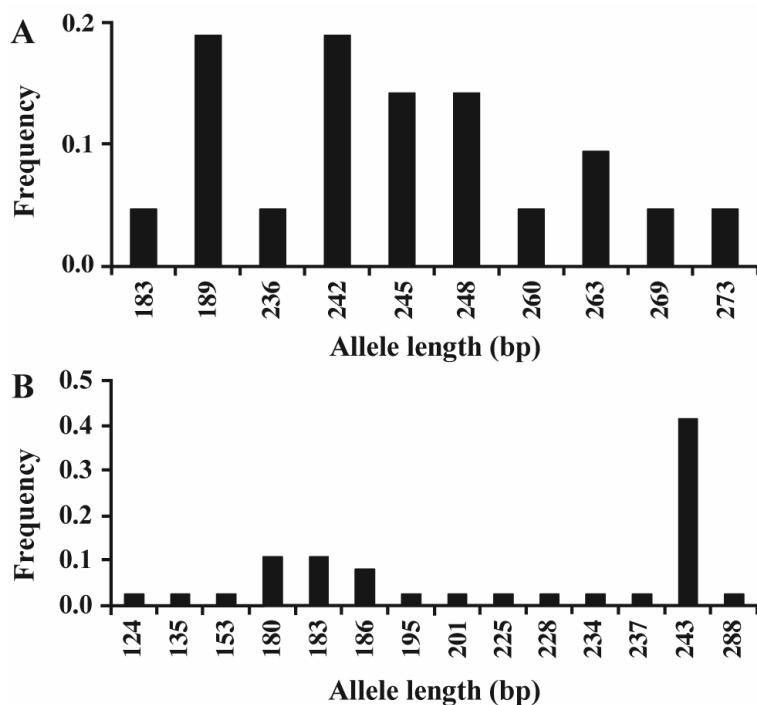
FIG. 2.5 Allelic frequencies of the microsatellite locus LP65. (A) Sample of 18 single cells from Southern California. (B) Sample of 24 single cysts from Sweden.

2.3.3 Sequence analysis of the ITS and partial 18S region of the rRNA gene

A set of fourteen 18S PCR products, originating from Southern California (cells 1-3 and cells 1-7 shown in Fig. 2 and Fig. 3 respectively), as well as 4 resting cysts isolated from Koljö Fjord north of Gothenburg, were sequenced. All fourteen sequences were identical and therefore only one sequence from Southern California (EU177124) and

one from Sweden (EU177125) have been submitted to GenBank. Based on their similarity with sequences published in GenBank, the fourteen single cells could be identified as *L. polyedrum*. Out of fourteen other published *L. polyedrum* 18S sequences, one (AF274269) was identical, the other thirteen sequences showed between 97 to 99% similarity.

Also a total of twenty ITS PCR products were sequenced comprising six clonal cultures isolated in the years 1998, 2003, and 2004 from Southern Californian waters, 10 single motile cells from La Jolla Bay, California collected in 2003, 2004, and 2005, as well as 2 single motile cells collected in 2006 off the coast of Plymouth, UK, and 2 resting cysts isolated from Koljö Fjord north of Gothenburg, Sweden. All twenty ITS sequences were identical and only one sequence from Southern California, Sweden, and the UK (EU177126, EU177127, and EU177128 respectively) have been submitted to GenBank. Based on their similarity with two other *L. polyedrum* ITS sequences published in GenBank the six cultures and fourteen single cells could be identified as *L. polyedrum*. The complete ITS sequence AM184208, originating from the Mediterranean Sea, was identical to the 20 sequences obtained in this study and the sequence AF377944, whose origin could not be traced, had a similarity of 99% compared to AM184208 and sequences reported above.

2.4 Discussion

2.4.1 Preparation of single cells for PCR

A new method for the preparation of single cells for genetic analysis was developed, in which a glass bead was used to grind a single cell during vortexing. This very quick method allowed the disruption of single cells and PCR amplification in the same tube without the need of any intermediate steps. Other methods such as the disruption of single cells by repeated freeze-thawing (Bolch 2001, Ruiz Sebastián and O'Ryan 2001) and the direct use of intact cells in PCR reactions (Edvardsen et al. 2003), were also tested, but did not result in good amplification success of microsatellite loci (results not shown). Other previously described methods were, on the other hand, unsuitable. For example, the enzymatic DNA extraction method by Marin et al. (2001) is too time-consuming to be applied to the large number of samples that are required for population

genetic studies. The Chelex extraction by Richlen and Barber (2005) is much quicker, but only makes use of an aliquot of the extracted DNA hence not the entire DNA from a single cell is made available for PCR amplification. Also the technique of crushing a single cell on a glass slide using a glass needle followed by the transfer of the crushed cell to a PCR tube (Takano and Horiguchi 2005) does not guarantee that the whole DNA is available for amplification. These last two approaches might therefore work well for the amplification of the rRNA gene and mitochondrial DNA, which are generally present in multiple copies in the genome (Prokopowich et al. 2003, McGrath and Katz 2004), but not for single copy microsatellite loci. The method presented here of physically breaking single cells using a glass bead resulted in efficient breakage of cells and ensured that the whole DNA from a single cell was available for amplification as the glass bead remained in the PCR tube until after the first step of the two-step PCR protocol. This method, which resulted in high amplification success rates for microsatellites and ribosomal gene regions from *L. polyedrum*, is inexpensive, suitable for processing large numbers of samples, and cells can be prepared in a very short time without the need for further processing.

2.4.2 Multiplex microsatellite genotyping of single cells

For the genetic analysis of multiple loci from single cells, these targeted loci have to be amplified simultaneously because the original template DNA can not be recovered for further analysis subsequent to the first PCR. This need for simultaneous amplification can be a challenge in the development of a single cell genotyping technique because increasing numbers of multiplexed primers also increase the chance for primer interactions and the amplification of undesirable products. This problem was addressed in the developed two-step PCR protocol by optimizing the conditions for the simultaneous amplification of the loci in the first amplification step to a degree that subsequently allowed the separate reamplification of the different loci during the second PCR. As a precaution to minimize the generation of fluorescently labelled undesirable products, labelled primers were only used during the reamplification step. The numbers of microsatellites used in population genetic studies of phytoplankton species are usually between three and ten loci (e.g. Evans and Hayes 2004, Rynearson and Armbrust 2005, Iglesias-Rodríguez et al. 2006). The results presented here showed that it is possible to amplify six microsatellite loci in combination with either the ITS or

partial 18S region of the ribosomal gene from single cells. This demonstrates that commonly used numbers of microsatellite loci in culture-based population genetic studies can also be analyzed from single cells. One clear limitation of the specific protocol described in this study was that only one of the six amplified microsatellite loci (LP65) could be successfully used as a population genetic marker. The reason for this limitation lies in the constructed microsatellite clone library, which only provided a fairly limited number of candidate microsatellite loci that were suitable for the necessary multiplexing of loci for single cell genotyping. It is therefore anticipated that, based on a larger number of microsatellite loci, it would be possible to develop a set of six or more informative microsatellite loci for single cell based population genetic studies. Using the single locus LP65 only one common allele was found between the two natural samples of *L. polyedrum* from California and Sweden. This indicates a significant genetic differentiation between the two populations and the observed gene diversities of 0.866 and 0.787 also showed high levels of genetic diversity within the two populations. Although more samples are needed for a robust statistical analysis, these initial results indicate that the microsatellite locus LP65 is a useful marker for population genetic diversity studies in *L. polyedrum*.

The genotyping of single motile cells showed that some of the cells had two products for locus LP65 (Fig. 2.3A). A likely explanation for obtaining two products could be that these were heterozygous diploid cells. Given that the only diploid motile stage in the life cycle of *L. polyedrum* is the planozygote, a product of sexual reproduction (reviewed in Lewis and Hallett 1997), microsatellite analysis of single cells may enable the direct detection of sexual reproduction in natural populations. However, as the two products could have also resulted from a second copy of LP65 within the haploid genome and because haploid vegetative cells and homozygous diploid cells would be indistinguishable by microsatellite analysis, further investigations are needed to assess the usefulness of microsatellite analysis of single cells in detecting life cycle changes in natural populations.

2.4.3 The benefits of co-amplifying ribosomal gene regions from single cells used in microsatellite genotyping

The amplification of the ITS or partial 18S rRNA gene region together with the microsatellite markers functioned as an internal positive control for the amplification

success of microsatellites. For example, the successful amplification of the 18S region from the three cells shown in figure 2 indicated that the single cell DNA template was not degraded and that the breaking of the single cells was effective. Any unsuccessful amplification of a microsatellite locus, e.g. failed amplification of LP2 from cell 2 (Fig. 2.2), therefore suggested a locus-specific amplification problem rather than a generic failure of amplification. Another example of how the amplification of the 18S rRNA gene aided in identifying the cause for failed amplifications is cell number 17 in figure 2.3A and 2.3B. This cell did not give products for either of the two loci and a generic PCR failure is therefore most likely.

Reliable species identification is crucial for any study of intraspecific genetic diversity, but even more so for single cell genotyping because the individual cells are lost for further morphological examination after being used for PCR analysis. Because this destruction of the sample is unavoidable, the amplification and sequencing of the ITS or partial 18S region had the other important function of providing an additional verification of species identity. Even though it may not be feasible to sequence the ITS or 18S PCR products of each single cell that is used in a population genetic study, the sequencing of a randomly chosen subsample of cells can function as a quality control for correct species identification. These quality control measures of both PCR amplification and species identification could become all-the-more important if future developments in single cell genotyping involve automatic sorting and isolation of single cells, for example via flow cytometry.

The ITS and partial 18S rRNA gene sequences obtained in the present study were also analyzed for variations that could be used to assess intraspecific genetic diversity in *L. polyedrum*. Although the samples originated from populations that are geographically widely separated both the 18S and ITS sequences did not show any sequence variation. For the 18S sequences this was less surprising, as this region of the ribosomal gene is usually highly conserved within a species and is generally not suitable for the assessment of intraspecific genetic diversity (reviewed in Parker et al. 1998, de Bruin et al. 2003, Nybom 2004). Less expected was the lack of any sequence variation in the ITS sequences. This region is generally less conserved than the large and small subunit of the ribosomal gene, and has been used previously for the assessment of the biogeography of dinoflagellate species and intraspecific genetic diversity (e.g. Edvardsen et al. 2003). With the exception of sequences published by Moorthi et al. (2006), which have unusually high levels of up to 3% sequence variation in the

corresponding 18S region , other published ITS and 18S sequences of *L. polyedrum* showed only minor variations. Therefore, both the ITS and the targeted partial 18S region of *L. polyedrum*, seem to be too conserved to function as markers for intraspecific genetic diversity within this species.

2.4.4 The benefits of single cell genotyping for population genetic studies

Single cell genotyping allows an unbiased assessment of natural population genetic diversity as any selection of culturable over unculturable strains, which could potentially give an incorrect measure of diversity, is eliminated. Moreover, a direct comparison between population genetic data obtained from cultures and single preserved cells from the same water sample offers a unique opportunity to study the selectivity of culturing on population genetic diversity. The analysis of single cells also enables population genetic diversity studies of unculturable species and has the advantage of a quick turnover time from sampling to genotyping.

The statistical analysis of population genetic data can be negatively affected by factors such as small sample size (Nei 1978, Ruzzante 1998) and unequal sample size between populations (Leberg 2002). For example, equal sample sizes are important for the assessment of allelic richness among populations and the error introduced by unequal sampling can only be dealt with by restricting the data set to the smallest available sample or by statistically estimating the introduced error (Leberg 2002). Because the described technique allows single cell genotyping of preserved natural samples, large sample sizes and equal sampling can both be easily achieved. Single cell microsatellite genotyping is therefore an important development for statistically rigorous genetic diversity studies in phytoplankton and other microorganisms.

This study represents a novel approach to population genetic studies of single cell phytoplankton species and other microorganisms. Garner (2002) has shown that the success rate in amplifying microsatellite loci is negatively correlated with genome size and suggested that this negative effect of large genomes on amplification success is probably due to the dilution of available primers by non-specific binding and a decrease in target to non-target DNA. Given that dinoflagellates, and in particular the species *L. polyedrum*, have very large genomes compared to other phytoplankton species (Spector 1984, Rizzo 2002), it is anticipated that similar single cell genotyping protocols could also be developed for other phytoplankton species and also other microorganisms.

Chapter 3

INFLUENCE OF PHOTON FLUX DENSITY ON THE LIFE CYCLE OF
LINGULODINIUM POLYEDRUM (DINOPHYCEAE) WITH FOCUS ON
NUTRIENT STRESS-INDUCED GAMETOGENESIS

Abstract

The influence of photon flux density (PFD) on the life cycle of two sexually compatible strains of *Lingulodinium polyedrum* (F. Stein) J.D. Dodge was examined. The study was conducted in batch cultures to investigate the effects of PFD under nutrient-replete and nutrient-deplete conditions. Different life cycle stages were identified microscopically and by crossing experiments between the two *L. polyedrum* strains. The identified life cycle stages differed markedly in cell size, which was utilized to distinguish the different cell populations using a Coulter counter. The only observed life cycle change during nutrient-replete exposure to the different PFDs was the formation of an ecdysal stage in the strain LP2810 under low light. However, nutrient stress induced gametogenesis was considerably enhanced in both strains under high light compared to medium light whereas low light prevented gametogenesis in the strain M22 and instead triggered the formation of an ecdysal stage. The observed effects of light give a first indication for the involvement of the photosynthetic apparatus in the induction of gametogenesis in *L. polyedrum*. Further, results presented indicate that gametes of *L. polyedrum* are formed by a single mitotic cell division but that these gametes have the ability to divide into a second, smaller class of gametes, resulting in the potential formation of four gametes per vegetative cell. These findings give new insights into the life cycle and its environmental control in *L. polyedrum*, providing the basis for a better integration of life cycle processes into the study of this important bloom-forming species.

3.1 Introduction

The complex interactions of phytoplankton species with their biotic and abiotic environment lead to equally intricate population dynamics. Understanding the population dynamics of harmful algal bloom (HAB) species is a particularly pressing issue as these species pose risks to human health and as a result the investigation of interactions of HAB species with their environment is advanced compared to other species (Donaghay and Osborn 1997, Roelke and Buyukates 2001). A major gap in understanding phytoplankton population dynamics is how their life cycle is interlinked with their population dynamics (Wyatt and Jenkinson 1997, Garcés et al. 2001). Life cycle changes, such as the transition from motile cells to ecdysal stages and the formation of resting cysts, affect species survival under different environmental conditions, protect from viruses, grazers or parasitic attack, contribute to the dispersal of a species and play key roles in the initiation, maintenance, and termination of blooms (Anderson and Morel 1979, Anderson et al. 1983, Dale 1983, Walker 1984, Ishikawa and Taniguchi 1996, Kremp and Heiskanen 1999, Montresor 2001, Zingone et al. 2001). The role of sexual *versus* asexual reproduction has been less studied but the available data suggest that the different forms of reproduction also strongly influence population dynamics (Anderson et al. 1983, Ishikawa and Taniguchi 1996).

In order to identify links between phytoplankton life histories and population dynamics, it is important to understand the role of the environment in triggering life cycle processes such as the transition from asexual to sexual reproduction (Zingone et al. 2001). In diatoms, for example, it has been shown that the sexual life cycle can be induced by nutrient stress, UV light and dim light, (reviewed in Garrison 1984) but the responsiveness to an induction trigger also depends on internal factors such as the cell cycle stage (Armbrust et al. 1990) and cell size (e.g. Drebes 1977). In dinoflagellates a number of environmental factors have been suggested to induce sexual reproduction including nutrient depletion, temperature, day length, dissolved gases and also irradiance (Pfiester and Anderson 1987). However, very little emphasis has been given to the systematic testing of these environmental factors and gametogenesis remains mainly attributed to nutrient stress (e.g. Pfiester 1976, 1977, Watanabe et al. 1982, Figueroa and Bravo 2005, Figueroa et al. 2006, 2007).

Detailed knowledge about the life history of a species is fundamental for the study of environmental control over life cycle transitions. A valuable model organism for the life cycle in dinoflagellates is *Lingulodinium polyedrum* (F. Stein) J.D. Dodge, an ecologically important bloom-forming dinoflagellate that produces yessotoxin (Paz et al. 2004, Armstrong and Kudela 2006). The three main processes that constitute the life cycle of *L. polyedrum* are: vegetative reproduction, formation of ecdysal stages, and sexual reproduction (reviewed in Lewis and Hallett 1997). The sexual life cycle starts with the formation of gametes, which appear lighter in colour and are slightly smaller then vegetative cells (Kokinos and Anderson 1995). In a recent study Figueroa and Bravo (2005) showed that *L. polyedrum* exhibits simple heterothallism (two sexual types, +/-). During sexual reproduction, gametes of opposite types fuse to form a diploid planozygote. Depending on nutrient availability, this planozygote either forms a resting cyst, which has a mandatory dormancy period, or a sexual ecdysal cyst. Both types of sexual cysts germinate into a planomeiocyte that returns to the vegetative state by dividing into two haploid cells (Lewis and Hallett 1997, Figueroa and Bravo 2005). The life cycle of *L. polyedrum* is typical for lower eukaryotes in that sex is facultative, occurs in response to stress, and results in the formation of a stress-resistant, diploid, dormant form which allows for meiotic recombinational DNA repair at the end of dormancy (Nedelcu and Michod 2003). As in most other dinoflagellate species, nutrient stress is the only factor shown to induce the sexual life cycle in *L. polyedrum* (Blanco 1995, Figueroa and Bravo 2005). In the present study we exposed two sexually compatible strains of *L. polyedrum* to three different PFDs under nutrient replete and increasingly nutrient limiting conditions to examine whether life cycle transitions in this dinoflagellate species are influenced by irradiance levels.

3.2 Material and Methods

Strains and pre-experimental culture conditions

Two clonal strains of *L. polyedrum*, both originating from Southern Californian waters, were used in this study. The strain LP2810 was isolated from a water sample collected at a depth of 4 m in La Jolla Bay on the 28/10/2004. The strain M22 was isolated from a surface water sample collected on the 01/11/2004 at Avila Beach. The cultures were

grown in F/2 medium (Guillard 1975) at $19 \pm 1°C$. Fluorescent lighting (Cool white Standard, Havells Sylvania, Charlestown, UK) was used to provide 130 µmol photons · $m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (PAR), on a 12h light/dark cycle. PFD was determined using a LI-189 light meter (LI-COR®, Lincoln, USA). In order to ensure that *L. polyedrum* cells were nutrient-replete at the start of the experiments, the two strains were maintained in exponential phase by regular sub-culturing for a period of 21 days, keeping cell densities below 3500 cells · mL^{-1}.

Experimental culture conditions

Three irradiance levels, high light (300 µmol photons · $m^{-2} \cdot s^{-1}$), medium light (130 µmol photons · $m^{-2} \cdot s^{-1}$) and low light (10 µmol photons · $m^{-2} \cdot s^{-1}$) were established by adjusting the number of fluorescent tubes and the distance of cultures to the light source. Ventilation of the growth room minimized local heating of cultures and ensured a temperature of $19\pm1°C$ under the different light conditions. The cultures were grown in 500 mL Erlenmeyer flasks containing 350 mL of f/2 medium. Inoculated cell densities were 250 cells · mL^{-1} and the cultures were given 24 h to allow for recovery due to possible physiological 'shock' caused by sub-culturing. The first sampling was conducted at 'time zero', followed by the transfer of cultures to HL and LL conditions. The control condition (ML) had the same PFD as the pre-experimental culture conditions. Sampling was conducted regularly over a period of 42 days to obtain a time series of life cycle changes under the three PFDs in nutrient replete, followed by increasingly nutrient limiting conditions. Cultures grown under the same irradiance were randomly redistributed after each sampling accompanied by measurements of irradiance to avoid shading effects between the flasks.

Measurements of photosynthetic efficiency

Measurements of variable fluorescence were performed using a fast repetition rate fluorometer (FRRF), configured for bench-top use (FAST$^{\text{tracka}}$ I, Chelsea Instruments, West Molesey, UK). The program configuration was set to provide 100 saturation flashes per sequence with a saturation flash duration of 4 µs, followed by 20 relaxation flashes of 4 µs, 120 µs apart. The sleep time between acquisition pairs was set to 60 ms. All FRRF measurements were made 7 to 9 h into the light cycle. To avoid sensor saturation, most samples had to be diluted. The required dilution factor was dependent

on cell density. Dilutions were performed using the batch of filtered, autoclaved sea water that was used to prepare the culture medium. The diluted samples were split into two sub-samples, one was used directly for measurements without dark relaxation, and the other half was stored in the dark for 20 min before measurements were taken. There were no marked differences between the direct measurements and the measurements after dark relaxation and only the latter measurements are shown as averages of five iterations ± SD of duplicate experiments. Statistical analyses were performed in SigmaStat 3.0. Based on the combined data of the duplicate experiments conducted with both strains a two way ANOVA, with light condition and time as variables, was performed to test the effects of PFD on photosynthetic efficiency over time and to compare the effects between the two strains M22 and LP2810. Pairwise multiple comparisons were performed using the Holm-Sidak method with an overall significance level of 0.05.

Cell counts, cell size determination and morphological examination

Samples of 15 mL were preserved by adding 300 μL of a 1:1 mixture of 37% formalin (containing 10 to 15% methanol) and glacial acetic acid. Cell counts and cell size measurements were performed using a Coulter Multisizer^{TM} II (Beckman Coulter Ltd., Luton, UK) fitted with a 200 μm orifice tube. Size calibrations were performed using 30 μm latex particles (L30 standard, Beckman Coulter). Three aliquots of 2 mL were measured in narrow range mode from 7 to 60 μm. When coincidence values of undiluted cultures exceeded recommended limits by the manufacturer, samples were diluted with Isotone (Beckman Coulter). Cell numbers and average cell diameters were determined using the software Multisizer AccuComp$^{®}$ version 1.19 (Beckman Coulter). Morphological examinations of cell types and manual cell counts were performed regularly in sub-samples of 200-1000 μL under the microscope using a Sedgwick-Rafter chamber. The manual counts were used to check the accuracy of the Coulter Counter measurements and showed a good correlation. Growth rates (μ (d^{-1})) were calculated using the equation $\mu = (d_2-d_1)^{-1} \cdot \ln (N_2/N_1)$ where d = day and N = cell density (Mouget et al. 1999, Tremblin et al. 2000).

Crossing experiments

Crossing experiments were conducted between cultures of identical light treatments by

combining 1 mL samples from both *L. polyedrum* strains in 24 well tissue culture plates for suspended cell cultures (Sarstedt, Beaumont Leys, UK). The behaviour of mixed cultures was usually observed using a dissecting microscope. Alternatively, for the documentation of life cycle stages and gamete fusion, an inverted microscope fitted with a digital camera was used.

3.3 Results

3.3.1 Effects of PFD on M22 and LP2810 under nutrient replete conditions: Phase I

The effects of three different PFDs: high light (HL), medium light (ML) and low light (LL) on photosynthetic efficiency (Fv/Fm), cell growth and average cell diameters (ACD) of M22 and LP2810 during nutrient-replete growth are shown in figure 3.1 A-F (phase I). At the start of the experiments all cultures were exposed to ML (day zero). Fv/Fm was 0.43 for M22 and 0.46 for LP2810 at this point, differing significantly (p <0.001) between the two strains. Within the first 48 h after transfer to the different light conditions Fv/Fm changed to new light-specific values. Under HL Fv/Fm decreased significantly (p <0.001) in both strains to 0.41 for LP2810 and to 0.38 for M22, and increased significantly (p <0.001) in both strains under LL to 0.51 for LP2810 and to 0.53 for M22. Fv/Fm between the two strains also differed significantly under HL (p <0.001) and LL (p =0.033). As expected, Fv/Fm of the control cultures under ML did not change significantly in either of the strains (Fig. 3.1 A and 3.1 B). After the change to light-specific values within the first 48 h, Fv/Fm remained fairly stable up to day six. On day six both strains reached early to mid exponential phase of vegetative growth under HL and ML and were still in lag-phase under LL (Fig. 3.1 C and 3.1 D). Maximum growth rates (μ) varied between the different PFDs and also between the two strains. The maximum growth rates of LP2810 under HL and ML were 0.246±0.009 d^{-1} and 0.229±0.004 d^{-1} respectively. Growth rates of M22 were lower under both irradiances with 0.191±0.003 d^{-1} under HL and 0.181±0.004 d^{-1} under ML. Maximum growth rates under LL were achieved at a later stage in the experiment and are described below.

The average cell diameter (ACD) at the beginning of the experiment was 28 μm for M22 and 27 μm for LP2810 (Fig. 3.1 E and 3.1 F). Within the first 24 h under ML and

HL the ACD of both strains increased to 29.7-30.9 μm and under LL decreased to around 26 μm for M22 and 24 μm for LP2810. Except LP2810 under LL, which continued to decrease to an ACD of about 21.5 μm, all other cultures returned to the initial ACD by day six.
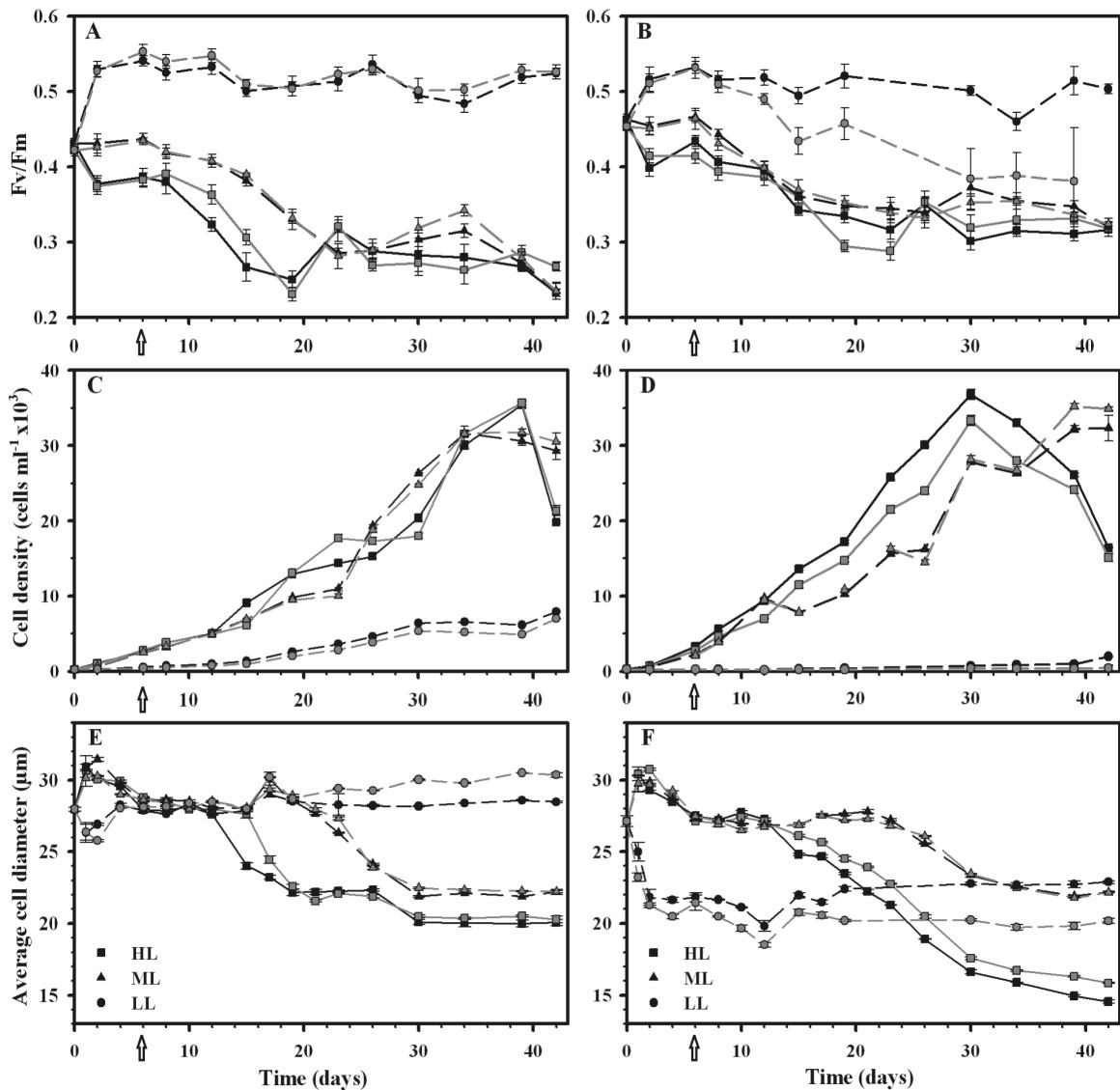
FIG. 3.1 Time series of Fv/Fm (A and B), cell density (C and D) and average cell diameters (E and F) of the two *Lingulodinium polyedrum* strains M22 (A, C, E) and LP2810 (B, D, F) during nutrient replete (phase I, up to day six) and increasingly nutrient limited exposure (phase II, after day 6) to high light (HL), medium light (ML) and low light (LL). The arrow marks the transition from phase I to phase II. Each graph shows the results of duplicate experiments (shown in black and grey) under each light condition. The colour scheme of duplicate experiments is continued consistently throughout figures 3.2-3.4 to enable the direct comparison of parameters measured within each experiment. Fv/Fm data are averages of five iterations (± 1 SD). Cell concentrations and average cell diameters are averages of triplicate measurements (± 1SD). Error bars are sometimes hidden by the data point symbols.

The corresponding cell size distributions showed that the temporary increase in ACD of both strains under ML and HL was due to a shift in size of the whole cell population (day 1 and day 6, Fig. 3.2 A/B and Fig. 3.3 A/B). Also the decrease in ACD of M22 under LL was due to a shift in cell size of the whole population (day 1 and day 6, Fig. 3.4 A), but the decrease in ACD of LP2810 was due to the development of a second population of smaller cells with a diameter around 15 μm (day 1 and day 6 onwards, Fig. 3. 4 B). Microscopic examination revealed that this second cell population in LP2810 consisted of ecdysal cysts (inset picture on day 6, Fig. 3.4 B). Crossing experiments between LP2810 and M22, conducted on day one, three and six for all PFDs gave negative results, showing that the motile cells were vegetative and that the temporary changes in cell size of motile cells during the first six days were not due to a life cycle change.
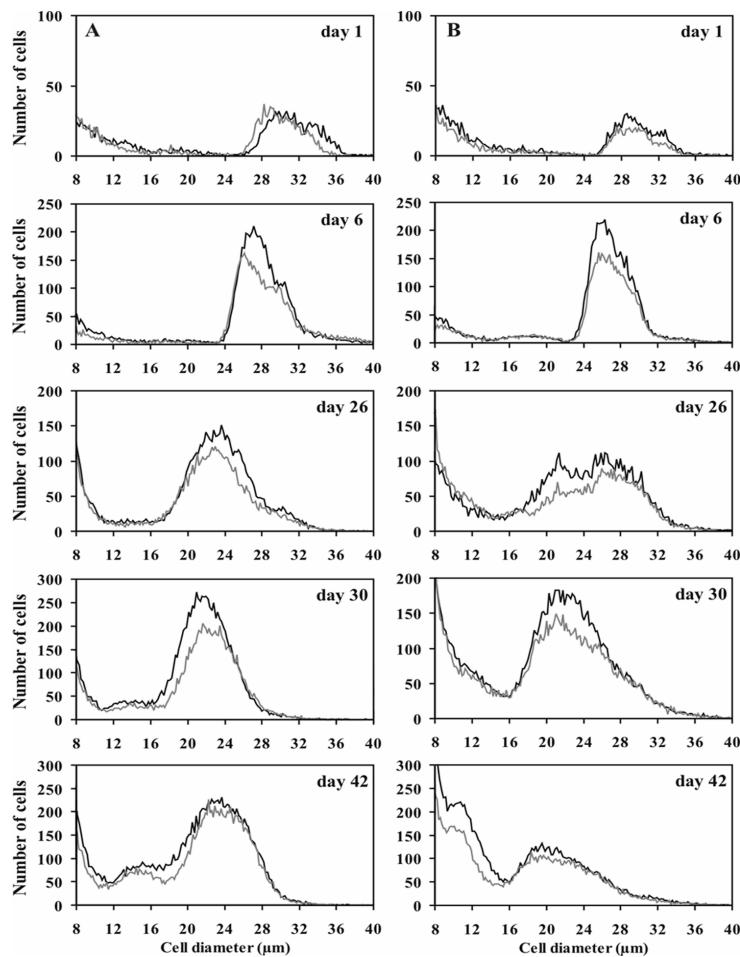
FIG. 3.2 Cell size distribution of M22 (A) and LP2810 (B) under medium light (ML) during key transition points between different life cycle stages. Day numbers correspond to the times shown in figures 1A to 1F. Each graph shows the results of duplicate experiments (black and grey). Individual cell size distributions are averages of triplicate measurements.
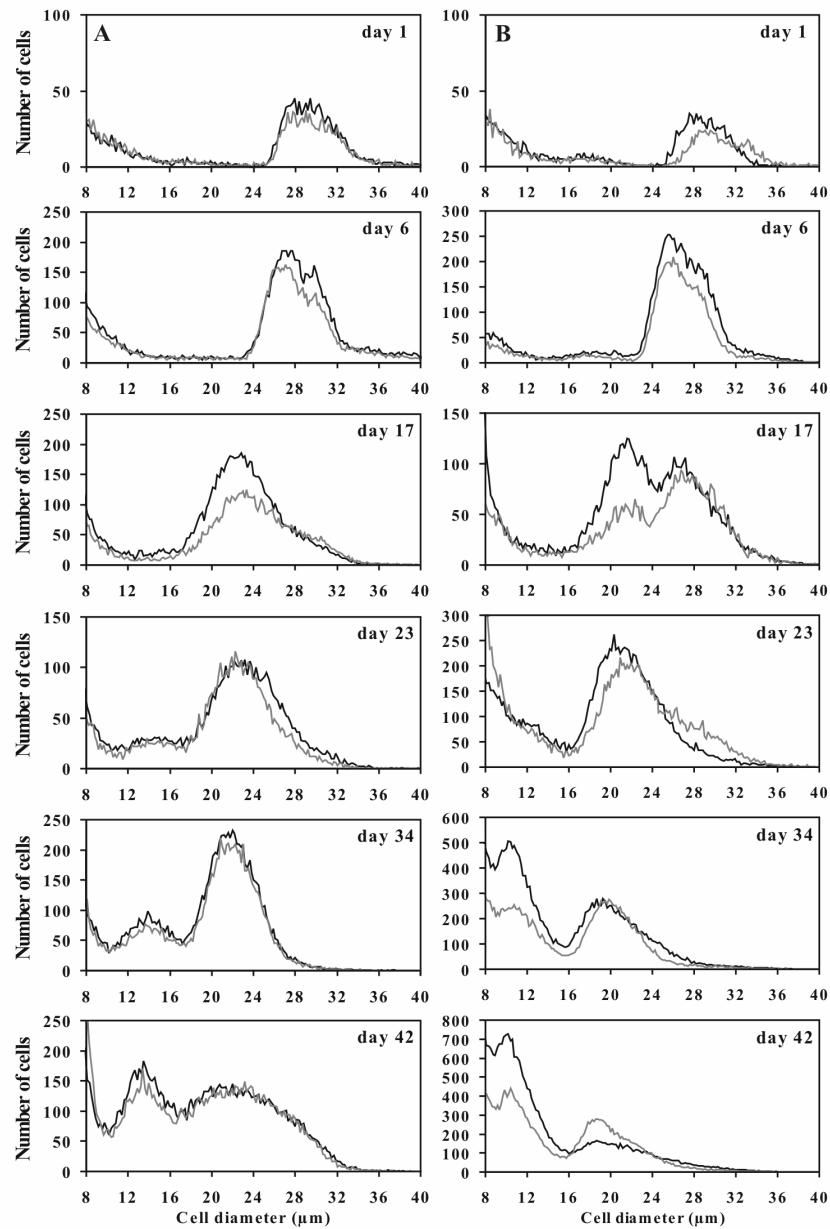
FIG. 3.3 Cell size distribution of M22 (A) and LP2810 (B) under high light (HL) during key transition points between different life cycle stages. Day numbers correspond to the times shown in figures 1A to 1F. Each graph shows the results of duplicate experiments (black and grey). Individual cell size distributions are averages of triplicate measurements.

3.3.2 Effects of medium light under increasingly nutrient limited conditions: Phase II

Photosynthetic efficiency, growth and average cell diameters (ACD) of M22 and LP2810 during increasingly nutrient limited growth are shown in figure 3.1 A-F (phase II). Under medium light, Fv/Fm started to decline after day six, indicating an early stage of nutrient limitation in both strains although this decline was more pronounced in

LP2810 (Fig. 3.1 A and 3.1 B). Growth rates of both strains were at a maximum on day six, showing that nutrient limitation affected photosynthetic efficiency before it affected cell division rates. The decline of Fv/Fm continued in M22 until day 23 to values of approximately 0.28 and until day 26 in LP2810 to values of 0.32. The lowest Fv/Fm values roughly coincided with cultures reaching a stationary phase with approximately 10,500 cells • mL^{-1} in M22 (day 19-23) and 16,000 cells • mL^{-1} in LP2810 (day 23-26) (Fig. 3.1 C and 3.1 D).
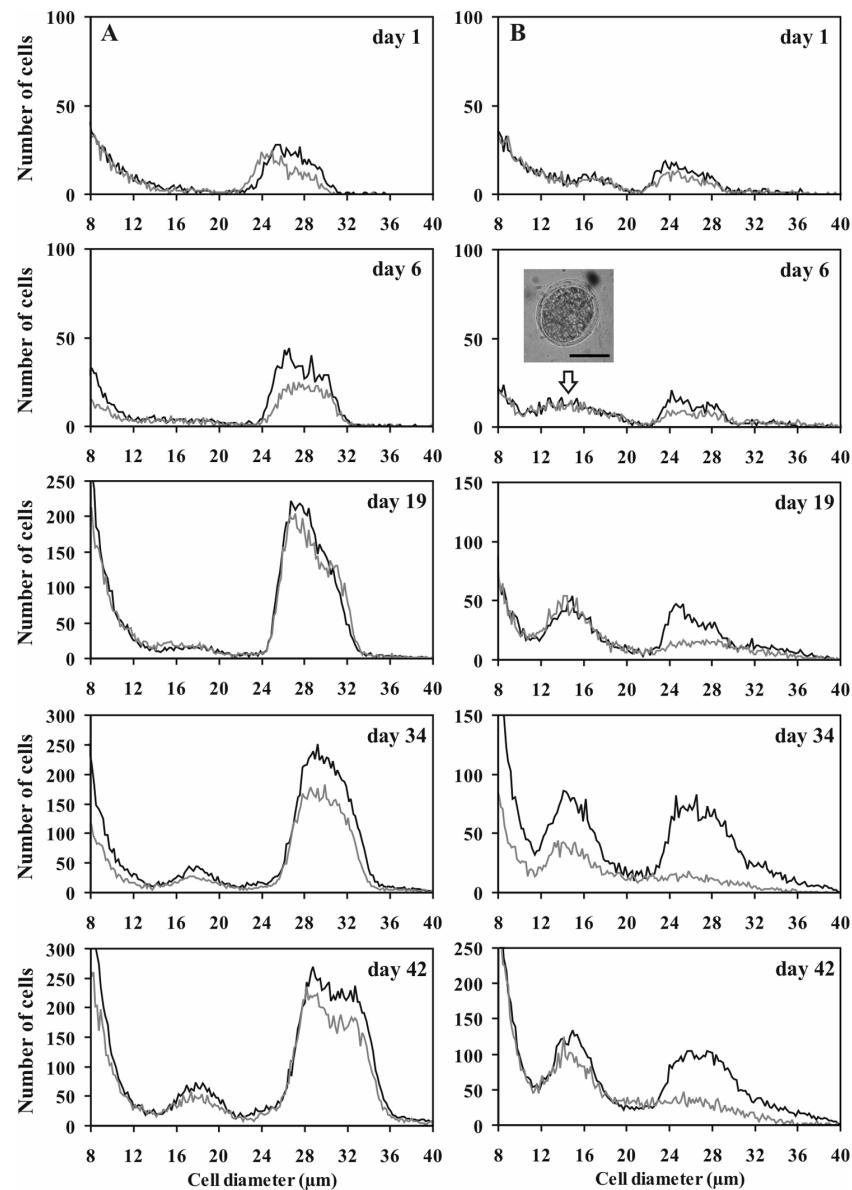
FIG. 3.4 Cell size distribution of M22 (A) and LP2810 (B) under low light (LL) during key transition points between different life cycle stages. Day numbers correspond to the times shown in figures 1A to 1F. Each graph shows the results of duplicate experiments (black and grey). Individual cell size distributions are averages of triplicate measurements. Scale bar in insert picture on day 6 = 10 μm.

After reaching stationary phase, cell numbers increased again, accompanied by a reduction of ACD (Fig. 3.1 E and 3.1 F). The decrease in ACD was due to the formation of a second population of cells with a diameter of approximately 23 μm in M22 and 21 μm in LP2810 (days 26 and 30, Fig. 3.2 A and 3.2 B). Coinciding with the formation of smaller cells, a recovery of Fv/Fm was observed in M22 between day 23 and day 34 and between day 23 and day 30 in LP2810 (Fig. 3.1 A and 3.1 B). Cell numbers of the second, smaller cell type reached a second stationary phase with 31,500 cells • mL^{-1} for M22 and 33,000 cells • mL^{-1} for LP2810 on days 34 and 39, respectively and remained stable to the end of the experiment on day 42 (Fig. 3.1 C and 3.1 D).

Crossing experiments between LP2810 and M22 at the time the cultures reached the second stationary phase were positive, showing that the cells of 21-23 μm were gametes. A time series of the fusion process of gametes observed during this crossing experiment is shown in figure 3.5. The outcome of a crossing experiment was positive (gametes present) if cells formed dense clouds followed by the pairing of individual couples of cells, and negative (no gametes present) when cells showed no sign of interaction. Towards the end of the ML experiment a third population of cells with yet smaller diameters of 11 μm in LP2810 and 13 μm in M22 started to be formed (day 42, Fig. 3.2 A and 3.2 B). The numbers of this third cell size class were however small compared to the gamete population and did not noticeably change overall cell numbers (Fig. 3.1 C and 3.1 D) or average cell diameter (Fig. 3.1 E and 3.1 F).
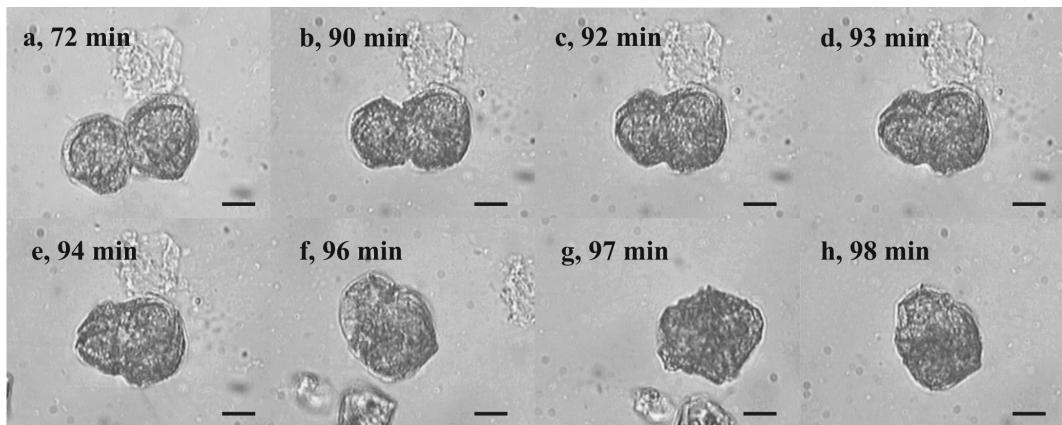
FIG. 3.5 Time series of gamete fusion between the *Lingulodinium polyedrum* strains M22 and LP2810. Approximately 72 min after gametes paired up, the fusion of cells began. The process from the first signs of fusion to the complete fusion of cells to a planozygote took less than 16 min. Scale bar = 10 μm.

3.3.3 Effect of high light under increasingly nutrient limited conditions: Phase II

Similar to the ML cultures, Fv/Fm in both strains started to decrease after day six. Fv/Fm in M22 declined more rapidly under HL compared to ML, while changes of Fv/Fm in LP2810 were generally similar under HL and ML (Fig. 3.1 A and 3.1 B). Maximum numbers of vegetative cells were reached between days 12 and 15, several days earlier compared to ML cultures and with approximately 7,000 cells · mL^{-1} for M22 and 9,000 cells · mL^{-1} for LP2810 maximum numbers of vegetative cells were lower than those observed under ML (Fig. 3.1 C and 3.1 D). A clear stationary phase of vegetative growth was not evident under HL. However, a reduction in ACD, due to the appearance of a second population of smaller cells (days 17 and 23, Fig. 3.3 A and 3.3 B) marked the end of vegetative growth and beginning of gametogenesis, comparable to observations under ML, and confirmed by positive results of crossing experiments. Fv/Fm in M22 reached a minimum on day 19 with values of approximately 0.24, and decreased to a less defined minimum in LP2810 on day 23 with values of 0.29-0.31. In contrast to the ML cultures, the lowest Fv/Fm values under HL did not coincide with cultures reaching the end of vegetative growth but occurred during an advanced stage of gametogenesis. However, similar to the ML experiments, gamete formation was accompanied by a recovery of Fv/Fm in both strains, which was most noticeable in M22, with Fv/Fm rising from 0.24 on day 19 to 0.32 on day 23 (Fig. 3.1 A and 3.1 B). Gametogenesis in M22 reached a stationary phase with approximately 16,000 cells · mL^{-1} around day 23; eleven days earlier than under ML, which was reflected in the ACD reaching approximately 23 μm (Fig. 3.1 E). LP2810 did not show a clear stationary phase of gamete formation but on day 23 most vegetative cells had turned into gametes (Fig. 3.3 B), sixteen days earlier than under ML. The cell concentration in LP2810 on day 23 was approx. 23,000 cells · mL^{-1} and the ACD approached 22 μm.

Comparable to observations under ML conditions, a third population of smaller cells was observed following the completion of gametogenesis. However, under HL this third cell size class was formed earlier, leading to a further increase in cell numbers (Fig. 3.2 A and 3.2 B), accompanied by a further reduction in ACD (Fig. 3.1 E and 3.1 F). On days 30 and 39 respectively this third growth phase led to approximately 35,000 cells · mL^{-1} in M22 and LP2810 followed by a rapid decline of cell numbers in both strains. Crossing experiments showed that this third population also consisted of gametes. The cell size distributions on day 34 in figures 3.3 A and 3.3 B show the transition of the first generation of gametes (21-23 μm) into this second population of gametes with

diameters of 11 μm in LP2810 and 13 μm in M22. The resulting ACD of the two gamete populations on day 34 was around 20 μm in M22 and 17 μm in LP2810 (Fig. 3.1 E and 3.1 F). A complete shift of the ACD to 13 μm in M22 did not occur as a large proportion of the first gamete generation remained up to the end of the experiment and larger cells, up to 32 μm in diameter, reappeared (day 42, Fig. 3.3 A). In LP2810 the reappearance of larger cells was not observed (day 42, Fig. 3.3 B) and the ACD continued to converge with the diameter of the second generation of gametes towards the end of the experiment (Fig. 3.1 F).

3.3.4 Effect of low light: Phase II

For the duration of the experiment Fv/Fm of the strain M22 remained stable around 0.5 (Fig. 3.1 A). Growth continued to be reduced in M22 under LL compared to the other light conditions with a maximum growth rate (μ) of 0.126±0.02 d^{-1}. M22 reached a stationary phase of vegetative growth around day 30 with 6,000 cells · mL^{-1}. Unlike in the ML and HL treatments the end of vegetative growth in M22 under LL was not reflected in a decrease of Fv/Fm. A second cell population with a diameter of approximately 18 μm was formed after M22 reached stationary phase (days 34 and 42, Fig. 3.4 A). Light microscopy examination showed that these cells were ecdysal cysts. The proportion of ecdysal cysts in the culture remained small and did not change the average cell diameter noticeably up to the end of the experiment (Fig. 3.1 E).

In contrast to the generally good reproducibility between duplicate experiments the results of the two tests conducted with LP2810 under LL differed in several ways. Firstly, the lack of growth observed in LP2810 under LL during phase I only continued in one of the experiments. In the replicate experiment, LP2810 achieved a small increase in cells (Fig. 3.1 D). The average maximum growth rate of both experiments was however still close to zero (0.019±0.02) d^{-1}. Secondly, the growing culture sustained high values of Fv/Fm whereas Fv/Fm of the stagnant culture declined rapidly after day eight (Fig. 3.1 B). Thirdly, the formation of an ecdysal stage, which was observed in both experiments with LP2810 under LL from an early stage on, continued at a higher rate in the stagnant culture compared to the growing culture leading to a larger proportion of the ecdysal stage in the former (day 19 onwards, Fig. 3.4 B). Gametes were not produced at any stage under LL as crossing experiments were negative throughout the duration of the experiments.

3.4 Discussion

3.4.1 Effects of PFD under nutrient replete conditions: phase I

Photosynthetic efficiency (Fv/Fm) is being used widely as a physiological indicator for nutrient limitation because variable fluorescence is sensitive to a wide range of nutrient limitations (e.g. Kolber et al. 1988, Geider et al. 1993, 1998). The stability of Fv/Fm, following the light-driven changes during the first 48 h, was therefore a clear sign for the nutrient-replete status of all cultures for at least the first six days of the experiments. For this reason day six was chosen as a conservative cut off point for phase I. The negative results of crossing experiments during phase I, indicated that nutrient-replete exposure to the three PFDs did not cause vegetative cells to undergo gametogenesis. This result confirms previous studies, in that nutrient limitation is an important requisite for the induction of gametogenesis in *L. polyedrum* (Blanco 1995, Figueroa and Bravo 2005). Consequently, the observed transient increase in cell size of both strains under ML and HL, and decrease in cell size of M22 under LL during phase I represent morphometric changes of vegetative cells. Given that the same increase in cell size was observed under ML and HL, this morphometric change was not due to the PFD, as the light regime for ML cultures remained unchanged between the pre-experimental and the experimental conditions. Most likely, the short-term increase in cell size occurred in response to the higher nutrient availability after sub-culturing. Hence, whilst Fv/Fm showed that cultures were generally nutrient replete during phase I, cell physiology in batch cultures already changed during early stages of growth. The early decline of Fv/Fm around day six, when growth was still maximal, is a further indication of the early onset of cell physiological changes in batch cultures. In contrast, the decrease in cell size of M22 under LL cannot be explained by higher nutrient availability after sub-culturing and was presumably a result of the lower PFD until M22 acclimated to the new light level and restored the initial cell size.

The only observed life cycle change during phase I was the formation of the ecdysal stage in low light cultures of LP2810. The ecdysal stage represents a mode to escape unfavourable or adverse conditions, and was previously described in *L. polyedrum* in response, for example, to low temperatures (Dürr 1979), short day length (Balzer and Hardeland 1991, Balzer 1996) and mechanical stress (Latz et al. 1994). Since LP2810 did not yield cell densities that could have caused nutrient-depletion under LL, and the

only variable manipulated in the experiments was irradiance, this stress response of LP2810 indicates that ecdysis in *L. polyedrum* can also be induced by low PFDs. The observed differences in growth rate and rate of ecdysis between duplicate experiments with LP2810 under LL were most likely a result of slight differences in PFD, which in one replicate experiment must have met the lowest light requirements for this strain. The decrease of Fv/Fm in the stagnant LP2810 culture could be a sign of the breakdown of physiological activity in vegetative cells due to the insufficient supply of light and/or photosynthetic inactivity of the large proportion of ecdysal stages found in this culture. The fact that M22 did not undergo ecdysis as an immediate stress response under LL was probably due to lower light requirements of M22, which is supported by the higher growth rate of M22 under LL compared to LP2810.

3.4.2 Effects of PFD under increasingly nutrient limited conditions: phase II

The much reduced growth of both strains under LL meant that nutrient limitation set in much later in M22 and probably did not occur in LP2810. Therefore, the division of growth stages into nutrient-replete (phase I) up to day six and increasingly nutrient-limited (phase II) thereafter did not apply to the LL cultures in the same way as for the ML and HL cultures. The division into the two phases on day six was therefore only adopted for the LL cultures for practical reasons in structuring the results.

Under ML and HL, early signs of nutrient limitation were indicated by the observed decline of Fv/Fm following day six while under LL, no decline of Fv/Fm occurred even during stationary phase of vegetative growth in M22. This was most likely due to the reduced damage in the light harvesting apparatus under low light rather than an indication of continued nutrient availability.

The formation of an ecdysal stage in M22 under low light following stationary phase has to be distinguished from the immediate stress response of LP2810 to low light, because ecdysis in M22 did not occur in response to low light itself, but only in response to nutrient limitation under low light. This suggests the existence of a range of PFD, which is high enough to not trigger ecdysis as an immediate stress response but is low enough to inhibit the gametogenic effect of nutrient limitation, changing the response to nutrient limitation from gametogenesis to ecdysis. The other major observed effect of light was that, under nutrient limitation, high light considerably enhanced gametogenesis compared to medium light. Depending on the strain, this acceleration of

gametogenesis resulted in a complete transition from vegetative cells to a first generation of gametes eleven to sixteen days earlier than under medium light and in an equally accelerated transition of this first generation of gametes into a second generation of gametes.

Prézelin (1982) showed that the cell volume of *L. polyedrum* in bright-light cultures, comparable to the HL condition used in this study, declined to about half by late stationary-phase. Similarly, another study by Prézelin et al. (1986) showed that high light exposure of exponential phase cultures had no effect on cell size, but exposure of stationary phase cultures to high light resulted in an immediate burst in cell division accompanied by a major reduction in cell volume. Prézelin and co-workers interpreted this reduction of cell size as part of a photoadaptive response to high light (Prézelin et al. 1986), and in later stages as an aging process (Prézelin 1982). However, based on the remarkable similarities with current results, it is suggested that their observations of increased cell division accompanied by a decline in cell sizes under high light were in fact a result of gamete formation. Under this assumption, the observation of Prézelin et al. (1986) that the nutrient status of smaller cells increased to a level comparable to that of nutrient-replete, exponential phase cells suggests that *L. polyedrum* has the ability to turn nutrient stressed vegetative cells into nutrient-replete gametes. Prézelin et al. (1986) showed that this regeneration of small cells was due to the fact that carbon and nitrogen loss were relatively small compared to the reduction in cell volume. This reconstitution of a higher nutrient status is equivalent to a re-supply of nutrients, and should therefore lead to a recovery of Fv/Fm (Geider et al. 1993, La Roche et al. 1993). Current observations that gametogenesis was accompanied by a recovery of Fv/Fm was therefore most likely a result of the regeneration of small cells described by Prézelin et al. (1986). A nutrient concentrating mechanism during gametogenesis, leading to an improved physiological health of cells, may increase the chances of gametes in finding a mating partner and could be of ecological importance for the survival of the species.

3.4.3 The process of gamete formation in Lingulodinium polyedrum

Figueroa and Bravo (2005) gave the first detailed description of the sexual life cycle in *L. polyedrum* but the process of gamete formation has not yet been investigated. The observation that gametogenesis resulted in approximately a doubling of cell numbers compared to vegetative cell numbers in stationary phase gives evidence that gametes of

L. polyedrum are formed by a single mitotic division, resulting in two gametes per vegetative cell. It has also been shown that this gamete population can divide into a second population of gametes of smaller size. Although the formation of this second gamete population did not reach an endpoint during the experiments, cell numbers again tended towards a doubling, suggesting that also these second, smaller gametes were formed by a single mitotic cell division of the first gamete generation.

Gametes of some dinoflagellate species have the ability to revert back to vegetative growth once nutrients get re-supplied (von Stosch 1973, Pfiester 1975). We observed that towards the end of the experiments large cells of the size of vegetative cells reappeared in M22 under HL. The nutrients for this growth of large, vegetative-like cells may have been supplied by bacterial nutrient recycling in the cultures. However, whether these larger cells originated from a small number of newly dividing vegetative cells that did not undergo gametogenesis or whether one or both types of gametes were able to revert back to vegetative growth is not clear. Future investigations, in which single gametes of both size classes are isolated and transferred to nutrient replete medium, are therefore needed to resolve this open question in the life cycle of *L. polyedrum*.

3.4.4 Intraspecific differences between M22 and LP2810

In general, M22 and LP2810 showed comparable life cycle changes and light had similar effects on life cycle processes. However, additional to the differences in ecdysis under low light, growth rates and the reappearance of large cells in M22, there were a number of other differences between the two strains. Firstly, the cell size of vegetative cells, gametes and the ecdysal stage varied between the two strains, being consistently smaller in LP2810 than M22. Compared to the literature, which reports vegetative cell sizes of *L. polyedrum* to be 38-45 μm in length and 35–45 μm in width (Kokinos and Anderson 1995) and gametes to be 22–40 μm in length and 18–35 μm in width (Figueroa and Bravo 2005), current measurements were generally smaller. The smaller cell sizes measured here are at least partly due to the measurement of particle diameter with the Coulter principal, which is calculated from the volume of the particle. In this calculation the particle is assumed to be a perfect sphere. For the polyhedral shape of *L. polyedrum* the reported diameters therefore represent a good approximation, but one that consistently underestimates the actual dimensions of the cells. Light-specific values

of Fv/Fm also differed between the two strains, M22 having a lower photosynthetic efficiency than LP2810 under ML and HL and achieving slightly higher values under low light. The differences in photosynthetic efficiency correlated well with the light specific maximum growth rates of both strains, suggesting that LP2810 is adapted to higher light intensities than M22. Whether the differences between the two strains are due to general intraspecific variability or represent sex-specific differences is not clear and additional investigations, comparing more *L. polyedrum* strains of both sexes, are needed to answer this open question.

3.4.5 A possible mechanism for the observed effects of light on gametogenesis

The cellular mechanism(s) that leads to the induction of sexual reproduction in dinoflagellates is unknown, and even the well documented effect of nutrient limitation on gametogenesis in dinoflagellates lacks an explanation on the mechanistic level. The finding that HL enhances and LL inhibits gametogenesis under nutrient limitation, give a first indication of the involvement of the photosynthetic apparatus in this process.

A multitude of environmental stressors, including both high light intensities and nutrient deficiency, can lead to an increase of reactive oxygen species (ROS) levels through the disruption of photosynthesis (reviewed in Asada 1996, Ledford and Niyogi 2005). Interestingly, recent studies have shown that the sexual life cycle in the green alga *Volvox carteri* is induced by a rise of ROS (Nedelcu and Michod 2003, 2004, 2005). Based on the above lines of evidence it can be argued that gametogenesis in *L. polyedrum* may be induced by elevated levels of ROS, caused by nutrient limitation and high PFDs. However, further tests are needed to validate this hypothesis.

3.4.6 Ecological relevance of high light-driven gametogenesis

The extent of asexual *versus* sexual reproduction in natural populations of *L. polyedrum* is unknown as is whether sexual reproduction occurs continuously in a fraction of the population or as a specific event in time and space. The frequent observation of onshore transport of dinoflagellate bloom populations during the relaxation of upwelling and downwelling events could be an effective mechanism to establish cyst beds in shallow water sediments (Donaghay and Osborn 1997), making blooms a likely stage in population development for sexual reproduction in *L. polyedrum*. Blooms of *L.*

polyedrum in Southern California are common from April to November and are associated with calm, warm weather conditions (e.g. Allen 1946, Holmes et al. 1967, Eppley and Harrison 1975). The resulting stratified waters are low in nutrients and high in irradiance (Eppley et al. 1978). Results presented here showed that in culture this combination of high irradiance and low nutrient concentrations is an effective trigger for gametogenesis. However, observations in deep tank experiments and in the field show that *L. polyedrum* overcomes the low availability of nutrients in surface waters by a diurnal vertical migration (DVM) to the nutricline (Holmes et al. 1967, Eppley et al. 1968, Walsh et al. 1974, Heaney and Eppley 1981. The fact that surface waters are low in nutrients does therefore not necessarily imply that bloom populations are nutrient limited.

If, as proposed here, the induction of the sexual life cycle in *L. polyedrum* is dependent on levels of ROS, could gametogenesis in natural populations be induced by high light without nutrient limitation? For example, in the southern California summer, PFDs reach values of approximately 1500 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} (Heaney and Eppley 1981). These irradiances in the field, which are approximately five times higher than the light conditions used in this study, might increase ROS levels sufficiently to induce gametogenesis without nutrient limitation. Another factor that could further increase ROS levels in a nutrient independent fashion is CO_2 limitation of bloom populations as shown in *Peridinium gatunense* (Vardi et al. 1999). However, because naturally occurring irradiance levels and the effect of CO_2 on gametogenesis were not tested in current experiments, the discussion is resumed under the prevalent theory that nutrient limitation is an important requisite for gametogenesis in *L. polyedrum*. As described above, DVM ensures the nutrient supply of *L. polyedrum* which would imply that natural populations do not experience the combination of high light and nutrient depletion. However, might *L. polyedrum* cease DVM at a certain stage of the bloom development, and subject itself to nutrient limitation and high irradiance in surface waters to induce gametogenesis in a controlled way? Previous studies do not give a clear indication whether *L. polyedrum* may control gametogenesis in such a way. A study by Eppley et al. (1968) showed that *L. polyedrum* stopped DVM under nutrient-deplete conditions, but this change in behaviour led to a even distribution of cells with depth. Contrastingly, Heaney and Eppley (1981) showed that *L. polyedrum* continued DVM when nitrogen depletion set in but avoided surface irradiance levels of 1130 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. However, the cell concentrations used in both these studies were low

compared to bloom populations, and a change in behaviour towards a 'deliberate' exposure to high light and nutrient limitation may also depend on cell densities. Still, under the current perception of ROS being harmful by-products of cell physiology, it is difficult to envisage why an organism would 'deliberately' expose itself to conditions that lead to an enhanced oxidative stress in order to induce the sexual life cycle. However, an increasing number of studies suggest that ROS are not just an inevitable harmful metabolic by-product, but that ROS play important roles in the regulation of cell proliferation, cell differentiation and signal transduction (reviewed in Aguirre et al. 2005). Further, the increased encounter rates of gametes resulting from a synchronization of sexual reproduction (Wyatt and Jenkinson 1997) may outweigh the negative effects of exposure to oxidative stress. Finally, the observed recovery of photosynthetic efficiency during gametogenesis in current experiments, suggests that *L. polyedrum* has the ability to mitigate some of the effects of nutrient deplete exposure to high light, which would explain the necessity for such a mechanism in the first place.

This study gives new insights into the life cycle of *L. polyedrum*, its environmental control, and gives a first indication for the involvement of ROS in the induction of gametogenesis. These results provide the basis for a better integration of life cycle processes into the study of population dynamics in this important bloom-forming species. Testing the role of ROS in the induction of gametogenesis and the study of natural *L. polyedrum* blooms with vertical resolution in the water column are important next steps to validate some of these assumptions and answer the open questions raised by these results.

Chapter 4

INTRASPECIFIC VARIABILITY OF LIGHT ACCLIMATION IN THE DINOFLAGELLATE SPECIES *LINGULODINIUM POLYEDRUM* (DINOPHYCEAE): A COMPARATIVE STUDY OF TWO CLONAL STRAINS FROM THE SOUTHERN CALIFORNIAN COAST

Abstract

The light acclimation of two strains of the dinoflagellate species *Lingulodinium polyedrum* (F. Stein) J.D. Dodge was examined over a twenty day period in semi-continuous cultures, followed by growth in batch cultures to stationary phase. During the acclimation period, changes in pigmentation and other photosynthetic parameters, measured by fast repetition rate fluorometry (FRRF), showed that the two strains acclimated to light by employing different mechanisms. The strain M22 acclimated to changes in photon flux density (PFD) by regulating concentrations of light harvesting pigments (chlorophyll a/c, peridinin) and β-carotene, whereas the strain LP2810 mainly regulated the amount of the photoprotective pigment diadinoxanthin. Further differences between the strains were evident in Fv/Fm and in maximum growth rates. Under high (HL) and medium (ML) light, LP2810 achieved significantly higher values of Fv/Fm and higher growth rates but under low light (LL) Fv/Fm was comparable between the strains and M22 achieved significantly higher growth rates. Taken together, these results indicate that M22 is better adapted to low, and LP2810 to high PFDs. In contradiction with the current model, light acclimation of nutrient-replete cultures did not involve a change in the size of photosynthetic units (PSU). Only under nutrient-depletion did a change in PSU size occur. FRRF measurements showed further that nutrient-depletion caused a slow down of the photosynthetic electron transport in LP2810, but not in M22. The two strains tested in this study share identical ITS sequences and are sexually compatible, suggesting that the here reported differences between M22 and LP2810 represent true intraspecific variability of light acclimation in *L. polyedrum*.

4.1 Introduction

The study of the marine dinoflagellate species *Lingulodinium polyedrum* (F. Stein) J.D. Dodge has substantially contributed to the understanding of photosynthesis and light acclimation as well as many other physiological, biological and ecological aspects of this distinctive group of chromophytes (Prézelin 1987, reviewed in Lewis and Hallett 1997). As members of the chromophyte algae, dinoflagellates possess a chlorophyll (chl) a-chl c carotenoid pigment system (Christensen 1989). *Lingulodinium polyedrum* possesses chl c_2 as accessory chlorophyll and peridinin as major accessory carotenoid (Boczar and Prézelin 1987). Photosynthetic pigments are arranged in form of a water-soluble, and several membrane-bound chl a-containing protein complexes. The water-soluble peridinin-chl a-protein (PCP) harbours 80 to 95% of total cellular peridinin in *L. polyedrum* (Prézelin and Sweeney 1978), and is the principal light-harvesting pigment-protein complex in *L. polyedrum* and other dinoflagellates (Prézelin and Haxo 1976, Haxo et al. 1976, Song et al. 1976). Additional carotenoid pigments in *L. polyedrum* include diadinoxanthin, diatoxanthin, dinoxanthin, and ß–carotene (Jeffrey et al. 1975, Jeffrey 1976). Both peridinin and ß–carotene have high rate constants of physical quenching of singlet molecular oxygen and it has been suggested that peridinin and to a lesser extent ß–carotene play important photoprotective functions in *L. polyedrum* (Pinto et al. 2000). The xanthophyll cycle is another mechanism that dissipates excess energy within the antenna system by non-photochemical quenching. In diatoms and dinoflagellates the de-epoxidation of diadinoxanthin to diatoxanthin under excess light and the reverse process under sub-saturating light conditions constitutes this important photoprotective mechanism (Hager and Starnsky 1970, Hager 1975, Olaizola and Yamamoto 1994, Casper-Lindley and Björkman 1998a).

Dinoflagellates generally photosynthesize and grow best at low photon flux densities (PFDs) and often experience photoinhibition at comparatively low irradiances (Richardson et al. 1983). The light requirements for growth and photosynthesis differ significantly between algal classes but within certain limits all species have the ability to acclimate to changing light conditions through a range of physiological responses. The speed at which these responses take effect varies over a wide range. State transitions and xanthophyll cycle inter-conversions occur in a matter of seconds or minutes and represent a short term response to changing irradiance levels whereas other

changes may take hours or days (Long et al. 1994, Raven and Geider 2003). One very common type of light acclimation is based on the up- and down-regulation of photosynthetic pigments (Richardson et al. 1983, Raven and Geider 2003). Low light acclimation of high light acclimated *L. polyedrum* cultures, for example, results in a doubling of chl a and chl c (Boczar and Prézelin 1987), and a doubling of peridinin concentrations (Prézelin and Sweeney 1978). Changes in pigmentation can be achieved in at least two ways; by increasing the relative size of light-harvesting complexes associated with a fixed number of photosynthetic units (PSUs) or by increasing the relative number of PSUs in the thylakoid membranes (Falkowski and La Roche 1991). Changes in pigmentation of *L. polyedrum* are thought to be the result of the former mechanism of increasing the relative size of PSUs (Prézelin and Alberte 1978, Prézelin and Sweeney 1978, Perry et al. 1981).

Intraspecific variation within phytoplankton species at the phenotypic as well as the molecular and genetic level is well established (Gallagher 1982, Krawiec 1982, Brand 1984, Cembella and Taylor 1986, Bomber et al. 1989, Scholin and Anderson 1994, Scholin et al. 1994a, b). To quote Wood and Leatham (1992): 'it is remarkable that for nearly every physiological character examined, significant interclonal variability is found essentially every time that strains from the same putative taxon are compared'. In *L. polyedrum*, the intraspecific diversity of photophysiology and light acclimation, or any other physiological process for that matter, has not been studied sufficiently.

Most photophysiological studies in *L. polyedrum* for example were based on one single strain (70A, UCSB Culture Collection No. 5M20) isolated from the Santa Barbara Channel, California in 1970 (e.g. Prézelin and Sweeney 1978, Prézelin et al. 1986, Boczar and Prézelin 1987). The importance of reaching a better understanding of intraspecific diversity in this species is all the more apparent considering the model status *L. polyedrum* has as a typical bloom-forming dinoflagellate species.

Differences in colouration between two clonal strains of *L. polyedrum*, isolated from the Southern Californian Coast in 2004, led to an investigation of how these strains acclimate to changing light conditions. The results indicate that within the species *L. polyedrum* strains with different light requirements exist, which employ fundamentally different light acclimation mechanisms.

4.2 Material and Methods

Strains and pre-experimental culture conditions

Two clonal strains of *L. polyedrum* from Southern Californian waters were used in this study. The strain LP2810 was isolated from a water sample collected at a depth of 4 m in La Jolla Bay on the 28.10. 2004. M22 was isolated from a surface water sample collected on the 01.11.2004 at Avila Beach. The cultures were grown in f/2 medium (Guillard 1975) at 19 ± 1ºC. Fluorescent lighting (Cool white Standard, Havells Sylvania, Charlestown, UK) was used to provide 130 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}, on a 12h light/dark cycle. PFD was determined using a LI-189 light meter (LI-COR®, Lincoln, USA). In order to ensure that *L. polyedrum* cells were nutrient-replete at the start of the experiments, the two strains were maintained in exponential phase by regular sub-culturing for a period of 21 days, keeping cell densities below 3500 cells $\cdot$ mL^{-1}.

Experimental culture conditions

Experimental light levels: high light (300 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), medium light (130 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), and low light (10 or 30 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) were established by adjusting the number of fluorescent tubes and the distance of cultures to the light source. Ventilation of the growth room minimized local heating of cultures and ensured a temperature of 19±1ºC under the different light conditions. All cultures were grown in 1L Erlenmeyer flasks containing 600 mL of inoculated f/2 medium. Cultures were inoculated to an initial cell density of 500 cells $\cdot$ mL^{-1} and were given 24 h to recover from any potential physiological 'shock' caused by sub-culturing. The first sampling was conducted at 'time zero', followed by the transfer of cultures to HL and LL conditions. The control condition (ML) had the same PFD as the pre-experimental culture conditions. For the first twenty days of the experiment the cultures were kept below 3500 cells $\cdot$ mL^{-1} by sub-culturing to ensure nutrient replete conditions during the acclimation to the different PFDs. The number of sub-culturing events during this acclimation period varied depending on growth rates of cultures under the different light levels. Under ML and HL conditions both strains were sub-cultured on days 4, 10 and 14. Under LL only M22 was sub-cultured once on day 14.

Sampling was conducted regularly over a period of 47 days to obtain a time series of the physiological changes under the different PFDs in nutrient replete conditions followed

by increasingly nutrient limiting conditions. Cultures, grown under the same PFD, were randomly redistributed after each sampling accompanied by measurements of irradiance to avoid shading effects between the flasks.

Cell counts and morphological examination

Culture aliquots of 15 mL were collected and preserved by adding 300 μL of a 1:1 mixture of 37% formalin (containing 10 to 15% methanol) and glacial acetic acid. Cell counts and cell size measurements were performed using a Coulter Multisizer^{TM} II (Beckman Coulter Ltd., Luton, UK) fitted with a 200 μm orifice tube. Size calibrations were performed using 30 μm latex particles (L30 standard, Beckman Coulter). Three aliquots of 2 mL were measured in narrow range mode from 7 to 60 μm. When coincidence values of undiluted cultures exceeded recommended limits by the manufacturer, samples were diluted with Isoton (Beckman Coulter). Cell numbers were determined using the software Multisizer AccuComp$^{®}$ version 1.19 (Beckman Coulter). Morphological examinations of cell types and manual cell counts were performed regularly in sub-samples of 200-1000 μL under the microscope using a Sedgwick-Rafter chamber. The manual counts functioned as a control for the accuracy of the Coulter Counter measurements and showed a good correlation. Growth rates ($\mu \cdot d^{-1}$) were calculated using the equation $\mu = (d_2 - d_1)^{-1} \cdot \ln (N_2/N_1)$ where d = day and N = cell density (Mouget et al. 1999, Tremblin et al. 2000). To test for statistically significant differences between strain and light specific growth rates the means of independent experiments were compared using a two-tailed t-test with a significance level of $\alpha = 0.05$.

FRR fluorometry

A Chelsea Instruments FAST^{tracka} I FRR fluorometer (Chelsea Technology Group, West Molesey, UK), configured for bench-top use, was used to acquire ST fluorescence transients for the determination of minimum (*Fo*) and maximum (*Fm*) fluorescence yields, PS II effective absorption ($\sigma_{PS\ II}$) and minimum turnover time of PS II photochemistry. The instrument configuration was set to provide 100 saturation flashes per sequence with a saturation flash duration of 4 μs, followed by 20 relaxation flashes of 4 μs, 120 μs apart. Sleep time between acquisition pairs was set to 60 ms. All FRRF measurements were made 7 to 9 hours into the light cycle. Depending on cell density

and nutrient status most samples had to be diluted to avoid sensor saturation. Dilutions were performed using the same batch of filtered, autoclaved sea water that was used to prepare the culture medium. The diluted samples were split into two sub-samples, one was used directly for measurements without dark relaxation, and the other half was stored in the dark for 20 min before measurements were taken. PS II photochemical efficiency was determined as $(Fm - Fo)/Fm$ $(= Fv/Fm)$. There were no fundamental differences between the direct measurements and the measurements after dark relaxation and only the later measurements are shown as averages of five iterations ± SD of two independent experiments. Statistical analyses were performed in SigmaStat 3.0 (Systat Software, San Jose, CA). Based on the means of duplicate experiments, a two way ANOVA, with light condition and time as factors, was performed to test the effects of PFD on photosynthetic efficiency over time and to compare the effects between the two strains M22 and LP2810. Pairwise multiple comparisons were performed using the Holm-Sidak method with an overall significance level of $\alpha = 0.05$.

Pigment analysis

Chlorophyll a and peridinin concentrations of *L. polyedrum* do not oscillate over the circadian cycle, but *ß–carotene* concentrations change on a circadian cycle (Hollnagel et al. 2002). Samples for pigment analysis were therefore always collected 3-4 hours into the light cycle to ensure reproducibility. 5 ml of culture was filtered onto a 25 mm GF/F filter (Whatman, Maidstone, UK) and immediately frozen at -80°C until analysis. All cultures were sampled in triplicates. Pigments were extracted from the filters on ice with 3 ml of 90% acetone and sonication for 30 seconds. Filter debris were separated by centrifugation at 14000 g for 10 minutes at 4°C. The pigment-acetone solution was filtered through a 0.22 μm GF/F filter cartridge (Whatman) into brown 3 ml glass bottles and immediately transferred to the HPLC auto sampler which was maintained at 4 °C. Pigment analysis was performed using a ThermoFinnigan Spectra HPLC System with a Thermo Separations AS3000 autosampler, Thermo Separations UV6000 and diode array absorbance detector and Chromquest 4 analytical software (Thermo Electron Inc., San Jose, CA, USA). The equipment was configured after the method described by Barlow et al. (1997). Calibration was done using external standards. Chlorophyll a standards were purchased from Sigma (UK). All other pigment standards were from the DHI Water and Environment Institute (Hørsholm, Denmark). Pigment peaks were identified by comparison with retention times and absorption spectra of

known standards (Jeffrey et al. 1997). Peak areas of pigments were integrated with the Chromquest 4 analytical software and were used to calculate pigment concentrations in $\mu g \cdot mL^{-1}$. The following pigments were recorded: Chlorophyll a, chlorophyll c_2, peridinin, β-carotene and diadinoxanthin.

Samples for carbon analysis were collected parallel to the sampling for pigment analysis. 5 ml aliquots of culture were filtered through 8 mm pre-combusted GF/F filters (Whatman). Filters were dried over night at 40°C and stored in a dessicator until analysis. Filters were folded and placed in pre-combusted tin-foil capsules. Particulate carbon analysis was performed using an Elemental Micro Analyser (EA1108, Carlo-Erba). Calibration of the instrument was performed using a CEF external standard. Blanks (tin-capsules with pre-combusted filters) were run routinely. The carbon content was calculated based on peak areas of standards and triplicate samples.

Pigment concentrations in $\mu mol/\mu g$ carbon were calculated by converting the pigment concentrations determined by HPLC ($\mu g \cdot mL^{-1}$ culture) into molar concentrations based on the molecular weights of the different pigments (Jeffrey et al. 1997) followed by normalization to carbon content of the samples. Statistical analyses were performed in SigmaStat 3.0. Based on the means of duplicate experiments, a two way ANOVA, with light condition and time as factors, was performed to test the effects of PFD on pigment concentrations over time. Pairwise multiple comparisons were performed using the Holm-Sidak method with an overall significance level of $\alpha = 0.01$.

Amplification of the ITS1-5.8S-ITS2 region of the rRNA gene

DNA extractions were prepared with a DNeasy™ Plant Maxi Kit (QIAGEN, Crawley, UK). The concentrations and the quality of DNA extracts were assessed by gel electrophoresis and spectophotometrically using a ND-1000 spectrophotometer (NanoDrop™, Wilmington, USA). Based on the *L. polyedrum* rRNA gene sequence AF377944 primers had been designed previously to amplify a 681 bp product including the complete 535 bp of the ITS1-5.8S rDNA-ITS2 region (Frommlet and Iglesias-Rodríguez 2008). The forward primer (5'GGAAGGAGAAGTCGTAACAAGG 3') and reverse primer (5'TCGCCGTTACTGAAGGAATC 3') were obtained from MWG (Ebersberg, Germany). PCRs were performed in 15 μl reactions on a PTC-200 gradient thermocycler (GRI). The PCR reagents were: Amplitaq Gold (5 U/μL), 10 x Gold buffer, MgCl$_2$ (25 mM) (Applied Biosystems, Warrington, UK); dNTPs (10 mM each)

(New England Biolabs, Hitchin, UK). PCR reactions contained 50 ng of DNA template and final concentrations of 1x Amplitaq Gold buffer, 1.5 mM MgCl$_2$, 0.625 units of Amplitaq Gold, 0.2 mM of dNTPs (each) and 1 μM of each primer. Amplification started with an initial denaturing step at 94ºC for 8 minutes followed by 35 cycles of denaturing at 94ºC for 30 s, annealing at 58ºC for 60 s, elongation at 65ºC for 60 s and a final elongation step at 65ºC for 2 min. The PCR products were gel purified (QIAGEN) and sequenced in forward and reverse direction by Geneservice (Cambridge, UK) using the above forward and reverse primers.

4.3 Results

4.3.1 Strain characterisation

The two strains M22 and LP2810 were morphologically identified as *Lingulodinium polyedrum* (F. Stein) J.D. Dodge. The morphological species identification was confirmed by sequencing the complete ITS1-5.8S-ITS2 (ITS) sequence of the rRNA gene of both strains. The sequences of M22 and LP2810 were identical and shared between 99.4 to 100% similarity with other *L. polyedrum* ITS sequences in GenBank (Fig. 4.1).
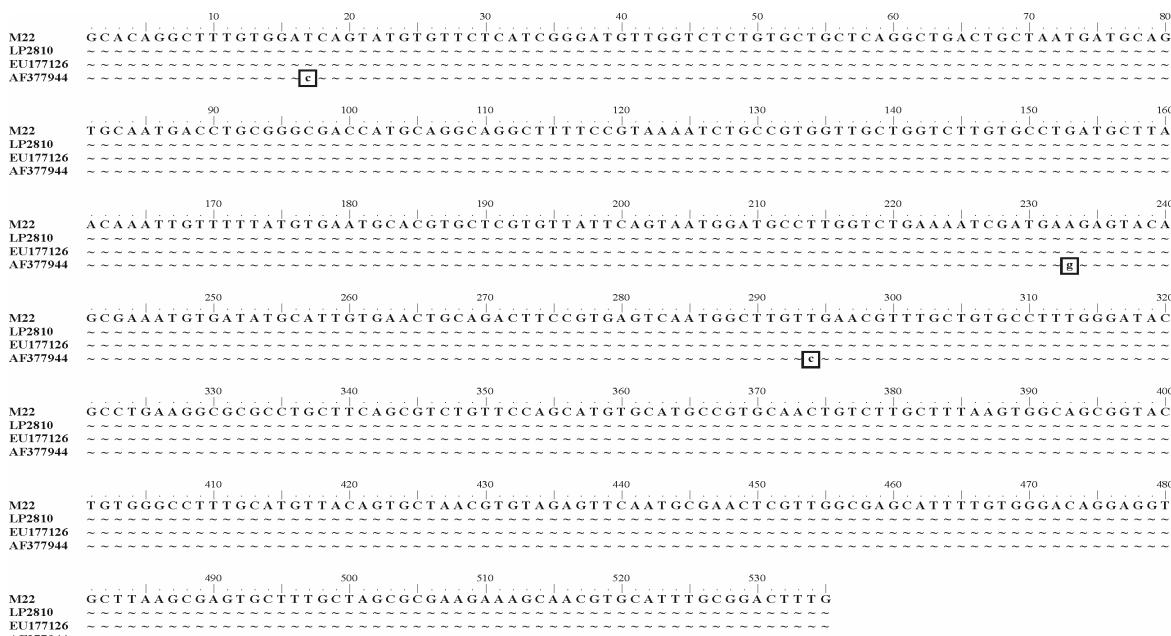
FIG. 4.1 Multiple alignment of the complete ITS1-5.8S-ITS2 (ITS) sequences of M22 and LP2810 with two other ITS sequences from GenBank. Apart from three substitutions in the sequence AF377944 (base pair position 17, 233 and 294) all four sequences were identical.

Crossing experiments between M22 and LP2810 resulted in the successful fusion of gametes to form planozygotes (results not shown), showing the reproductive compatibility of the two strains and further supporting their affiliation to the same species. Differences in colouration between the two strains were apparent by visual examination of cultures with the naked eye; LP2810 appearing overall darker in colour than M22. Quantitative pigment analysis of ML-acclimated cultures showed that LP2810 contained significantly higher concentrations of chl a, peridinin and diadinoxanthin compared to M22, while chl c_2 and $ß$-carotene concentrations were comparable between the two strains (Fig.4.2).
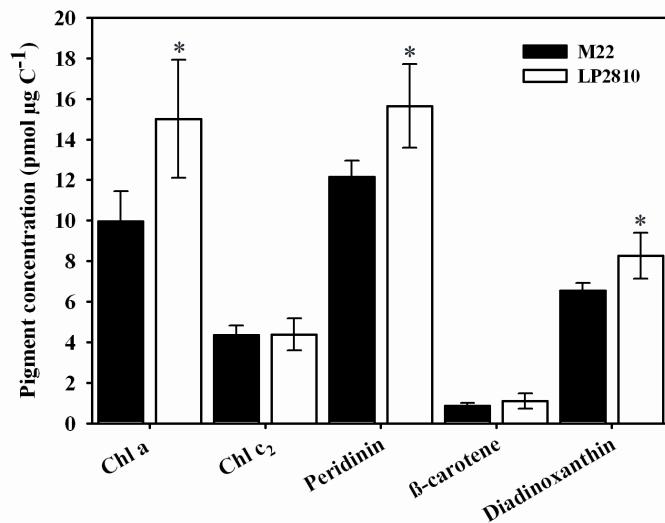
FIG. 4.2 Pigment concentration of the Lingulodinium polyedrum strains M22 and LP2810 acclimated to ML (n = 6). Error bars equal ± 1 SD. * denotes a statistically significant difference (p <0.05).

4.3.2 Light acclimation of M22 and LP2810 under nutrient-replete conditions

Growth

Growth of the strains M22 and LP2810 during a twenty day acclimation period to HL, ML and LL are shown in figure 4.3 A-C, respectively. When cell densities approached 3500 cells · mL^{-1}, cultures were diluted to 500 cells · mL^{-1}. Under ML and HL this was performed on days 4, 10 and 14. Under LL only M22 was sub-cultured once on day 14. Growth of both strains resulted in approximately 9-10 generations under HL and ML. Growth under LL was much reduced in M22 and resulted in approximately 2 generations while growth of LP2810 was reduced to almost zero.
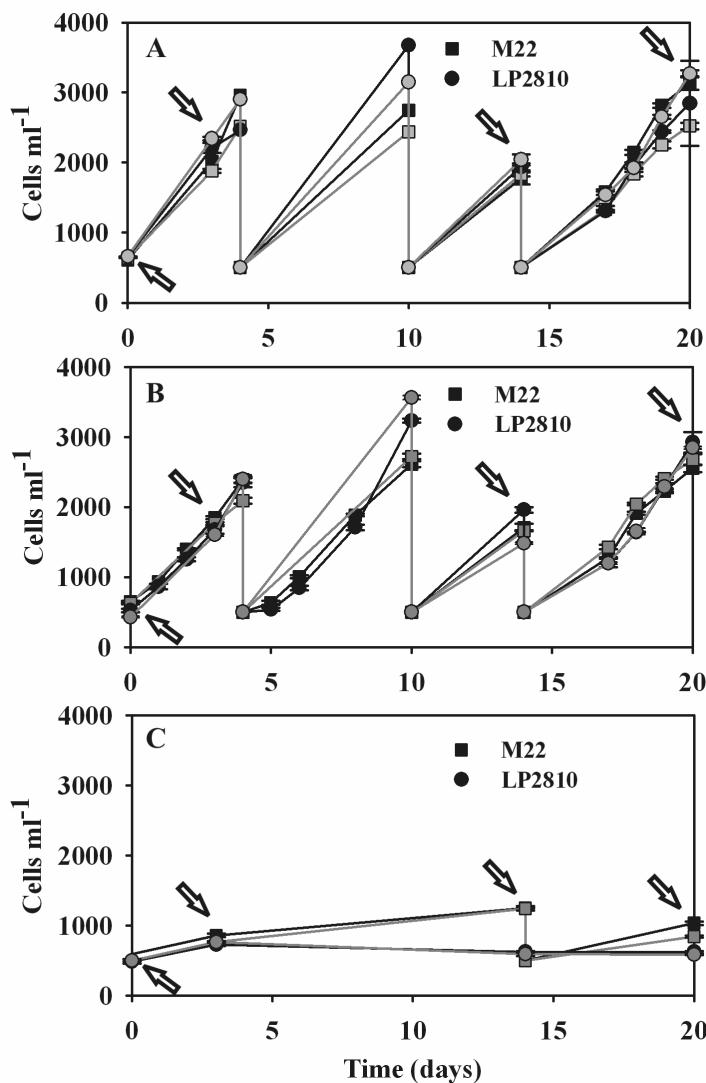
FIG. 4.3 Growth of the *L. polyedrum* strains LP2810 and M22 during a twenty day acclimation period to (A) HL, (B) ML and (C) LL. Arrows indicate the days when samples for pigment and carbon analysis were collected. The ML and HL cultures were sub-cultured on days 4, 10 and 14.

Photochemical efficiency of PS II (Fv/Fm)

The photochemical efficiency of photosystem II at the start of the experiments (day zero), with all cultures being exposed to ML, was 0.42 for M22 and 0.45 for LP2810, differing significantly ($p < 0.001$) between the two strains (Fig. 4.4 A and 4.4 B). Within the first 24-48 h after transfer to the different light conditions Fv/Fm changed to new light-specific values. Under HL Fv/Fm decreased significantly ($p < 0.001$) in both strains to 0.36 for M22 and to 0.42 for LP2810, and increased in both strains significantly ($p < 0.001$) under LL to 0.52. Fv/Fm under HL also differed significantly between the two strains ($p < 0.001$) but not under LL. As expected, Fv/Fm of the control cultures under ML did not change significantly in either of the strains (Fig. 4.4 A and 4.4 B). After the

change to light-specific values within the first 48 h, Fv/Fm remained fairly stable. However, over the course of the acclimation period, Fv/Fm of both strains under HL slowly recovered to values that were not significantly different from those of ML cultures on day twenty.
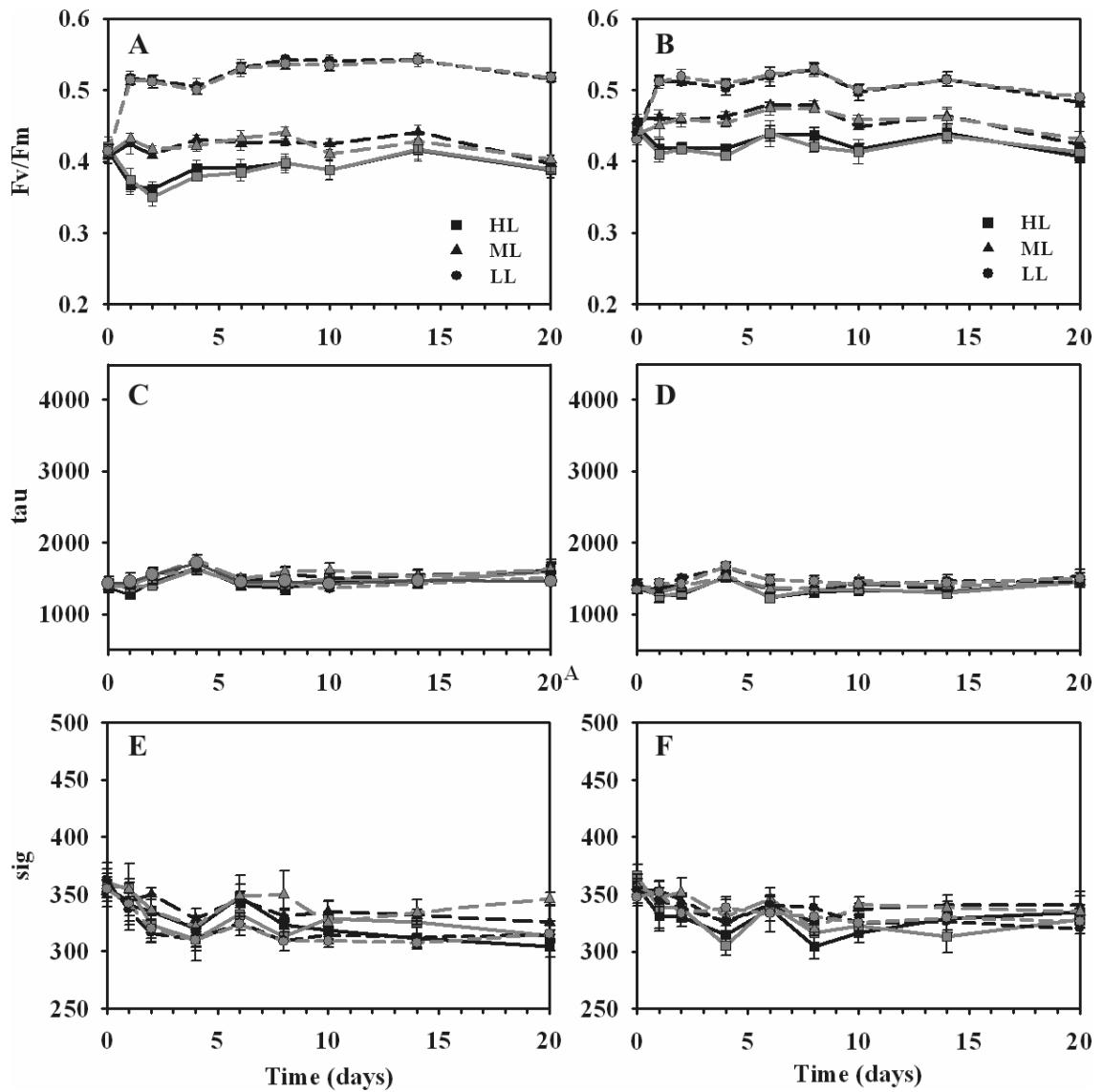
FIG. 4.4 Time series of Fv/Fm (A and B), τ (tau) (C and D) and σ (sigma, sig) (E and F) of the two *Lingulodinium polyedrum* strains M22 (A, C, E) and LP2810 (B, D, F) during a twenty day acclimation period to high light (HL), medium light (ML) and low light (LL). Each graph shows the results of duplicate experiments under each light condition shown in black and grey. The colour coding of duplicate experiments is consistent with figures 4.3 and 4.7 to enable the direct comparison of the different parameters measured within each experiment. All data points are means of five iterations ± 1 SD. Error bars are shown for all data points but are sometimes hidden by the data point symbols.

Minimum turnover time of PS II photochemistry (τ) and the effective cross-section of PS II, $\sigma_{PS\,II}$

The photophysiological variables τ (Fig. 4.4 C and 4.4 D) and $\sigma_{PS\,II}$ (Fig. 4.4 E and 4.4 F) did not change in either of the two strains during the acclimation period, nor did the absolute values of both τ and $\sigma_{PS\,II}$ show strain specific differences between M22 and LP2810.

Changes of pigment concentrations

Changes in pigment concentrations during the twenty day acclimation period to different PFDs are shown in figure 4.5. On day three of the acclimation period, chl a and peridinin concentrations in both strains were significantly higher under LL than under ML. In LP2810 also the concentration of chl c_2 was significantly higher under LL compared to ML and M22 showed significantly lowered concentrations of peridinin under HL. Diadinoxanthin concentrations in M22 were unaffected by PFD whereas LP2810 displayed significantly lower levels of diadinoxanthin under LL. On day fourteen, chl a and peridinin concentrations under LL had further increased in M22 but in LP2810 the initially increased values of these pigments on day three had declined back to levels that were not significantly different from the two higher PFDs. Similarly, the chl c_2 concentrations of LP2810 LL cultures had dropped back to levels that were comparable to those of the cultures under ML and HL. In contrast, the chl c_2 concentration in M22 was now significantly higher under LL and lower under HL compared to the ML cultures. The peridinin concentrations in M22 were fairly stable between day three and day fourteen, remaining significantly reduced under HL and elevated under LL. Also the diadinoxanthin concentrations in M22 stayed unchanged between day three and day fourteen and remained comparable between the different PFDs. In LL cultures of LP2810 the concentrations of diadinoxanthin remained significantly lowered. The pigment changes between day fourteen and day twenty where generally smaller than between day three and day fourteen. Chlorophyll a and peridinin concentrations in M22 remained significantly elevated under LL and both pigments were now significantly lower under HL compared to ML. In LP2810 these pigments did not differ significantly between ML and LL. Chlorophyll c_2 and peridinin concentrations in LP2810 under HL were however significantly lowered. In M22 chlorophyll c_2 concentrations remained significantly elevated under LL and reduced

under HL. Diadinoxanthin concentrations remained comparable between all PFDs in M22 and remained significantly lowered in LP2810 under LL.
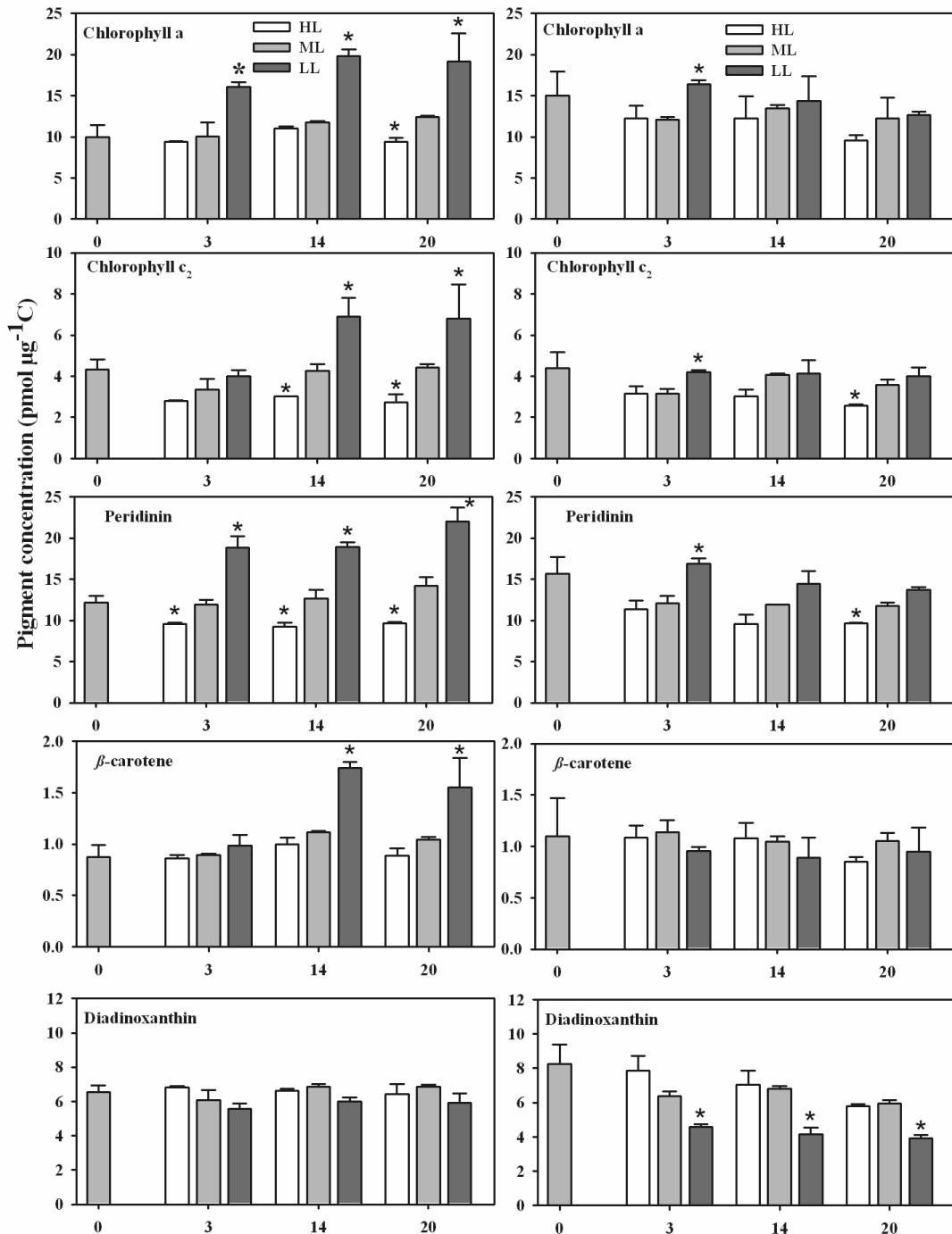
FIG. 4.5 Time series of chlorophyll a, chlorophyll c2, β-carotene, peridinin and diadinoxanthin concentrations during a twenty day acclimation period of M22 (left) and LP2810 (right) to high light (HL), medium light (ML) and low light (LL). Error bars equal ± 1 SD of duplicate measurements. * denotes a statistically significant difference of pigment concentrations compared to the corresponding ML control (p <0.01).

4.3.3 Growth of light acclimated strains in batch cultures

Growth rates

Growth rates of the light acclimated strains M22 and LP2810 differed under all PFDs (Fig. 4.6). Under ML, LP2810 (μ=0.27 d^{-1}) grew significantly faster than M22 (μ=0.21 d^{-1}) (p <0.05). Also under HL, LP2810 (μ=0.31 d^{-1}) achieved slightly higher growth rates than M22 (μ=0.29 d^{-1}) but this difference was not statistically significant. Contrastingly, under 30 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} and under the lowest PFD (10 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), M22 (μ=0.19 and 0.10 respectively) grew significantly faster than LP2810 (μ=0.15 d^{-1} and 0.001 d^{-1} respectively) (p <0.05). Growth rates of LP2810 under ML and HL did not vary significantly, showing that growth was light saturated above ML. Contrastingly, growth of M22 was not light saturated under ML as the growth rate of M22 under HL was significantly higher than under ML (p <0.05).
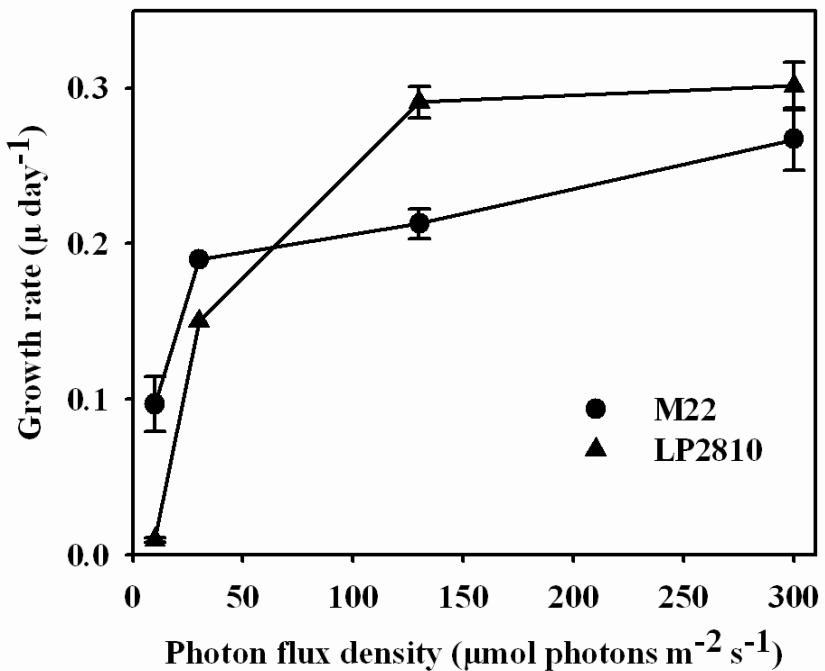
FIG. 4.6 Maximum growth rates (μ) of the light acclimated strains M22 and LP2810 under four different PFDs. Data points at 130 and 300 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} are means of triplicate experiments. Data at 10 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} are means of duplicate experiments and data at 30 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} is from a single experiment. Error bars of data at 10, 130 and 300 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} equal ± 1 SD.

Photosynthetic efficiency of PS II (Fv/Fm)

The Fv/Fm in batch cultures decreased in both strains under ML and HL, probably due to nutrient limitation (Fig. 4.7 A and 4.7 B). Fv/Fm of LP2810 in both HL and ML decreased at a very similar rate and reached a minimum of 0.30 on day 39. Contrastingly, Fv/Fm in M22 decreased earlier under HL than under ML. The stability of Fv/Fm at a higher level under ML was followed by a more rapid decline to values comparable to those under HL of approximately 0.27. Under LL conditions Fv/Fm of both strains remained stable between 0.5 and 0.54 for the duration of the experiments.
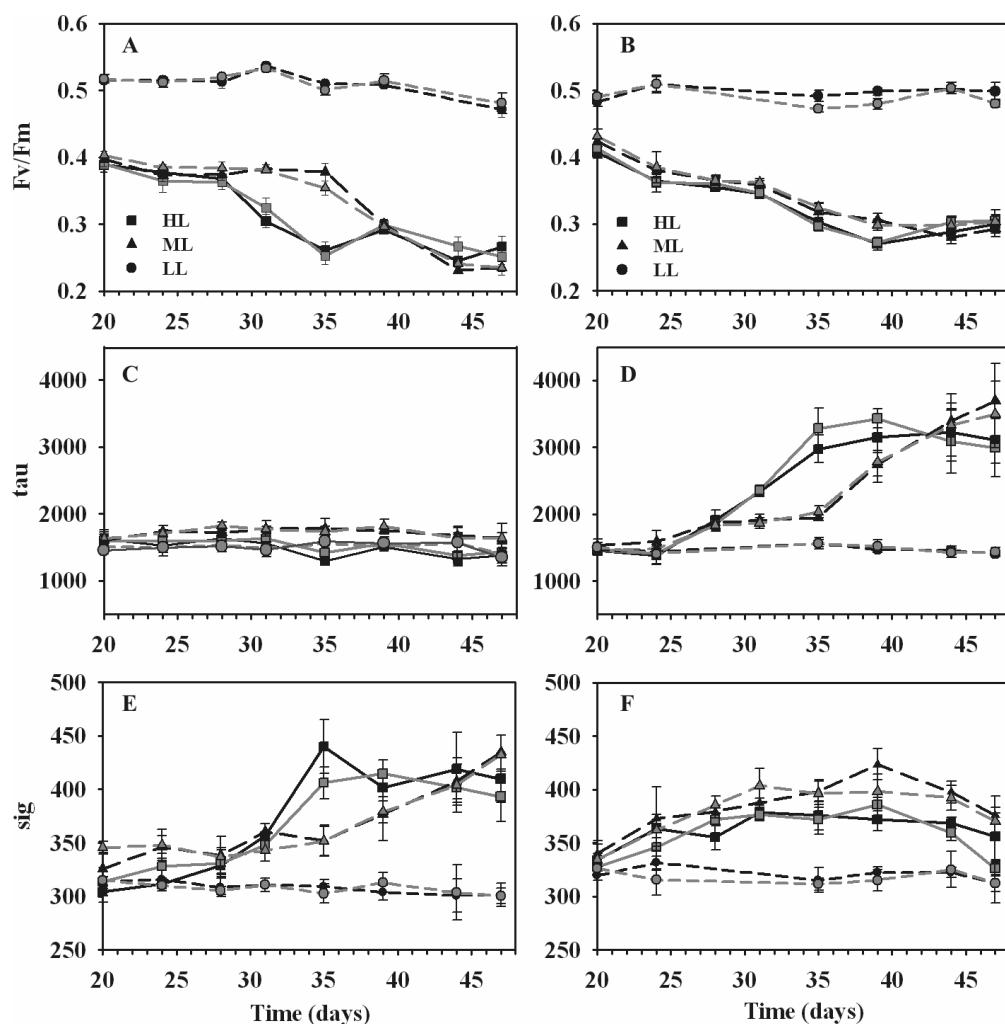


FIG. 4.7 Time series of Fv/Fm (A and B), τ (tau) (C and D) and σ (sigma, sig) (E and F) of the two *Lingulodinium polyedrum* strains M22 (A, C, E) and LP2810 (B, D, F) during 27 days in batch cultures under high light (HL), medium light (ML) and low light (LL) following the acclimation period of 20 days shown in figure 4.4. Each graph shows the results of duplicate experiments under each light condition shown in black and grey. The colour coding of duplicate experiments is consistent with figures 4.3 and 4.4 to enable the direct comparison of the different parameters measured within each experiment. All data points are means of five iterations ± 1 SD. Error bars are shown for all data points but are sometimes hidden by the data point symbols.

Minimum turnover time of PS II photochemistry (τ)

The values of τ did not change in M22, values remaining tightly grouped between 1200 and 1700 and showing no correlation with PFD (Fig. 4.7 C and 4.7 D). In contrast, τ in LP2810 increased markedly starting from day 24. The rapid increase of τ under HL led to peak values of approximately 3300 on day 39. Under ML, τ increased initially more slowly but the maximum values were comparable to the HL treatment. Under LL conditions τ remained unchanged in both strains throughout the experiments.

Effective absorption cross-section of PS II ($\sigma_{PS\,II}$)

The values of $\sigma_{PS\,II}$ increased in both strains under ML and HL conditions, indicating an increase in antenna size (Fig. 4.7 E and 4.7 F). A trend towards a quicker rise of $\sigma_{PS\,II}$ in LP2810 was observed but maximum values were higher in M22. In both strains was a tendency for $\sigma_{PS\,II}$ being lower in HL cultures compared to ML cultures. Further, $\sigma_{PS\,II}$ in ML and HL cultures of M22 remained at a higher level throughout the experiments while $\sigma_{PS\,II}$ in LP2810 showed a tendency for reduced values from day 39 onwards. Under LL conditions, $\sigma_{PS\,II}$ remained unchanged in both strains throughout the experiments.

4.4 Discussion

4.4.1 Species identification

The biological and ecological interpretation of physiological differences, such as those between the two strains M22 and LP2810, depend critically on whether these two strains belong to same species. The identification of a species is however not trivial, since the different species concepts, e.g. the morphological, biological, genetic species concept follow their own definitions of a 'species' and all species concepts have certain limitations (see review by Manhart and McCourt 1992). For example, molecular studies repeatedly revealed cryptic or pseudo-cryptic speciation within phytoplankton morpho-species, showing that the morphological species concept is not always sufficient to distinguish between different phytoplankton species (Montresor et al. 2003, Sáez et al. 2003, Sarno et al. 2005, Amato et al. 2007). Since cryptic or pseudo-cryptic species do

not share a common gene pool, physiological differences within a morpho-species should therefore not be interpreted as intraspecific diversity, unless there is evidence that the entities build a reproductively isolated group and share a common gene pool. In the present study this issue was addressed by employing three different approaches. First, the two strains M22 and LP2810 were identified morphologically as *Lingulodinium polyedrum* (F. Stein) J.D. Dodge. Second, the morphological species identification was confirmed by sequencing the ITS1-5.8S-ITS2 region of the rRNA gene of both strains. Third, crossing experiments showed that the two strains are reproductively compatible. Hence, the strains M22 and LP2810 belong to the same morphological, biological and genetic species, which suggests that their differences in pigmentation and light acclimation represent genuine intraspecific physiological diversity.

4.4.2 Differences in light acclimation mechanisms of M22 and LP2810

The optimization of photosynthesis during light acclimation involves the modification of components of the light and dark reaction such as: pigment concentration and/or composition, the number of photosynthetic units (PSUs) per cell, the optical cross-section of PSUs, and photosynthetic carbon fixation parameters (Falkowski and La Roche 1991, Geider et al. 1998). A common type of light acclimation is based on the up- and down-regulation of photosynthetic pigments (Richardson et al. 1983, Raven and Geider 2003). Low light acclimation of previously high light-acclimated *L. polyedrum* cultures, for example, results in a doubling of chl a, chl c and peridinin concentrations (Prézelin and Sweeney 1978, Boczar and Prézelin 1987). In the present study, M22 and LP2810 acclimated to LL and HL conditions employing distinctly different mechanisms. The strain M22 up-regulated chl a, chl c, peridinin and *ß*-carotene under LL, down-regulated the same pigments under HL, and hence reacted to changes in PFD as expected based on previous data. The strain LP2810 on the other hand mainly down-regulated diadinoxanthin under LL and up-regulated diadinoxanthin under HL. Previous studies on *L. polyedrum* did not include measurements of diadinoxanthin and so a direct comparison of results is not possible. In diatoms, the photoprotective function of diadinoxanthin has been shown to depend on its pool size, and diatoms increase the concentration of diadinoxanthin under HL (Willemoes and Monas 1991, Arsalane et al. 1994, Olaizola and Yamamoto 1994). The observed up-regulation of diadinoxanthin in

LP2810 under HL may therefore have an important photoprotective function. Since photoprotective and light harvesting pigments compete for the absorption of light, an increased pool size of diadinoxanthin may increase photoprotection not only through non-photochemical quenching but also by reducing the amount of light energy that is transferred to the photosystems (Fujiki and Taguchi 2001). *Vice versa*, the observed down-regulation of diadinoxanthin in LP2810 under LL may therefore increase the amount of light that is available to drive photosynthesis. Taken together, these results suggest that the diadinoxanthin-based light acclimation of LP2810 may represent an effective alternative to the 'strategy' of up- and down-regulating photosynthetic pigments as observed in M22 and previously described in *L. polyedrum* (Prézelin and Sweeney 1978, Boczar and Prézelin 1987). The effectiveness of the diadinoxanthin-based acclimation mechanism is further supported by the fact that LP2810 achieved a higher photosynthetic efficiency (Fv/Fm) under ML and HL than M22 and achieved significantly higher growth rates than M22 under ML. Thus, under HL and ML the diadinoxanthin-based mechanism of LP2810 did not represent an inferior light acclimation 'strategy' compared to the conventional 'strategy' of M22. The acclimation mechanism of LP2810 seemed however less suitable to acclimate to LL (< 30 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}), leading to much reduced growth rates of LP2810 compared to M22.

The presented results support a classification of M22 as a strain that has advantages under low PFDs and LP2810 as a strain that seems to be better adapted to higher PFDs. However, growth rates under HL were not significantly higher in LP2810 compared to M22 and growth of LP2810 was light saturated at lower PFDs than that of M22. It is therefore possible that M22 may achieve higher growth rates than LP2810 under higher PFDs than used in this study. An important next step would be to study the acclimation of both strains to such light conditions. In case M22 would show higher growth rates under higher PFDs, the strain may have to be considered rather a light generalist than a low light adapted strain. *Vice versa*, LP2810 would then represent an opportunist or perhaps specialist for medium light conditions.

4.4.3 Time course of pigment changes during light acclimation

Light harvesting pigments in *L. polyedrum* are arranged in the water soluble peridinin-chl a-protein (PCP) and up to five pigment protein complexes bound in the thylakoid membrane. The pigment composition of pigment protein complexes and their relative

abundance depend on the light history of the cell (Boczar and Prézelin 1987, Knoetzel and Rensing 1990). Pigment protein complexes were not characterised in the present study and the limited number of data points throughout the light acclimation phase does not allow the determination of an exact sequence of pigment changes. However, current results showed that the concentrations of the different pigments did not change simultaneously, which gives an indication of the timing of some of the processes during light acclimation.

For example, during low light acclimation of M22, chl a and peridinin concentrations increased earlier than those of chl c_2 and β–carotene. Based on previous data on *Glenodinium* sp. (Prézelin 1976) and *L. polyedrum* (Prézelin and Sweeney 1978) that showed that most of the peridinin is bound in PCP, the change of peridinin concentrations early into the acclimation phase indicates that PCP is up-regulated rapidly during low light acclimation. Since chl a and peridinin concentrations remained approximately equimolar during the increase of both pigments, but PCP contains peridinin and chl a in a ratio of 4:1, other chl a-protein complexes must have also been up-regulated quickly upon LL-exposure.

As mentioned above, light acclimation in *L. polyedrum* also involves changes in the up to five membrane bound pigment protein complexes (Boczar and Prézelin 1987, Knoetzel and Rensing 1990). Together, these different complexes contain essentially the entire pool of chl c_2, of which the largest amount is bound in complex II (Boczar and Prézelin 1987). The delayed rise of chl c_2 concentrations compared to chl a and peridinin therefore suggests that, during LL-acclimation, membrane bound chl a/c protein complexes are up-regulated slower than PCP. Since only membrane bound pigment protein complexes contain β–carotene (PCP is devoid of β–carotene), the simultaneous rise of β–carotene with chl c_2 further supports the notion that membrane bound chl a/c protein complexes are up-regulated after PCP.

Also observed was a statistically significant reduction of diadinoxanthin under LL and a tendency for an increased concentration under HL early into the light acclimation phase. The rise of diadinoxanthin under HL was not statistically significant, but was consistent with results of an earlier trial experiment (data not shown). As mentioned above, diadinoxanthin has important photoprotective functions in the xanthophyll cycle, which take effect within minutes of high light exposure (Long et al. 1994). The early increase of diadinoxanthin under HL therefore suggests that the xanthophyll cycle is an

important mechanism during early stages of high light acclimation in *L. polyedrum*. The reduction of diadinoxanthin back to levels comparable to that of ML cultures on day fourteen implies further, that during the course of light acclimation the xanthophyll cycle becomes less important and may get replaced or complemented by other mechanisms. The slow but steady recovery of Fv/Fm in both strains during HL acclimation supports the notion that, additional to the changes in photosynthetic and photoprotective pigments, other, slower acclimation processes took place.

4.4.4 Light acclimation through changes in PSU size or PSU number?

A change of photosynthetic pigment concentrations and/or composition is a common response of phytoplankton to changes in PFD (Richardson et al. 1983, Raven and Geider 2003). The regulation of photosynthetic pigments can be a result of changes in the number of photosynthetic units (PSUs) per cell, the average size of the PSUs, or both (Falkowski and LaRoche 1991). The established theory is that *L. polyedrum* acclimates to changing PFDs by regulating PSU size (Prézelin and Alberte 1978, Prézelin and Sweeney 1978, Perry et al. 1981). A change in PSU size is often accompanied by changes in pigment molar ratios (e.g. Prézelin 1976), not so in *L. polyedrum* though, which is thought to be the result of a balanced change of both LHC components (PCP and chl a/c-proteins) (Prézelin and Sweeney 1978).

Using FRRF, the effective absorption cross-section of PS II ($\sigma_{PS\ II}$) was measured, which depends on PSU size, the efficiency of energy conversions and the pigment composition (Kolber et al. 1998). The parameter $\sigma_{PS\ II}$ therefore gives an indication of the PSU size of PS II, but is not a direct measure of the PSU size of PS II (Kolber et al. 1998). In both strains, $\sigma_{PS\ II}$ was remarkably stable during nutrient-replete exposure to different PFDs. As discussed earlier, light acclimation of LP2810 was not based on changes in photosynthetic pigment concentrations. Hence, the stability of $\sigma_{PS\ II}$ in LP2810 is in agreement with the pigment data of this strain. Following, the focus is therefore mainly on the strain M22. The stability of $\sigma_{PS\ II}$ in M22 suggests that the pigment changes in this strain during light acclimation were achieved by a change of PSU number rather than PSU size. Current results are therefore in conflict with the established theory that *L. polyedrum* regulates pigment levels mainly through changes in PSU size. The discrepancy between the established model and current results may partly be explained by a different nutrient status of the tested cultures. The established model

of light acclimation in *L. polyedrum* was based on cultures that were harvested between mid exponential (Prézelin and Sweeney 1978) and late exponential phase (Prézelin and Haxo 1976, Prézelin and Alberte 1978, Perry et al. 1981). As shown by the decline of Fv/Fm early into the second half of exponential growth (Fig. 4.4 A/B), nutrient availability affects the photophysiology of *L. polyedrum* already during early stages of batch culturing and hence previous studies may have reported light acclimation of cultures that were in an early stage of nutrient limitation.

In this case, current measurements of $\sigma_{PS\ II}$ during increasingly nutrient limiting conditions ought to be in agreement with previous results, but this is also not the case. Whereas previous studies reported a decrease of PSU size under HL (Prézelin and Alberte 1978, Perry et al. 1981), measurements of $\sigma_{PS\ II}$ in this study during nutrient-deplete conditions indicated an increase of PSU size under HL and ML. A possible explanation for this discrepancy could lie in the different methods that were applied to infer the PSU size of PS II. Previous studies used the ratio of accessory chlorophyll to chlorophyll in the reaction centre of photosystem I (chl a/P $_{700}$) to infer PSU size of PS II (Prézelin and Alberte 1978, Perry et al. 1981). This method relies on the assumption that chlorophyll ratios of PS I and PS II are equal and constant, which is however not always the case (Prézelin 1987). FRRF based measurements give direct information about the effective absorption cross-section of PS II ($\sigma_{PS\ II}$), but since this parameter is also influenced by the efficiency of energy conversions and the pigment composition, $\sigma_{PS\ II}$ is also not a direct measure for PSU size (Kolber et al. 1998). Therefore, both approaches only allow an indirect inference of PSU size of PS II and depend on reply on different assumptions.

Interestingly, a recent study by Suggett et al. (2007) showed that the effective absorption cross-section of PSI and PS II in *Emiliania huxleyi* can change independently and in opposite directions during light acclimation. If similar mechanisms are involved in the light acclimation of *L. polyedrum*, PSU size of PS I may decrease at the same time that PSU size of PS II increases, which may explain the seemingly discrepancy between results of this study and previous observations in *L. polyedrum*.

4.4.5 Photosynthetic electron transport under nutrient limitation

The time constant for photosynthetic electron transfer on the acceptor side of PS II (τ_{Qa}) reflects the minimum turnover time for electron transport (Kolber et al. 1988). The

stability of τ_{Qa} during nutrient replete light acclimation of both strains indicated that exposure to different PFDs did not affect the photosynthetic electron transport. In contrast, exposure to HL and ML under increasingly nutrient limited conditions resulted in a dramatic increase of τ_{Qa} in LP2810, indicating a slowdown of electron transfer, but had no effect on M22. For a detailed interpretation of these results we do not have enough data at this stage and the difference of τ_{Qa} between the strains merely shows that the photophysiology of the two strains under nutrient stress is markedly different. In order to determine the cause(s) for the observed difference between the two strains M22 and LP2810, measurements of pigment changes and parameters of the dark reaction during nutrient limitation may give important clues.

4.4.6 Ecological Implications

If the results of this study were to be simply extrapolated to the environment, one would expect that in a low light environment the strain M22 would very quickly out-compete LP2810 and *vice versa* under medium light. However, aquatic environments exhibit a wide range of light intensities over different temporal and spatial scales, and it appears that, among other processes, it is this variability of the environment that maintains genetic diversity within microalgal populations and prevents individual clonal lineages from becoming numerically dominant (Bell 1997, Rynearson and Armbrust 2005).

An assessment of the relevance of the described intraspecific differences between M22 and LP2810 with respect to the ecophysiology and population ecology of *L. polyedrum* in its natural environment is also very difficult because: 1) intraspecific differences between the two strains may span over numerous other, untested physiological characteristics; 2) especially in a motile phytoplanktonic group like the dinoflagellates, the optimization of photosynthesis is not limited to photophysiological changes but can also include behavioural mechanisms such as vertical migration (e.g. Heaney and Eppley 1981), which are difficult to study in the laboratory; and 3) it is not certain whether the two strains coexist and compete for resources in the same habitat or whether they occupy different ecological niches. The pronounced differences in light requirements and light acclimation 'strategies' between the two strains may, for example, be reflected in temporally and/or spatially distinct distributions of the two strains. In this context the origin of the two strains is of interest. Between the two locations, from which M22 and LP2810 were isolated, lies a sharp hydrographical and

biological boundary at Point Conception (U.S. GLOBEC 1994, Strub and James 2000). Surface waters north of this boundary are typically much higher in nutrients than surface waters to the South, which is also reflected in a higher productivity and stronger light attenuation north of Point Conception (Garrison 1976, Eppley et al. 1978, U.S. GLOBEC 1994). The hydrographical boundary at Point Conception may therefore restrict genetic exchange and/or the distinct nutrient and light regimes of the two regions may strongly select for different genotypes. The recent development of a single cell microsatellite genotyping protocol for *L. polyedrum* (chapter 2; Frommlet and Iglesias-Rodríguez 2008) may provide the opportunity to genetically distinguish M22 and LP2810 and to study how the intraspecific differences between the two strains may affect the distribution of their respective clonal lineages over time and space in their natural environment.

Chapter 5

POPULATION DEVELOPMENT AND POPULATION GENETIC
DYNAMICS OF *LINGULODINIUM POLYEDRUM* (DINOPHYCEAE)
IN SOUTHERN CALIFORNIA: A STUDY EMPLOYING
MICROSATELLITE GENOTYPING OF SINGLE CELLS

Abstract

Previously, a method for the single cell-based microsatellite genotyping of the dinoflagellate species *Lingulodinium polyedrum* (F. Stein) J.D. Dodge was developed. In the present study this novel approach was employed in the first population genetic analysis of *L. polyedrum*. The aim was to assess the population genetic distance/differentiation between geographically and hydrographically distinct sampling sites of *L. polyedrum* in Southern California and to study how population genetic parameters including the abundance of individual genotypes change over time and space and in relation to environmental change, population development and sexual reproduction. Bloom samples of *L. polyedrum* were collected in the years 2003 and 2005. In 2004, sampling of non-bloom populations was mainly conducted in La Jolla Bay over a period of two months and samples were also collected from four other sites along the Californian Coast. While *L. polyedrum* did not reach bloom concentrations in 2004, signs of sexual reproduction and a storm event represented the opportunity to study the development and the genetic dynamics of *L. polyedrum* populations in the context of environmental change and life cycle dynamics. Genotyping of 648 individual cells resulted in the identification of 52 distinct genotypes and consistently high gene diversity within populations (0.7-0.93). An UPGMA dendrogram, based on Nei's unbiased genetic distance, did not show a correlation between genetic distance and geographic distance, nor did it show a direct effect of a sharp hydrographic boundary in the study area. The clustering of samples rather support the idea that population genetic exchange in the Southern Californian Bight is tied to water circulation patterns. However, the results also showed that genetic distance between samples from the same location can exceed that of samples from widely separated sites. Frequencies of individual genotypes further indicated that habitat structure and environmental change strongly affect the population genetic composition of *L. polyedrum*.

5.1 Introduction

Lingulodinium polyedrum (F. Stein) J.D. Dodge is a bloom-forming dinoflagellate species of marine coastal waters (Lewis and Hallett 1997). In Southern California, *L. polyedrum* blooms are common from April to November and are associated with calm, warm weather conditions (Allen 1946, Holmes et al. 1967, Eppley and Harrison 1975). The development of dinoflagellate blooms in coastal environments is governed by interactions of biological and physical processes over a broad range of spatial and temporal scales (Donaghay and Osborn 1997). Coastal upwelling and the stratification of surface waters during relaxation periods influence nutrient availability, the alongshore and cross-shore transport, and the accumulation or dissipation of phytoplankton blooms (e.g. Fraga et al. 1988, Franks and Anderson 1992, Pitcher et al. 1993, 1998). Smayda and Reynolds (2001, 2003) defined nine types of mixing-irradiance-nutrient habitats in the pelagic marine environment that select for specific dinoflagellate life-form types. According to this classification system, *L. polyedrum* is a typical bloom-forming dinoflagellate of the type V (upwelling relaxation taxa). Type V species are R-strategists (= ruderal), characterized by their tolerance to physical disturbance, and by physiological, morphological and behavioural adaptations that allow them to acclimate to changing environmental conditions and capture sufficient light energy for photosynthesis at relatively low irradiance levels (Smayda and Reynolds 2003).

Based on this knowledge on the principal environmental factors that govern the population dynamics of bloom-forming dinoflagellates, and an understanding of the adaptations that allow a species to flourish under certain environmental conditions, one may conclude that dinoflagellate population dynamics should be predictable. Lewis and Hallett (1997) suggested that based on the exceptional knowledge and understanding of *L. polyedrum*, the population ecology of this species ought to become close to being predictable. However, even the population dynamics of well studied dinoflagellate species remain unpredictable (Smayda, 1997b, Smayda and Reynolds 2001, 2003). According to Smayda and Reynolds (2001, 2003), the selection of species within specific dinoflagellate life-form types is stochastic, and modelling showed that the outcome of phytoplankton multispecies competitions for limiting resources was fundamentally unpredictable (Huisman and Weissing 2001). Predictability may

therefore be an unachievable goal in the study of phytoplankton population dynamics.

Nonetheless, an improved understanding of the principles at work in shaping phytoplankton populations dynamics can be achieved but depends greatly on the study of the complex interactions of biological and physical systems in their entireness. Two of the main challenges in the study of phytoplankton population ecology are: 1) to link the life cycle and its environmental control with the population dynamics of a species (Wyatt and Jenkinson 1997, Garcés et al. 2001) and 2) to understand the reciprocal interactions between intraspecific genetic and phenotypic diversity and the dynamics of phytoplankton populations. Previously reported were: The development of a novel approach that provides the molecular and technical tools for a population genetic study of *L. polyedrum* (chapter 2; Frommlet and Iglesias-Rodríguez 2008), new insights into the life cycle and its environmental control in *L. polyedrum* (chapter 3, Frommlet et al. 2008) and first data on intraspecific physiological variability in *L. polyedrum* (chapter 4). The aim of the present study was to employ the developed genotyping technique to assess the population genetic dynamics of *L. polyedrum* in Southern California over a range of different spatial and temporal scales. The main questions addressed in this study are: does the population genetic distance/differentiation of *L. polyedrum* in Southern California correlate with geographic distance? Is population genetic exchange in Southern California restricted by a strong hydrographic boundary in the region at Point Conception? How do natural populations of *L. polyedrum* in La Jolla Bay develop over time and in relation to environmental conditions, and how do population genetics change in relation to population dynamics?

5.2 Material and Methods

Sampling along the Southern Californian Coast

For the assessment of population genetic diversity of *L. polyedrum* along the Southern Californian Coast, surface bucket samples of seawater were collected from five locations between Avila Beach and La Jolla Bay on the 20/10/2004 (Fig. 5.1 and Table 5.1). For details on the processing of samples see the section on sampling in La Jolla.
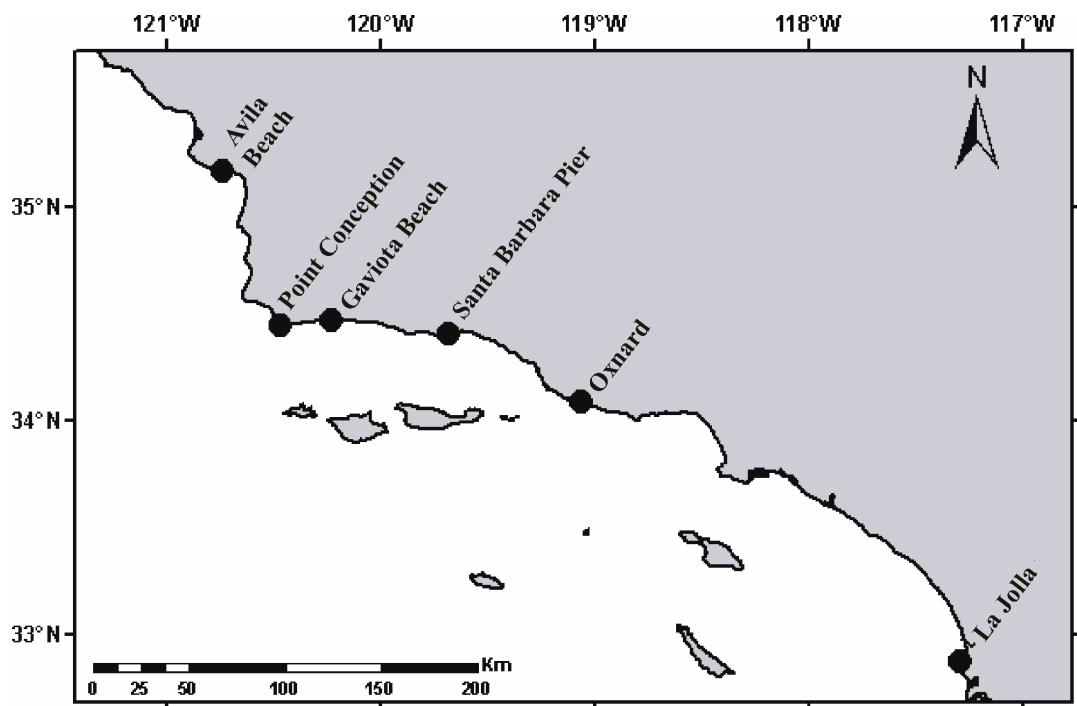
FIG. 5.1 Map of Southern California, USA.

Sampling in La Jolla Bay

The majority of samples for this study were collected in La Jolla Bay. A map of specific sampling sites is shown in figure 5.2. Two blooms of *L. polyedrum*, one in August 2003 the other in July 2005, were each sampled once from Scripps Pier (Table 5.1). In 2004, samples were collected weekly (weather permitting) from mid September to mid November. Sampling was conducted by boat at two stations in La Jolla Bay with vertical resolution in the water column. Prior to the collection of water samples, conductivity-temperature-depth (CTD) profiles, including measurements of *in vivo* fluorescence were taken, using a SEACAT 19 profiler (Sea- Bird Electronics, Bellevue, Washington, USA) fitted with a pumped WETStar fluorometer (WETLabs, Philomath, Oregon, USA). Samples were collected from the water surface and, depending on the station and real-time temperature and fluorescence profiles, down to the sea floor or below the thermocline, with intermediate depths being chosen to sample prominent features in the *in vivo* fluorescence profile. Detailed information about sampling sites, sampling dates and other sampling specifications, such as CTD cast numbers and depths of collected water samples are given in Table 5.1. Processing of CTD data involved alignment according to manufacturer's instructions, including the correction of fluorescence profiles based on the pump speed, and processing of derived parameters such as salinity and density. Fluorescence profiles are expressed in relative units due to

out of date calibration of the fluorometer at the time the instrument was used.

Water samples for the enumeration of plankton were collected using a 3 L Niskin bottle. Samples were gently transferred to 1 L plastic bottles and were kept in the shade until they were processed in the laboratory. Samples were concentrated by gentle inverse filtration through a 10 μm mesh, preserved in formalin (see chapter 3 for recipe and final concentrations), and stored in the dark at 4 ºC. A Sedgwick-Rafter chamber was used to identify and enumerate the different *L. polyedrum* life cycle stages. Since plankton distributions in the Sedgwick-Rafter chamber were found to be heterogeneous, entire 1mL aliquots of concentrated samples were counted to increase the accuracy of enumerations.
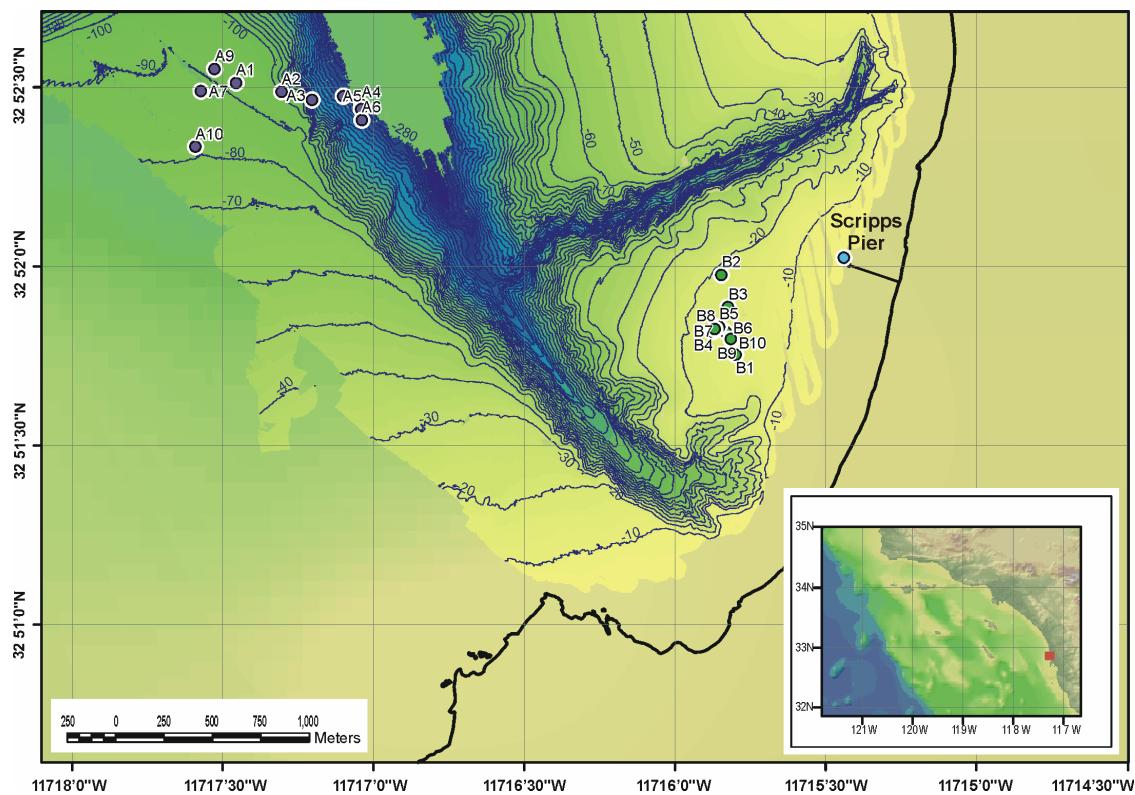
FIG. 5.2 Map of La Jolla Bay, Southern Californian Bight, showing the sampling sites at Scripps pier, Station A and Station B. Numbers next to station ID refer to the temporal order of sampling and correspond to data listed in Table 5.1.

Due to the very low abundance of *L. polyedrum* at the beginning of the sampling campaign in 2004, additional samples for the isolation of clonal cultures and the genetic analysis of single cells were collected using a 10 μm plankton net. The depths that were sampled by Niskin bottle and by plankton net where always identical. To minimize the

collection of plankton from water other than that at the aimed depths, the plankton net was fitted with a lead weight at the frame of the net opening and a graduated cord for towing the net at a specific depth. This setup minimized the amount of water that entered the net on the way down to, and up from the desired sampling depth because the net descends and ascends at an angle of 90 º to its opening and the drag on the net itself sealed the opening. The net was then drift towed at depth for approximately 5 min. Back on board, the samples were gently filtered through a 100 μm mesh to remove large zooplankton and stored in the shade in 1 L plastic bottles.

Table 5.1 Sampling in La Jolla Bay, CA, U.S.A.

Date & Time	Julian day	Scripps Pier		Station A		Station B	
		CTD cast	Sampling depth (m)	CTD cast	Sampling depth (m)	CTD cast	Sampling depth (m)
09-09-2003 [a]	252		0 – 6 [b]				
10-09-2004 12:00-15:00	254			A1	2, 10, 20	B1	3, 7, 15
17-09-2004 11:00-14:00	261			A2	3, 5, 7, 9, 11	B2	2, 5, 7, 10
23-09-2004 11:00-14:30	267			A3	3, 5, 7, 9, 11	B3	2, 5, 7, 10
30-09-2004 11:30-16:00	274			A4	0, 10, 18, 23, 27	B4	0, 4, 9, 16
07-10-2004 11:30-16:00	281			A5	0, 10, 15, 18, 27	B5	0, 4, 9, 16
14-10-2004 11:30-16:00	288			A6	0, 10, 15, 18, 27	B6	0, 4, 9, 16
28-10-2004 10:30-14:00	302			A7	0, 10, 15, 18, 27	B7	0, 4, 9, 16
03-11-2004 12:00-17:00	308			A8	0, 18, 22, 27, 36	B8	0, 4, 9, 16
11-11-2004 11:00-15:30	316			A9	0, 10, 20, 30, 36	B9	0, 4, 9, 16
21-11-2004 10:30-13:30	326			A10	0, 10, 20, 25, 36	B10	0, 4, 9, 16
23-07-2005 [a]	204		0				

[a] Samples were collected around midday but exact sampling times were not recorded. [b] Sample was collected using a plankton net (mesh size 10μm).

In the laboratory the samples were concentrated by inverse filtration through a 10 μm mesh and split into two subsamples. One part was gently pelleted at 700 g, the supernatant removed, the pellet preserved in 90% molecular grade ethanol (Sigma-

Aldrich), and stored at -20 ℃ for later genotyping of single cells. The second subsample was diluted in sterile f/2 medium (Guillard 1975) and transferred to a Sedgwick rafter chamber from where single cells of *L. polyedrum* were picked using a finely drawn out glass capillary. Picked single cells were washed once in f/2 medium, transferred to 96-well plates (Sarstedt, Beaumont Leys, UK), sealed with parafilm to minimize evaporation and incubated at 19 ℃, and 130 μmol photons $\cdot$ m^{-2} $\cdot$ sec^{-1}, under a 12h light/dark cycle to establish clonal cultures. Two of the cultures that were isolated this way were M22 and LP2810. Both strains were used in studies on the influence of irradiance on life cycle control (chapter 3; Frommlet et al. 2008) and light acclimation (chapter 4) in *L. polyedrum*.

Microsatellite genotyping of single cells

A detailed description of the development of microsatellite markers and their characteristics is given in chapter 2 (Frommlet and Iglesias-Rodríguez 2008). In short, DNA from nine clonal cultures of *L. polyedrum* was pooled and used to construct a partial genomic library, enriched for microsatellite regions by selective hybridization. The employed methods were derived from Armour et al. (1994), Kandpal et al. (1994), Toonen (1997) and Hammond et al. (1998). The screening of the microsatellite enriched library resulted in the identification of 17 microsatellites of which six could be successfully amplified from single cells in multiplex PCR together with the entire ITS1-5.8S-ITS2 region or a partial region of the 18S rRNA gene. However, only the microsatellite marker LP65 had the important attributes of being polymorphic at the intrapopulation genetic level and having a high amplification success rates. Therefore, in the present study the marker LP65 was employed to assess population genetic diversity in *L. polyedrum*. Together with primers for the amplification of a partial region of the 18S rRNA gene, LP65 could be routinely amplified in multiplex PCRs from single cells. For details on the primers, PCR conditions and the preparation of single cells for PCR see chapter 2 (Frommlet and Iglesias-Rodríguez 2008).

Analysis of genetic data

Microsatellite PCR products were analyzed on a CEQ 8800 capillary sequencer (Beckman-Coulter, High Wycombe, UK) using a fluorescently-labelled 400 bp internal size standard (Beckman-Coulter). The PCR products of the 18S rRNA gene were run on 2% agarose gels and gels were photographed for assessing amplification success. A

total of sixteen randomly selected 18S PCR products were gel purified (QIAGEN) and sequenced by Geneservice (Cambridge, UK) in forward and reverse direction.

Routinely, the microsatellite locus LP65 and the partial 18S rRNA gene were co-amplified from 55 single cells of each sample. When more than 32 cells resulted in positive amplification of LP65, still only 32 microsatellite amplicons were randomly chosen for genotyping to ensure even sample size. Uncorrected success rates for the amplification of LP65 were calculated based on the total number of single cells analysed. Since failed amplifications of both loci (18S rRNA gene and microsatellite) suggested that the template DNA was degraded, the species was wrongly identified during single cell isolation or amplification failure occurred, a corrected success rate for the amplification of LP65 could be calculated by excluding these cells from the analysis.

For each of the 22 samples and across all samples, allelic frequencies and gene diversity were calculated using POPGENE (ver. 1.32) (Yeh et al. 1997). According to Nei (1987) the formula for calculating gene diversity (h) is:

$$h = 1 - \sum_{i=1}^{m} \chi_i^2$$

Where m is the number of alleles and χ_i the population frequency of the ith allele at a locus. This measure of population genetic diversity is defined as the probability that two randomly chosen genotypes from a population are different, which is equivalent to the expected heterozygosity for diploid data (Nei 1987). To study spatial and temporal relationships between samples, an unweighted pair-grouping method using an arithmetic averages (UPGMA) dendrogram was constructed in TFPGA (Miller 1997). The dendrogram was based on bootstrapping 1000 permutations of Nei's unbiased genetic distance (Nei 1978). Also in TFPGA, an exact test between pairs of samples was performed to test for genotypic differentiation using Fisher's combined method (Raymond and Rousset 1995). Specified Markov chain parameters were 1000 dememorization steps followed by 10 batches of 2000 iterations per batch. Original significance levels ($\alpha = 0.05$) were adjusted for multiple tests using the Dunn-Sidàk method (Sokal and Rohlf 1998). For example, the significance level for pair-wise comparisons of all 22 samples was adjusted for 230 independent tests ($\alpha = 0.00022$).

5.3 Results

5.3.1 Single cell microsatellite genotyping

A summary of descriptive microsatellite genotyping indices is shown in Table 5.2. Based on 704 genotyping results from 22 samples, a total of 52 genotypes could be identified. Genotype numbers ranged from 9-21 per sample. Gene diversity (Nei 1972) ranged from 0.70-0.93 with an average of 0.89. Uncorrected amplification success rates for the microsatellite locus LP65 ranged from 32 to 90% with an average of 63%.

Table 5.2 Summary of descriptive statistics for microsatellite genotyping

Sample	No of genotypes	Uncorr. success rate (%)	Corr. success rate (%)	Gene diversity (h)	Cells with two 'alleles' of LP65 (%)
Avila Beach	18	74	94	0.91	20
Gaviota	13	82	89	0.87	6
Santa Barbara	10	67	77	0.83	10
Oxnard	12	57	69	0.84	9
La Jolla					
2003 Bloom	21	32	70	0.93	13
2005 Bloom	14	90	96	0.84	7
17/09/2004 A 0m	9	38	60	0.70	7
17/09/2004 A 11m	11	39	62	0.76	0
23/09/2004 A 0m	14	62	100	0.84	0
23/09/2004 A 11m	18	42	100	0.92	3
30/09/2004 A 0m	12	84	100	0.87	0
30/09/2004 A 27m	15	75	100	0.88	6
07/10/2004 A 10m	17	69	85	0.92	13
07/10/2004 A 27m	12	84	93	0.89	9
14/10/2004 A 0m	13	69	92	0.89	6
14/10/2004 A 27m	12	69	92	0.86	9
28/10/2004 A 0m	14	76	84	0.86	16
28/10/2004 A 27m	15	85	91	0.87	13
03/11/2004 A 0m	15	71	100	0.90	9
03/11/2004 A 27m	15	68	100	0.89	16
11/11/2004 A 0m	10	62	80	0.78	0
11/11/2004 A 30m	14	62	80	0.82	9
All samples	**52**	**63**	**86**	**0.89**	**8**

A total of sixteen partial 18S PCR products were sequenced; eight randomly chosen from reactions that resulted in a product for LP65 and eight from reactions that did not yield a LP65 PCR product. All sixteen sequences were identical and a blast search confirmed that all sixteen sequences originated from *L. polyedrum*. Sequencing results are not shown, since all sixteen sequences were identical with the sequences with NBCI accession numbers EU177124 and EU177125, previously published in GenBank. Corrected amplification success rates for LP65, calculated by excluding cells that did not give any PCR products, were between 60 to 100% with an average of 86%. On average, 8% of single cells resulted in the amplification of two PCR products for LP65, but percentages varied substantially (0 to 20%) depending on the sample.

5.3.2 Population genetic distance/differentiation of L. polyedrum in Southern California

Figure 5.3 shows a UPGMA dendrogram of six samples from five locations along the Southern Californian Coast based on Nei's unbiased genetic distance (Nei 1978). Samples from Avila Beach, Gaviota, Santa Barbara and Oxnard were collected on a single day in October 2004. Samples from La Jolla Bay could not be collected on the same date due to bad weather conditions. Instead, two samples from La Jolla Bay (one collected a week before, the other a week after the sampling at the other four sites) were included in the analysis (see figure 5.1 and Table 5.1 for sampling locations and specific dates of sampling).
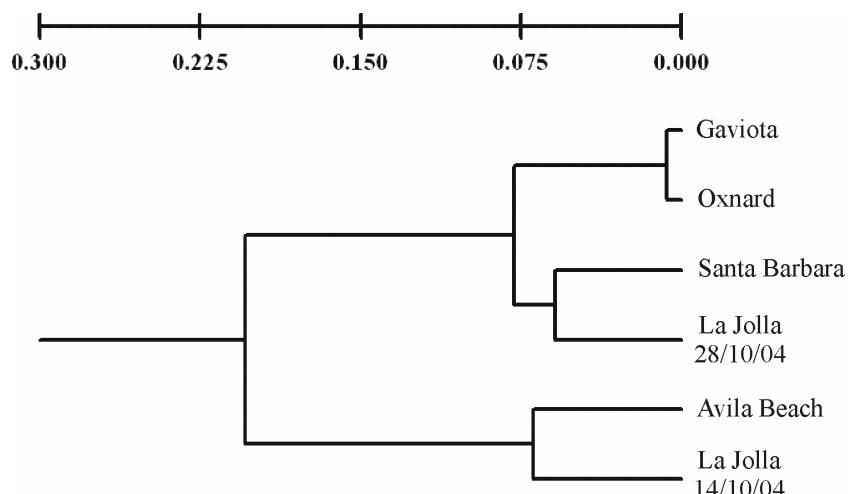
FIG. 5.3 Unweighted pair-grouping method using an arithmetic averages (UPGMA) dendrogram based on Nei's unbiased genetic distance (Nei 1978) among 6 samples of *Lingulodinium polyedrum* from 5 locations in Southern California.

Based on the UPGMA dendrogram two main clusters could be identified: (1) Gaviota, Oxnard, Santa Barbara and La Jolla (14/10/04); and (2) Avila Beach and La Jolla (28/10/04). Cluster 1 could further be divided into two pairs: Gaviota/Oxnard and Santa Barbara/La Jolla (14/10/04). A Fisher's test of genotypic differentiation showed on the other hand that genetic differences between pairs of samples were not statistically significant (Table 5.3). *P*-values between Avila Beach and Oxnard as well as Avila Beach and Santa Barbara were however relatively low compared to the other thirteen pair-wise comparisons.

Table 5.3 Probability values for Fisher's combined test of genotypic differentiation

	Gaviota	Oxnard	Santa Barbara	Avila Beach	La Jolla 14/10/2004	La Jolla 28/10/2004
Gaviota						
Oxnard	0.6977					
Santa Barbara	0.4414	0.1410				
Avila Beach	0.1860	0.0066	0.0065			
La Jolla 14/10/2004	0.1309	0.1481	0.0498	0.5814		
La Jolla 28/10/2004	0.6976	0.0495	0.2443	0.1581	0.1240	

Original significance level ($\alpha = 0.05$) after adjustment for multiple tests using the Dunn-Sidàk method (Sokal and Rohlf 1998) was $\alpha = 0.0036$. All samples were collected from surface waters.

5.3.3 La Jolla Bay: Environmental conditions and chlorophyll fluorescence

Figure 5.4 shows a set of time series of temperature (left) and chlorophyll (right) at station A (bottom) and station B (top) in La Jolla Bay from the 10/9/2004 (day 254) to 21/11/2004 (326). For a period of several weeks before the sampling campaign commenced, weather conditions in La Jolla Bay were sunny and calm, resulting in highly stratified waters at the beginning of sampling on day 254. A shallow mixed surface layer was separated from deeper, colder waters by a steep thermocline at a depth of around 8 m at station B and 12 m at station A, further offshore. At both stations, sea surface temperatures were approximately 24 ºC and chlorophyll fluorescence peaked just below the thermocline with maximum values being slightly higher onshore than further offshore. One week later (day 261), sea surface temperatures at both stations had decreased to 23 ºC but, at the same time, the layer of warm surface waters had deepened. At station B the depth of the 19 ºC isotherm had deepened to 16 m and the themocline was less defined compared to the previous sampling. At station A, the

deepening of warm surface waters was less pronounced and the thermocline remained well defined. Another six days later (day 267), warm surface waters were much shallower again and sea surface temperatures at both stations had decreased to 21.5 ºC, probably due to a gradual decrease of solar irradiance with the progressing season.

While stratification at station B became less defined, a strong stratification of the water column remained at station A. Unfortunately, fluorescence measurements could not be made on days 261 and 267. CTD profiling on day 274 showed a further decline of sea surface temperatures, a further loss of stratification at station B and an upwards movement of the thermocline at station A to approximately 10 m. Chlorophyll fluorescence at station B did not show any well defined peak, whereas chlorophyll fluorescence at station A peaked slightly deeper, at around 20 m, than on day 254. Over the following two weeks (days 281 and 288) sea surface temperatures at both stations declined further, to approximately 19-20 ºC. Water stratification at station B remained weak, and at station A became somewhat less pronounced. Chlorophyll fluorescence on day 281 peaked between 2.5 - 7.5 m at station B and between 10-20 m at station A. On day 288, chlorophyll fluorescence peaked deeper in the water column, between 15 m and the seabed at station B and between 25-35 m at station A. Environmental conditions changed dramatically between the 20/10/2004 and the 23/10/2004, due to a storm with heavy rainfalls that stretched along the Southern Californian Coast. Strong westerly winds resulted in enhanced mixing and presumably downwelling. Sampling could not be conducted during the storm. The first sampling after the storm (day 302) showed the pronounced effects of the strong winds had had on the physical conditions of the water column and on the phytoplankton community. At station B, the water temperature down to the seabed was homogenous at approximately 18.5 ºC. The temperature profile at station A was similarly homogenous down to a depth of about 52 m. Chlorophyll fluorescence at station B indicated elevated numbers of phytoplankton just above the seabed, whereas at station A, no clear fluorescence peak could be detected. Temperature profiles on days 308, 316 and 326 showed a slow and generally weak re-stratification of the water column. Chlorophyll fluorescence at station B continued to peak just above the seabed. Further offshore, at station A, a fluorescence peak reappeared on day 308 at a depth of around 30 m and over time moved upwards in the water column to about 20 m. Density and salinity time series in La Jolla Bay at station A (bottom) and station B (top) are shown in Figure 5.5. Over the entire sampling period, density profiles largely reflected the described patterns in temperature. Before the storm, salinity generally

decreased with depth. After the storm, salinity was noticeably lower across the whole water column, indicating that the strong winds had effectively mixed the direct rainwater input into the sea and the rainwater from surface runoff with the saltier seawater. Contrastingly, sampling on days 308 and 316 revealed a thin layer of fresh water, characterised by its very low density and salinity. This thin layer of fresh water was only measurable further offshore at station A and not at station B, indicating that following the local fresh water input, a plume of rainwater from other regions was transported along the Coast at some distance to the shore.
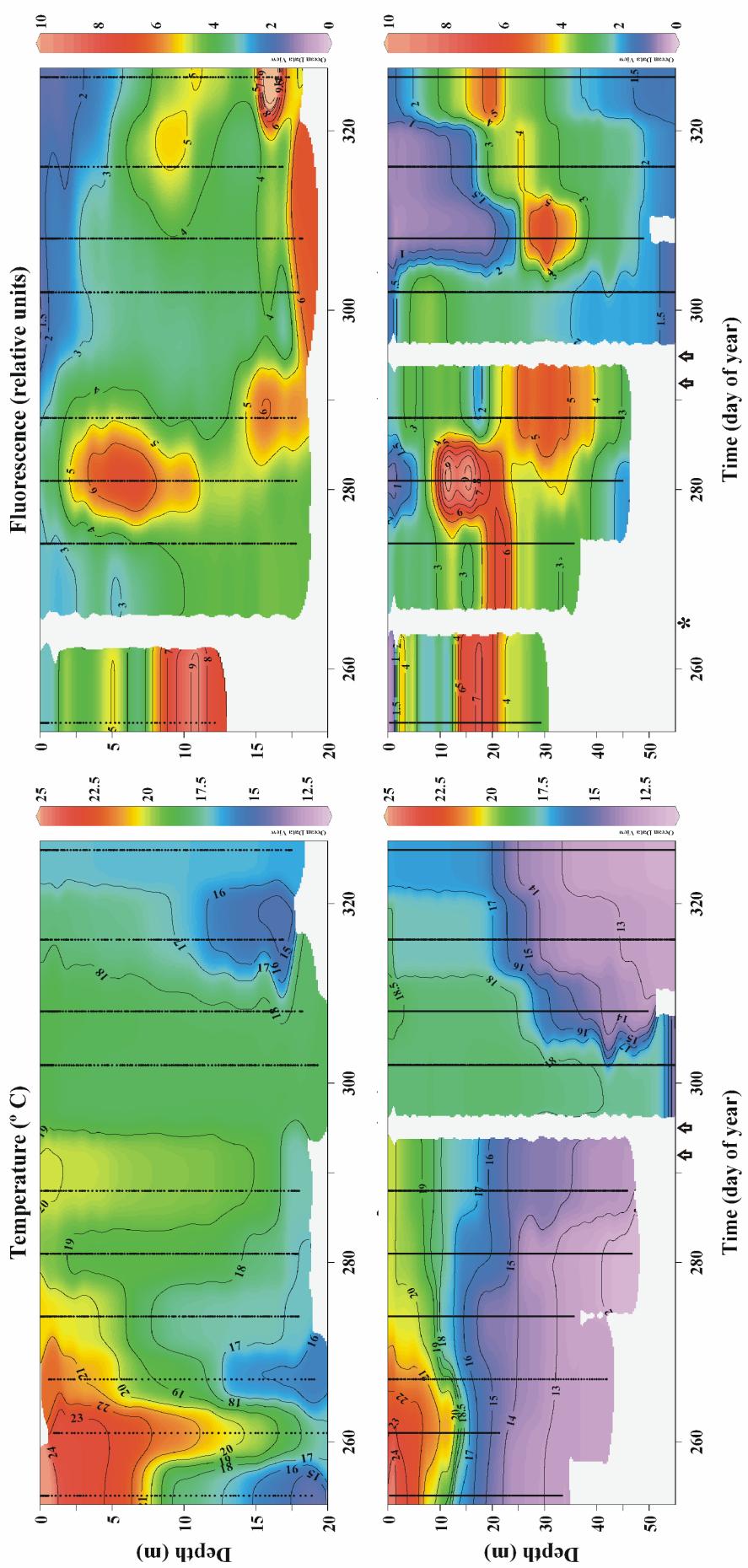
Fig. 5.4 Time series of temperature (left) and chlorophyll a fluorescence (right) at station A (bottom) and station B (top) in La Jolla Bay. Arrows indicate the storm between the 20/10/2004 and the 23/10/2004. * missing fluorescence data.
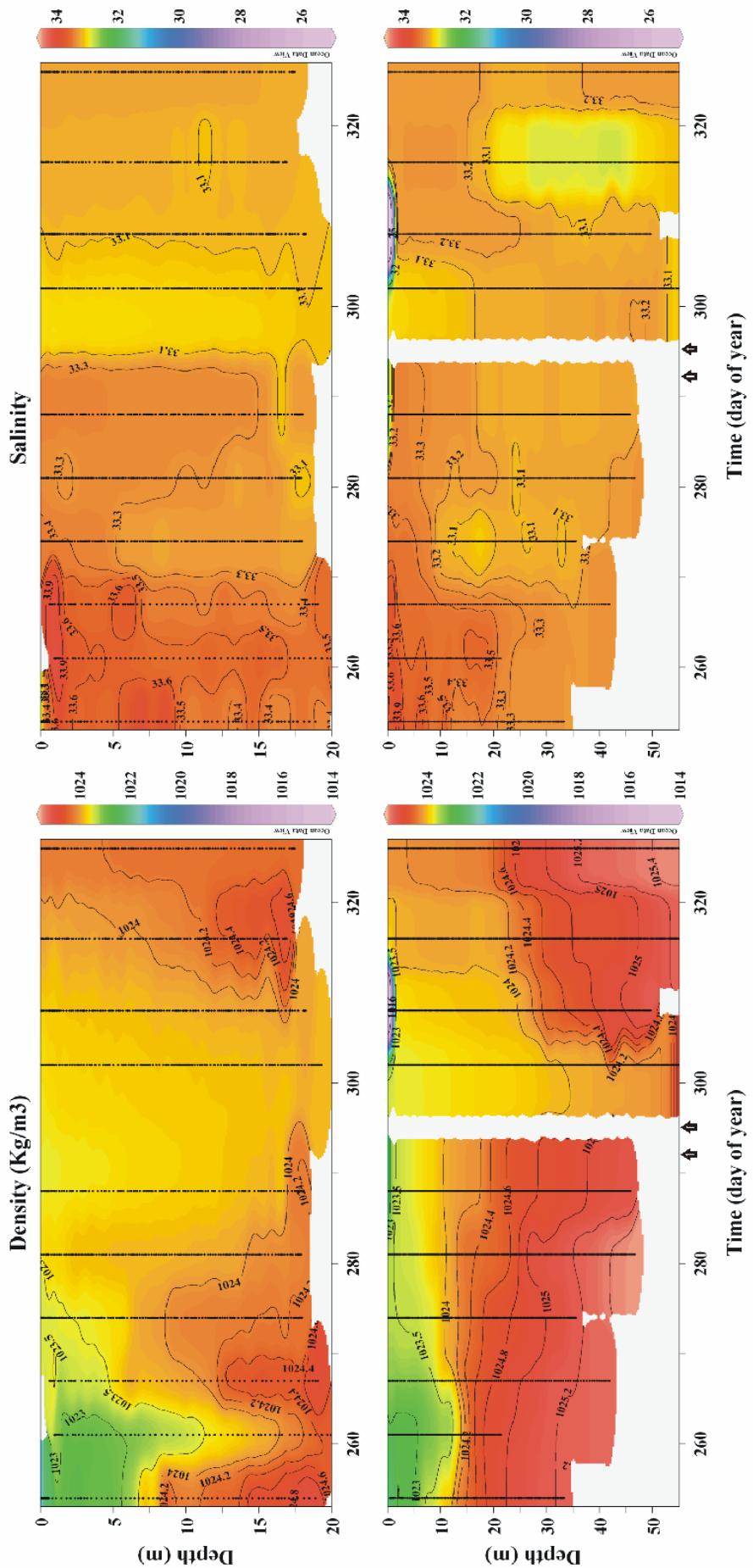
FIG. 5.5 Time series of density (left) and salinity (right) at station A (bottom) and station B (top) in La Jolla Bay. Arrows indicate the storm between the 20/10/2004 and the 23/10/2004.

5.3.4 La Jolla Bay: Lingulodinium polyedrum population dynamics

Figure 5.6 shows time series of the abundance of motile cells (A) and resting cysts (B), figure 5.7 of ecdysal cysts (C) and empty resting cysts (D) of *L. polyedrum* at station A (bottom) and station B (top) in La Jolla Bay from the 10/9/2004 (day 254) to the 21/11/2004 (day 326). Motile cells of *L. polyedrum* were present in most water samples and their abundance and distribution changed over time and space. On day 254, their peak in abundance (32 cells L^{-1}) at station B was observed at 15 m. One week later (day 261), a peak in cell density of 134 cells L^{-1} was found at 10 m but the vertical distribution of *L. polyedrum* was more homogenous compared to the previous sampling. After a further seven days (day 268), *L. polyedrum* numbers had increased to a maximum of 240 cells L^{-1} at 5 m. At 3 m and 7 m, cell numbers were 170 and 150 cells L^{-1} respectively and decreased to 87 cells L^{-1} at 10 m. During the same period, the highest abundance of motile *L. polyedrum* cells further offshore, at station A, was found to be close to the surface with 152, 258 and 161 cells L^{-1} on days 254, 261 and 268 respectively and, in contrast to station B, cell numbers generally dropped more noticeable with depth.

Over the following three weeks (days 274, 281 and 288), *L. polyedrum* numbers increased substantially at both stations. At station B, a maximum number of 2,168 cells L^{-1} were counted at 9 m on day 274 and the following week (day 281) cell numbers had increased to 46,888 cells L^{-1} at a depth of 4 m. However, cell numbers at the surface and at 9 m were also high with more than 24,000 and 34,000 cells L^{-1} respectively. Another week later (day 288), with cell numbers staying high, the vertical abundance of *L. polyedrum* showed a bimodal distribution with approximately 30,000 cells L^{-1} at the surface, more than 39,000 cells L^{-1} at 16m and a minimum at 9 m with 8,800 cells L^{-1}. At station A, maximum cell numbers on day 274 were 2,023 cells L^{-1}, comparable to the numbers further onshore, but in contrast to station B, cell numbers peaked again close to the surface and declined sharply with depth. The following week (day 281), cell abundance at station A had increased to more than 34,000 cells L^{-1} but in contrast to previous counts, which showed highest abundances of *L. polyedrum* close to the surface, now only 100 cells L^{-1} were found at the surface and the bulk of the population was at a depth of 15 to 18 m. The following week (day 288), cell numbers were again highest at the surface but were lower (15,899 cells L^{-1}) compared to the previous week. The abundance of *L. polyedrum* in La Jolla Bay decreased sharply at both stations after the storm. Cell numbers initially dropped to values in the low hundreds per litre (day

302) and did not show noticeable variation with depth. This trend of declining cell numbers continued over time with the exception of a slight recovery of numbers on the last sampling day (day 326) with 121 cells L^{-1} at 9 m.

Resting cysts of *L. polyedrum* were first detected at station B on day 274 at a depth of 9 m with numbers of 8 cysts L^{-1}. At the same station, numbers peaked in a single maximum of 64 cysts L^{-1} at a depth of 9 m the following week (day 281). Another week later (day 288), cyst distributions showed two maxima (at 4 m and 16 m) with numbers of 36 and 29 cysts L^{-1}, respectively. Cyst numbers were very low (0 - 2 cysts L^{-1}) in the two weeks following the storm and increased again to a peak of 37 cysts L^{-1} a week later (day 316). At station A, the appearance of resting cysts was confined to day 288 with a maximum of 21 cells L^{-1} at the surface. The temporal distribution pattern of empty resting cysts reflected well the distribution of intact resting cysts, but peaks of empty resting cysts were generally found closer to the surface.

Ecdysal cysts of *L. polyedrum* were only found at station B, where numbers peaked on day 281 at a depth of 16 m with 46 cysts L^{-1}. A week later (day 288), ecdysal cysts were most abundant at the surface with 33 cysts L^{-1} and following the storm, were found at a concentration of 24 cells L^{-1} at a depth of 9 m.
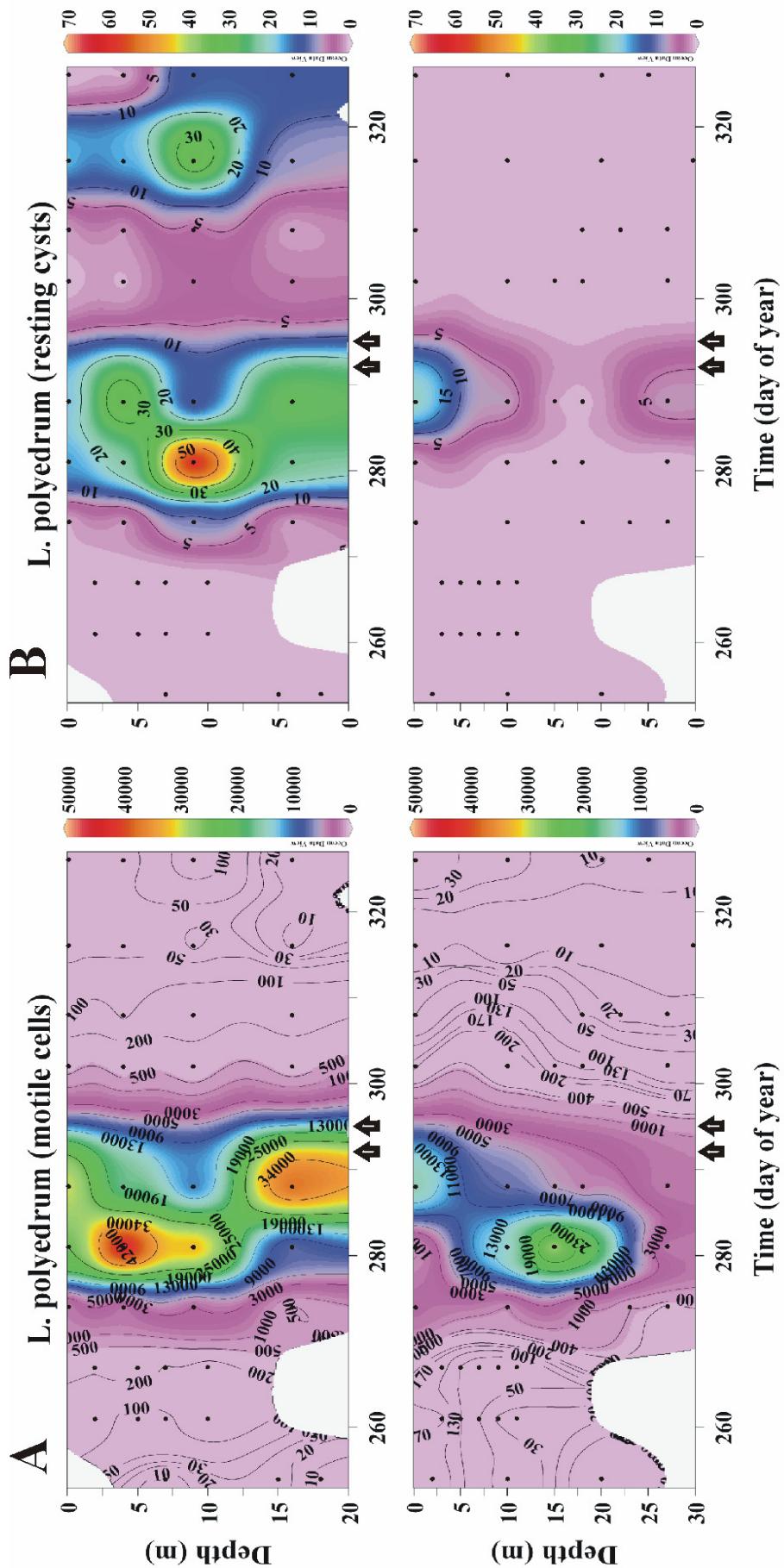
FIG. 5.6 Population development of *L. polyedrum* in La Jolla Bay. (A) motile cells and (B) resting cysts of *L. polyedrum* at station A (bottom) and station B (top). Cell concentrations are given in cells L^{-1}. Arrows indicate the storm between the 20/10/2004 and the 23/10/2004.
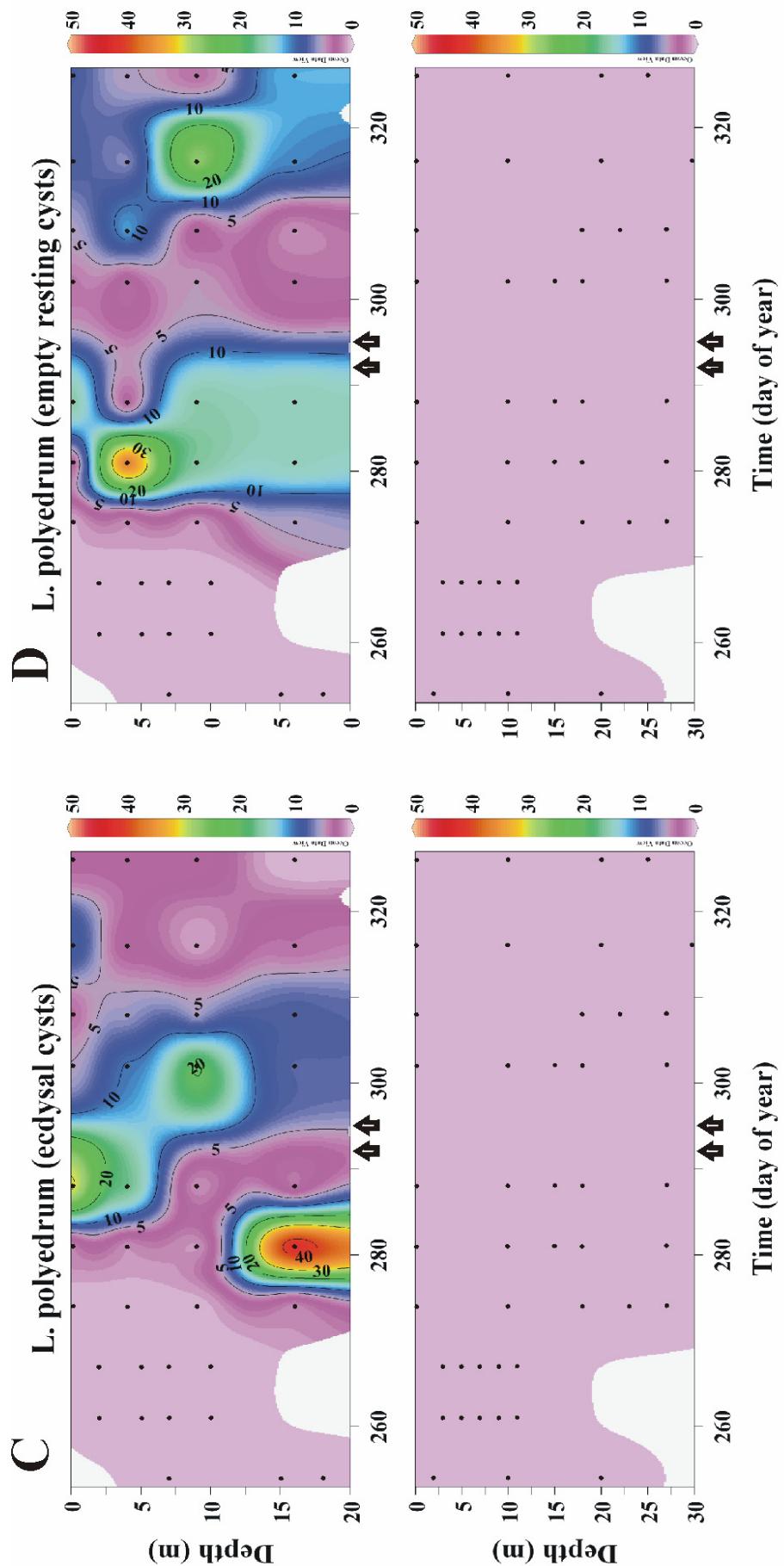
FIG. 5.7 Population development of *L. polyedrum* in La Jolla Bay. (C) ecdysal cysts and (D) empty resting cysts of *L. polyedrum* at station A (bottom) and station B (top). Cell concentrations are given in cells L^{-1}. Arrows indicate the storm between the 20/10/2004 and the 23/10/2004.

5.3.5 La Jolla - Station A: microsatellite amplification success, 'diploid' cells and gene diversity

Figure 5.8 A-D shows time series of four of the indices listed in Table 5.2 from samples collected at station A in La Jolla Bay from the 17/09/04 (day 261) to the 11/11/04 (day 316). Uncorrected LP65 amplification success rates (A) were noticeably lower from samples that were collected at the beginning of the sampling campaign. Corrected success rates (B) were generally more homogenous over time and space but remained lower at the beginning of the sampling campaign. The percentage of cells that resulted in two PCR products for LP65 (C) varied noticeable. Elevated numbers were first recorded on day 281 at a depth of 9 m. Following the storm, the percentage of these cells in the population remained high and they were abundant across the water column. Percentages in the surface sample of day 316 were comparable with the low values recorded at the beginning of sampling whereas at a depth of 30 m the percentage remained at approximately 10%.

Gene diversity (Nei 1972) was generally high and relatively homogenous (D). However, lower levels of gene diversity were found in the samples that had reduced amplification success rates for LP65. The highest gene diversity was recorded on day 281 at a depth of 9 m, coinciding with the highest number of motile cells that were recorded at station A and also coinciding with the first recording of elevated numbers of cells that resulted in two PCR products for LP65.
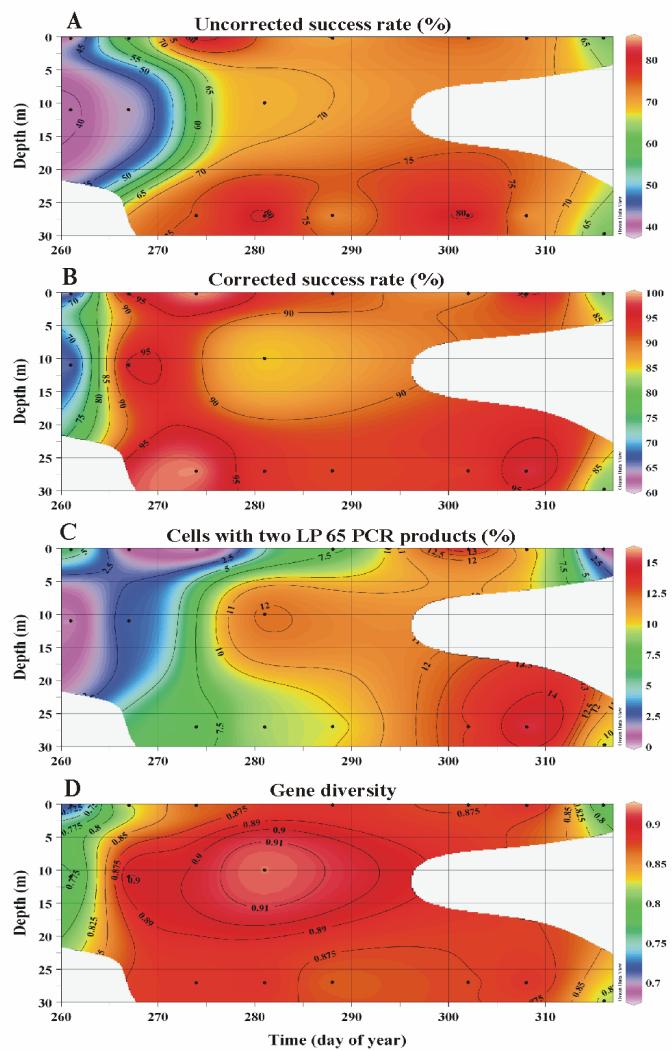
FIG. 5.8 Time series of uncorrected (A) and corrected (B) LP65 amplification success rates, the percentage of cells that resulted in two PCR products for LP65 (C) and gene diversity (Nei 1972) (D) at station A in La Jolla Bay.

5.3.6 La Jolla - Station A: Population genetic distance/differentiation of L. polyedrum

Figure 5.9 shows a UPGMA dendrogram, based on Nei's unbiased genetic distance (Nei 1978), of all 22 samples that were analysed in the present study. Samples that were previously shown in figure 5.3 were included in the dendrogram for comparison. Based on the UPGMA dendrogram several clusters could be identified. Bloom samples from 2003/2005 and a non-bloom sample from September 2004 produced the most distinct cluster (1) compared to all the other samples. A second cluster comprised the Avila Beach sample, the two earliest samples from La Jolla and one of the last samples from La Jolla. All of the samples in cluster 3 originated from samples collected before the storm, and most were from the phase (30/09/04 – 14/10/04) during which cell numbers of *L. polyedrum* increased substantially in La Jolla Bay.
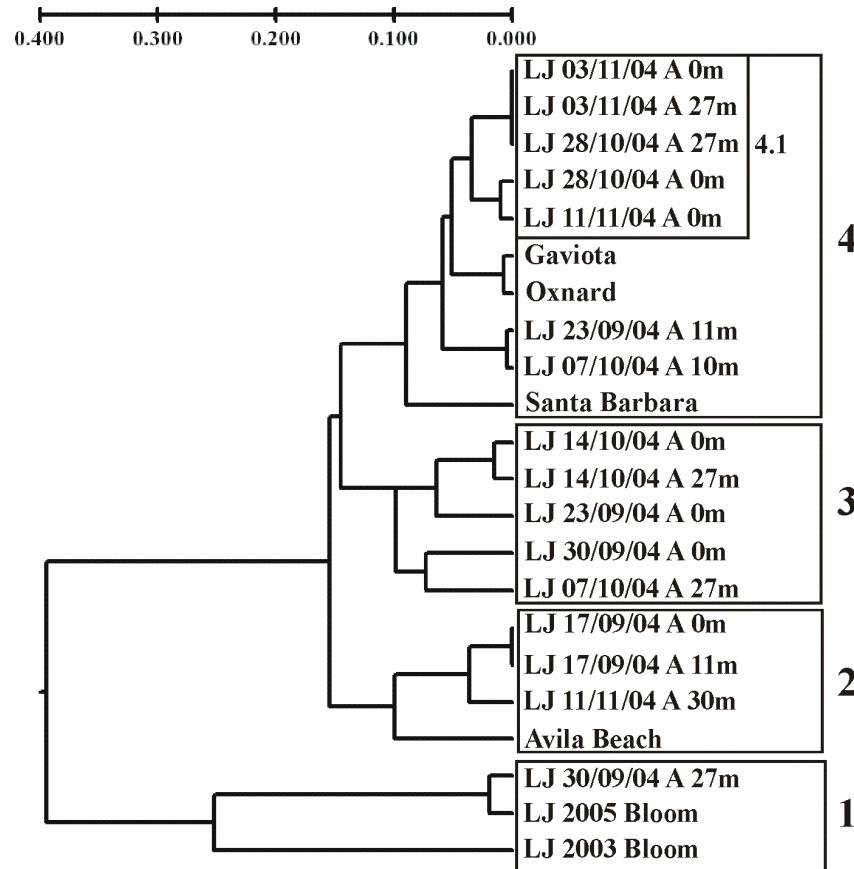
FIG. 5.9 Unweighted pair-grouping method using an arithmetic averages (UPGMA) dendrogram based on Nei's unbiased genetic distance (Nei 1978) among all 22 samples of *Lingulodinium polyedrum* analysed in this study.

Cluster 4 was less defined and contained a diverse set of samples from different locations, depths and dates of sampling. One subgroup in this cluster (4.1) was however exclusively comprised of samples that were collected after the storm. The other five samples in cluster 4 could be grouped into two pairs: 1) Gaviota and Oxnard and 2) Two samples from La Jolla that originated from a similar depth. The sample from Santa Barbara was the most distinct sample in cluster 4. A Fisher's test of genotypic differentiation showed that genetic differences between most pairs of samples were not statistically significant (Table 5.4). Only between the 2005 bloom sample and the 2004 samples from Santa Barbara and Avila Beach there was significant genetic differentiation detectable with p-values below 0.00022.

Table 5.4 Probability values for Fishers combined test of genotypi c differentiation for all 22 samples analysed in this study.

	Gav.	Oxn.	St. Bar.	Avila B.	17/09 0m	17/09 11m	23/09 0m	23/09 11m	30/09 0m	30/09 27m	07/10 10m	07/10 27m	14/10 0m	14/10 27m	28/10 0m	28/10 27m	03/11 0m	03/11 27m	11/11 0m	11/11 30m	2003 Bl.	2005 Bl.
Gaviota																						
Oxnard	0.7041																					
St. Bar.	0.4090	0.1539																				
Avila B.	0.2123	0.0086	0.0030																			
17/09 0m	0.1941	0.1962	0.0089	0.0442																		
17/09 11m	0.2572	0.1431	0.0163	0.4492	0.7777																	
23/09 0m	0.1757	0.1517	0.0048	0.0731	0.2290	0.1330																
23/09 11m	0.7355	0.4963	0.4779	0.3698	0.0286	0.1832	0.3903															
30/09 0m	0.3382	0.0232	0.0069	0.1413	0.1227	0.0488	0.3402	0.0680														
30/09 27m	0.2613	0.1010	0.0042	0.1972	0.0020	0.0186	0.4121	0.6128	0.4493													
07/10 10m	0.8603	0.2466	0.2409	0.8220	0.0912	0.2536	0.3745	0.9541	0.1714	0.8753												
07/10 27m	0.5422	0.0989	0.0850	0.1070	0.0761	0.0570	0.2099	0.7537	0.4064	0.7451	0.6028											
14/10 0m	0.1306	0.1645	0.0620	0.5597	0.0760	0.2008	0.2975	0.5040	0.1927	0.2595	0.7018	0.2368										
14/10 27m	0.7659	0.4347	0.1748	0.2843	0.1580	0.1875	0.6417	0.2671	0.6306	0.5553	0.6548	0.2462	0.6032									
28/10 0m	0.6818	0.0280	0.2015	0.1576	0.2191	0.1333	0.2793	0.6996	0.1464	0.0376	0.7909	0.3187	0.1002	0.1247								
28/10 27m	1.0000	0.9145	0.4743	0.3325	0.5818	0.3130	0.7111	0.8376	0.5141	0.5713	0.9632	0.7328	0.3823	0.9671	0.9082							
03/11 0m	0.8563	0.1349	0.0632	0.5734	0.0376	0.0597	0.2461	0.8157	0.0158	0.1623	0.9322	0.1881	0.0562	0.3496	0.7054	0.9589						
03/11 27m	0.7841	0.5408	0.0789	0.2633	0.2499	0.1364	0.6400	0.7316	0.2712	0.1929	0.7906	0.4056	0.2563	0.4760	0.7056	0.9896	0.9365					
11/11 0m	0.5633	0.6846	0.5607	0.0175	0.2372	0.2812	0.1676	0.6515	0.0023	0.0176	0.3925	0.1126	0.1690	0.2213	0.6634	0.7908	0.2995	0.4544				
11/11 30m	0.3301	0.0592	0.0629	0.5560	0.2599	0.4420	0.1708	0.1062	0.0235	0.0185	0.5489	0.0556	0.1811	0.6480	0.4042	0.5791	0.5052	0.2473	0.2928			
2003 Bl.	0.0163	0.0008	0.0004	0.4368	0.0003	0.0062	0.3357	0.1500	0.0779	0.1802	0.2920	0.0052	0.6076	0.2433	0.0207	0.0516	0.0243	0.0792	0.0037	0.0306		
2005 Bl.	0.0103	0.0262	0.0000	0.0002	0.0033	0.0064	0.3987	0.0702	0.0126	0.6317	0.1179	0.0493	0.0169	0.0574	0.0013	0.0298	0.0030	0.0216	0.0078	0.0038	0.0033	

Original significance level ($\alpha = 0.05$) after adjustment for multiple tests was $\alpha = 0.00022$. Gav. = Gaviota; Oxn. = Oxnard; St. Bar. = Santa Barbara; Avila B. = Avila Beach; Bl. = Bloom sample. For samples from La Jolla in 2004 day/month and depth in meters at station A are shown.

5.3.7 La Jolla - Station A: Dynamics of individual genotypes over time and space

During the 2004 sampling campaign in La Jolla Bay, 37 microsatellite genotypes were present out of the total of 52 identified genotypes in the temporal and spatial Californian groups. Figure 5.10 A-H shows time series of the eight genotypes with the highest peak frequencies at station A between the 17/09/04 (day 261) and the 11/11/04 (day 316).
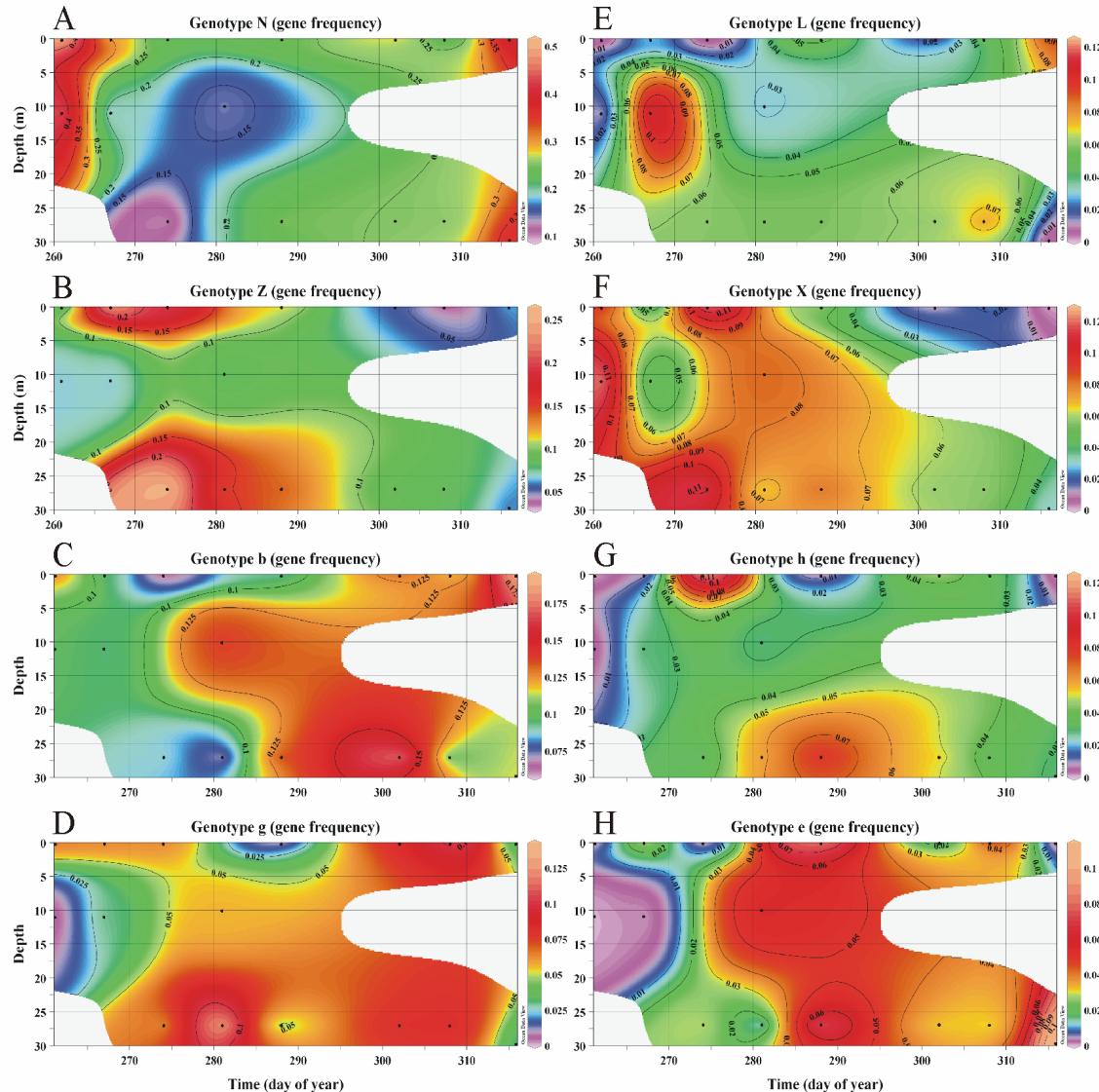
FIG. 5.10 Gene frequencies of the eight most common genotypes (A-H) in La Jolla Bay during sampling in 2004.

Genotype N was exceptional, in that it was by far the most abundant genotype. Its frequency in the population was always above 0.1 and reached peak values close to 0.5. The relative abundance of this common genotype generally did not change with depth but noticeably over time, being highest at the beginning and at the end of the sampling

campaign. Frequencies of Genotype Z peaked in the first half of the sampling period (before the storm) with values close to 0.25. Its vertical frequency distribution was bimodal with higher values at the water surface and below the thermocline. The lowest frequencies were observed towards the last two weeks of the sampling period. The frequency of genotype b reached values above 0.17 and its peak distributions complemented the pattern that was seen in the distribution of genotype Z in several features. Peak frequencies of the genotypes g, L, X, b, and e were fairly close between 0.1 and 0.125 but the frequency distributions of these five genotypes also had distinct temporal and spatial features. For example, the frequency distributions of the genotypes L and X showed a complementary pattern, similar to that between genotypes b and Z.
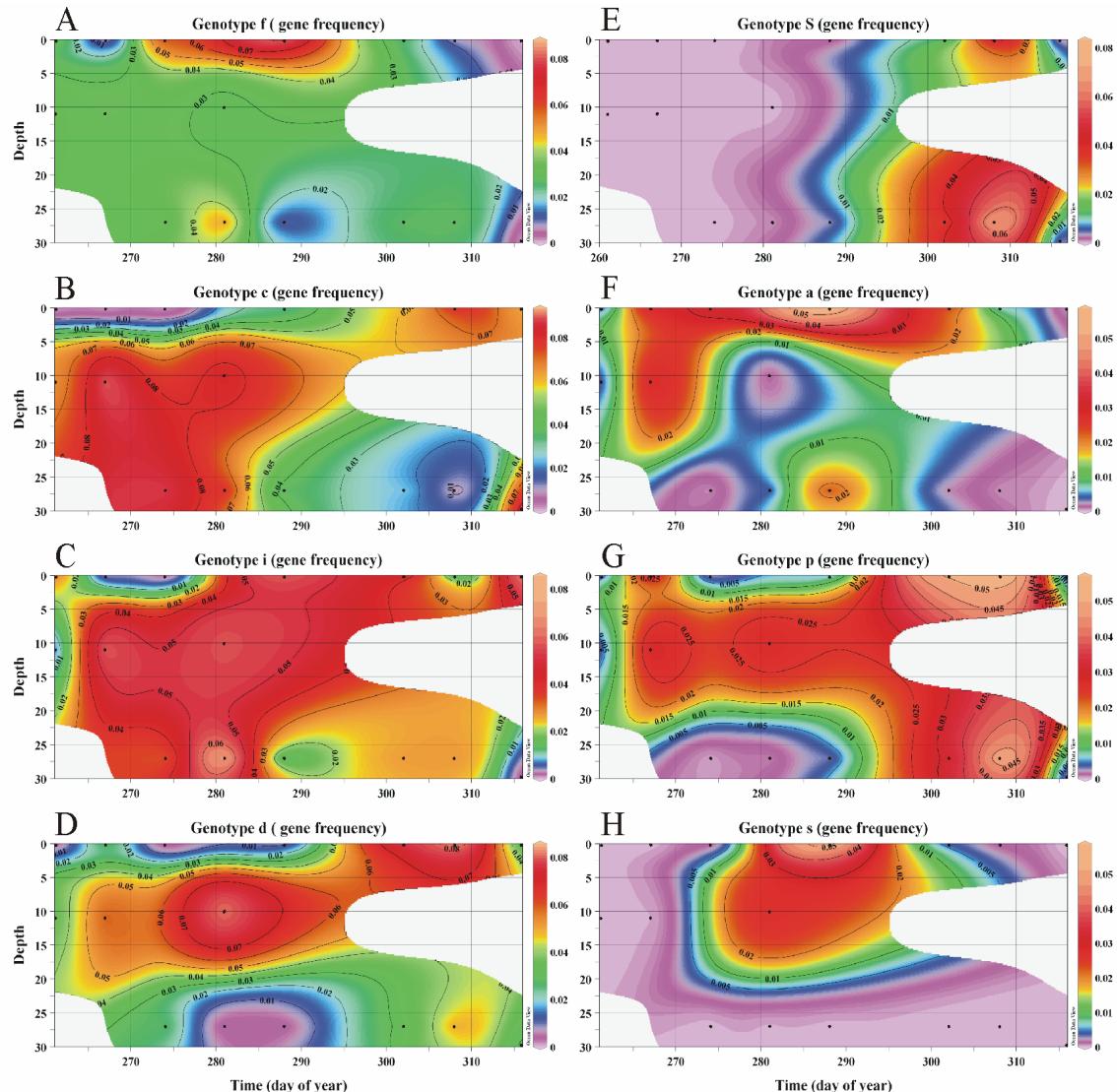
FIG. 5.11 Gene frequencies of the eight most common genotypes (A-H) in La Jolla Bay during sampling in 2004 after the eight genotypes shown in figure 5.9.

Figure 5.11 A-H shows time series of the eight genotypes that had peak frequencies below 0.1 and down to 0.05. Frequencies of genotype f peaked before the storm and at the water surface, whereas pre-storm frequencies of genotype c were consistently lower at the surface compared to those at greater depth. Only after the storm the latter genotype was found at an increased frequency at the surface. Genotype i showed little change over time and space, while pre-storm frequencies of genotype d peaked around the thermocline and at the surface following the storm. An interesting temporal development could also be seen in genotype S. This genotype was below the detection limit before the storm but following the storm its frequency increased quickly.

A selection of nine low frequency genotypes (maximum gene frequencies below 0.05) is shown in figure 5.12 A-I. The genotypes are arranged to show the temporal succession of different genotypes from left to right and the succession with depth from top to bottom. For example, genotypes A, r and W exemplify the three main patterns of vertical frequency distributions that were encountered: Surface bound genotypes, genotypes that were most frequent around the thermocline, and genotypes that were only found at depth. Pattern distributions for genotypes t, q and F exemplify how genotypes like A, r and W were displaced over time by other genotypes that occupied similar niches. Genotypes o, j and J exemplify how some of the genotypes that were most abundant at a certain depth before the storm, after the storm were found throughout the entire water column and how others were still only found at certain depths after the storm. One general observation in this group of low abundance genotypes was that their occurrence in the population was normally patchier than that of genotypes with higher frequency in the population.
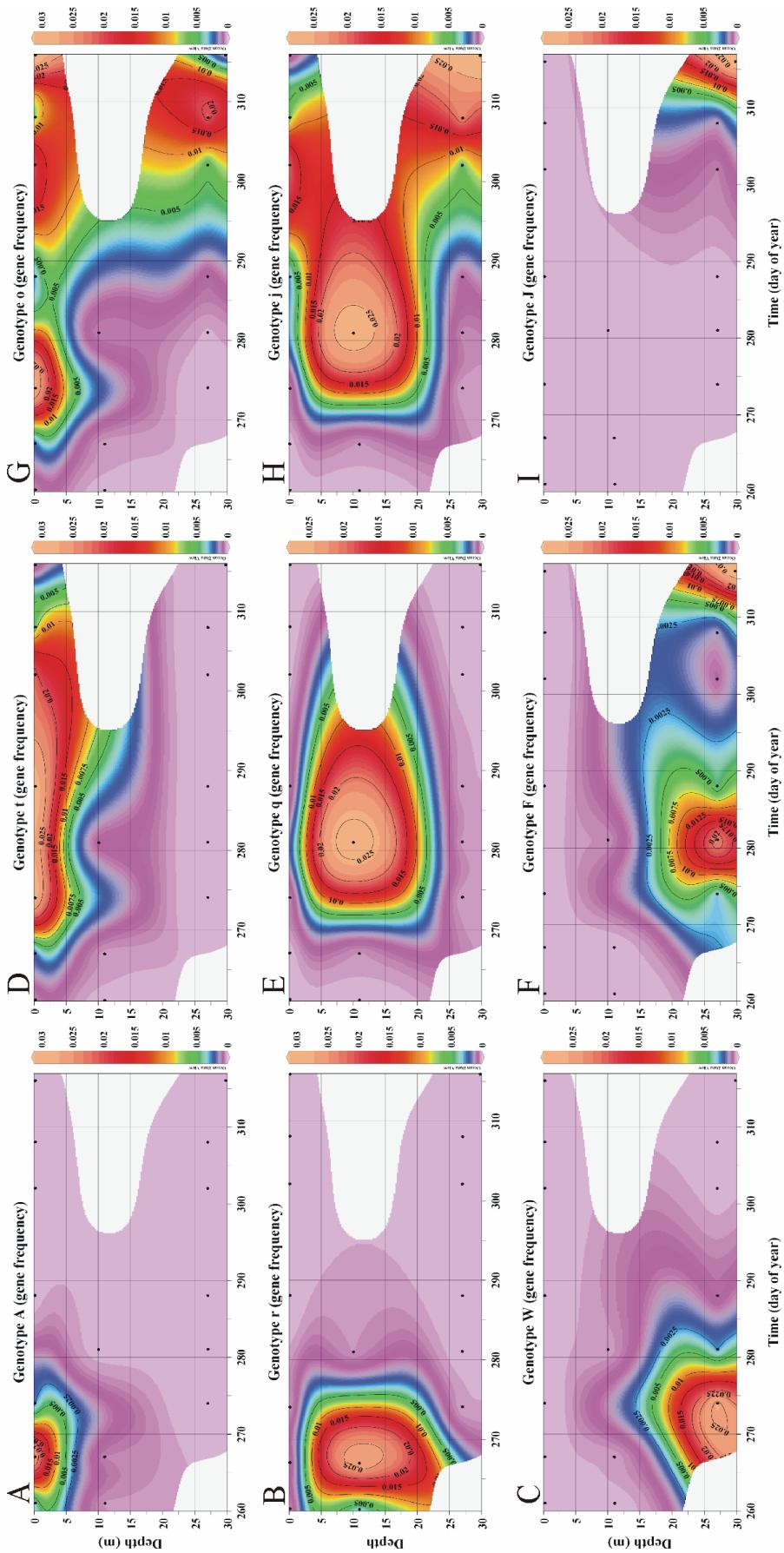
FIG. 5.12 Gene frequencies of nine rare genotypes (A-I) in La Jolla Bay during sampling in 2004 showing the temporal and vertical variability of relative genotype abundances.

5.4 Discussion

5.4.1 Single cell microsatellite genotyping: method evaluation

A first evaluation of the developed method of single cell microsatellite genotyping is given in chapter 2 (Frommlet and Iglesias-Rodríguez 2008). However, since this previous evaluation was based on a relatively small number of samples, the aim of this section is to compare previous findings with the results of the present study. Previous data suggested that the microsatellite locus LP65 is a highly polymorphic marker, suitable for diversity studies at the population genetic level. In the present study LP65 allowed the identification of 52 genotypes in a sample of 704 genotyping results. Gene diversity (Nei 1972) ranged from 0.70-0.93 with an average of 0.89. The large number of genotypes that could be identified with LP65 and the similarly high gene diversity of 0.89 compared to a previously found value of 0.87 confirm the usefulness of LP65 as a marker for population genetic diversity in *L. polyedrum*.

Uncorrected amplification success rates for LP65 in the present study varied widely from 32 to 90% between individual water samples with an average of 63%. Previous amplification success rates were comparable to the upper values found in the present study (see chapter 2).

As discussed in more detail in chapter 2, the amplification and sequencing of ribosomal regions has two very important functions in the developed single cell genotyping protocol: 1) as an internal positive control for the amplification success of microsatellites and 2) as an independent means of verifying morphological species identification. Sequencing of the hundreds of 18S PCR products that were generated in the present study was not feasible. Therefore, sequencing of a subset of samples was aimed as a random quality control. For this purpose, a random selection of eight 18S PCR products from reactions that resulted in a product for LP65 and eight 18S PCR products from reactions that did not yield a product for LP65 resulted in sixteen identical, *L. polyedrum* specific 18S sequences, indicating that morphological species identification was conducted at a high standard and that unsuccessful microsatellite amplifications from samples that resulted in 18S products were most likely not due to wrong species identification. The unsuccessful amplification of LP65 from, on average, 14% of single cells therefore suggests the presence of null alleles, due to e.g. mutations of primer binding regions, leaving these cells inaccessible to genetic analysis.

The most likely causes for unsuccessful amplifications of both targeted loci (18S and LP65) were: 1) wrong morphological species identification; 2) degraded single cell DNA template; and/or 3) inefficient breaking of single cells prior to PCR. Regardless of the cause, the cells that did not yield any PCR products should not be considered in the calculation of a LP65-specific amplification success rate. A corrected success rate, excluding these cells, was between 60 to 100% with an average of 86%. This success rate is very high, even compared to culture-based microsatellite genotyping of phytoplankton (e.g. Rynearson and Armbrust 2005, 2006, Nagai et al 2007). In section *5.4.4* water sample-specific amplification success rates are discussed in more detail and in the context of the population development of *L. polyedrum* at the time of sampling.

On average, 8% of single cells resulted in the amplification of two PCR products for LP65, but this percentage varied widely between 0 to 20% depending on the water samples. Similar results have been obtained previously (see chapter 2). A likely explanation for obtaining two products could be that these cells were heterozygous diploid cells. Given that the only two diploid motile stages in the life cycle of *L. polyedrum* are the planozygote (the product of sexual reproduction) and the planomeiocyte (the diploid cell that emerges from resting cysts) (Lewis and Hallett 1997, Figueroa and Bravo 2005), microsatellite analysis of single cells may enable the detection of sexual reproduction and excystment events in natural populations. For a discussion of these results in the context of the *in situ* population development of *L. polyedrum* at the time of sampling see section *5.4.4*.

5.4.2 Population genetic distance/differentiation of L. polyedrum in Southern California

The physical and chemical characteristics of Californian Coastal waters are strongly determined by the California Current System (CCS), an eastern boundary current affected by seasonal coastal upwelling during spring and summer in response to strong and persistent northwesterly winds (Reid et al. 1958, Hickey 1989, Strub and James 2000). One of the sharpest hydrographical and biological boundaries in this region occurs at Point Conception due to an anticlockwise circulation of water masses in the Southern California Bight, the California Countercurrent (U.S. GLOBEC 1994) (Fig. 5.12). Blooms of *L. polyedrum* are most common south of Point Conception in the Southern California Bight (Allen 1946, Holmes et al. 1967, Eppley and Harrison 1975). Interestingly, the occurrence of *L. polyedrum* does however not stop sharply at Point

Conception but extends approximately 100 km further north to 35° N (Wall 1986). The occurrence of *L. polyedrum* across this hydrographic boundary raises questions concerning the population genetic structure of *L. polyedrum* in this region. To test whether distinct populations of *L. polyedrum*, separated by geographic distance or hydrographic boundaries, exist in Southern California, samples from five locations along the Coast were genotyped. The hypothesis was that the hydrographic boundary at Point Conception may represent an effective barrier for population genetic exchange between *L. polyedrum* north and south of this point. If that was the case, the sample collected at Avila Beach, which roughly marks the northern limit for the occurrence of *L. polyedrum* along the Californian Coast, would be expected to be genetically the most distant to all the other samples that were collected south of Point Conception (Fig 5.13).
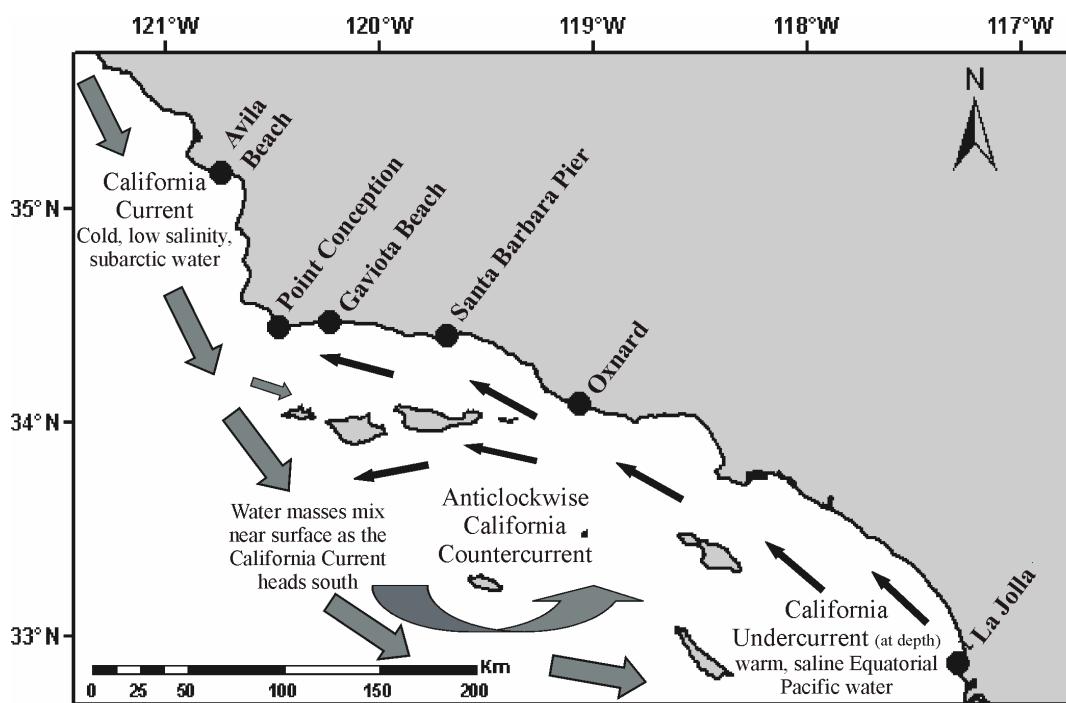
FIG. 5.13 Characteristic oceanic circulation in the Southern California Bight and sources of water masses (redrawn after: Minerals Management Service (MMS OCS Draft EIS/EA 2001-046).

Not considering the samples from La Jolla, the UPGMA dendrogram shown in figure 5.3 may support this hypothesis. However, when the data from La Jolla are included, this separation of the Avila Beach sample is no longer supported. Quite contrary, the Avila Beach sample clustered closest with one of the samples from La Jolla Bay, which, following the coast line, is approximately 500 km further south and geographically represents the location that is farthest away from Avila Beach. Also the comparatively

small genetic distance between samples from Gaviota and Oxnard compared to the Santa Barbara sample did not reflect their relative geographic location to each other. However, a pattern in the clustering of samples emerges when large scale water circulation patterns in the Southern California Bight are considered (Fig. 5.13). For example, following the deflection of the CCS at Point Conception off the coast by the California Countercurrent, the CCS moves back onshore further south (U.S. GLOBEC 1994, Strub and James 2000). The small genetic distance between the samples from Avila Beach and La Jolla Bay may therefore be due to the southward transport of *L. polyedrum* from Avila Beach in the CCS. From La Jolla, a northward gene flow into the California Countercurrent may then be controlled by the California Undercurrent. This central position of La Jolla may explain why one sample from La Jolla clustered with the Avila Beach sample, while the other sample from La Jolla clustered with the samples from within the California Countercurrent. The results of an exact test shown in Table 5.3 generally support these findings, since the largest population genetic differentiation was found between Avila Beach and the samples from Oxnard and Santa Barbara. However, the fact that population genetic differentiation was not statistically significant between any pairs of samples, also indicates that genetic exchange within the species *L. polyedrum* in the whole region must be high. An entrainment of *L. polyedrum* in the circular California Countercurrent and the pronounced seasonality of the CCS and California Countercurrent dynamics (Strub and James 2000) may contribute to this low population genetic differentiation by enhancing genetic exchange along the coast. The meroplanktonic life cycle of *L. polyedrum* may further contribute to the population genetic exchange, since the formation of stress tolerant ecdysal cysts and resting cysts allows dispersal of the species under conditions that would not support motile, vegetative cells (Wyatt and Jenkinson 1997).

In summary, these results suggest: 1) that the hydrographic boundary at Point Conception does not prevent population genetic exchange of *L. polyedrum*; 2) that genetic distance of *L. polyedrum* in Southern California is not related to geographic distance; 3) that large scale circulation patterns in the Southern California Bight drive population genetic exchange of *L. polyedrum*; 4) that *L. polyedrum* in the Southern Californian Bight and possibly beyond Point Conception to the North forms one large population; and 5) that the low population genetic differentiation is most likely a result of the combined effects of the meroplanktonic life cycle of *L. polyedrum* and the water circulation patterns that favour genetic exchange in *L. polyedrum*.

5.4.3 Environmental dynamics and L. polyedrum population development in La Jolla Bay

Since sampling of *L. polyedrum* blooms was restricted to a single sample from 2003 and 2005 respectively, the focus of this section is to discuss the development of the more frequently sampled 2004 *L. polyedrum population* in La Jolla Bay. The environmental conditions at the start of the sampling in 2004 were typical for late Southern Californian summers in that the weather was calm and sunny, resulting in warm, well stratified and shallow surface waters, overlaying a sharp thermocline at a depth of around 8-12 m. Based on previous studies, these are ideal conditions for *L. polyedrum* to form blooms in Southern California (Allen 1946, Holmes et al. 1967, Eppley and Harrison 1975). The rapid development of the 2004 *L. polyedrum* population in La Jolla Bay from cell numbers close to the detection limit to a peak abundance exceeding 46,000 cells L^{-1} in a matter of 4 weeks (10/09/04 - 07/10/04) confirms the importance of these environmental factors for the development of *L. polyedrum*.

The storm described in section *5.3.3* reduced *L. polyedrum* abundance to numbers in the low hundreds. At a mechanistic level it is not clear how the storm decreased *L. polyedrum* numbers so dramatically. Principally, two mutually not exclusive scenarios are plausible: 1) displacement of the population out of La Jolla Bay and/or 2) a direct negative effect of the storm on the population, possibly through the wind-driven mixing, resulting in increased shear stress and mixing of the population to depths that did not provide sufficient irradiance for photosynthesis. In either case, the storm was most likely the reason why in 2004 the initially rapid development of *L. polyedrum* did ultimately not result in a bloom. Based on this argument, it is reasonable to consider the large population of *L. polyedrum* immediately before the storm as a pre-bloom stage of population development.

The results presented in chapter 3 (Frommlet et al. 2008) showed that irradiance levels can affect the induction of the sexual life cycle in *L. polyedrum*. Specifically, it was shown that high irradiance levels enhance the induction of gametogenesis under nutrient-deplete conditions. One possibility is that *L. polyedrum* may actively change its vertical position in the water column at certain stages during population development in order to induce the sexual life cycle in a temporally and spatially synchronised and environmentally controlled way.

A comparison of vertical cell distributions between the two stations in La Jolla Bay during the rapid increase of *L. polyedrum* shows that cell numbers onshore peaked at a depth of several meters, while further offshore cell numbers repeatedly peaked at the water surface. Since each individual sampling took approximately 4 h (see Table 5.1) and station B had consistently been sampled after station A, this difference in the vertical distribution of *L. polyedrum* may however have been a sampling artefact due to advanced stage of diurnal vertical migration (DVM) of *L. polyedrum* at the time of sampling station B (Eppley and Harrison 1975, Harrison 1976, Heaney and Eppley 1981). The observed temporal changes in the vertical position of *L. polyedrum* at both stations were however not affected by the same systematic time lag and are likely to represent actual changes in the vertical position of the population. The question is whether these *in situ* observations of vertical changes in population distribution are consistent with the previously suggested link between an active change in the vertical position of the population and an induction of sexual reproduction (chapter 3; Frommlet et al. 2008). In order to address this question, first it has to be established whether sexual reproduction occurred at any stage during sampling in 2004. At station B, resting cysts, the product of sexual reproduction, were first found in the water column on the 7/10/04, coinciding with the highest cell densities of motile *L. polyedrum* cells at this station. This could be interpreted as a sign for sexual reproduction to have occurred at this stage of population development. However, at the same time also empty resting cysts were found. Because *L. polyedrum* resting cysts have a dormancy period of several months (Figueroa and Bravo 2005), these empty resting cysts could not have originated from recent sexual reproduction and hence were most likely introduced into the water column by resuspension of already empty resting cysts from the sediment and/or intact resting cysts that excysted upon being resuspended. The close proximity of station B to the shore and the shallow water depth make this station susceptible to inputs of sedimentary material into the water column and the temperature profile at the time supports the idea that upwelling of colder water may have caused a resuspension of cysts. Based on this argument the detection of resting cysts at station B does therefore not prove that sexual reproduction had occurred at the time the resting cysts were found. Nonetheless, strong evidence that sexual reproduction occurred during the pre-bloom stage before the storm comes from the detection of resting cysts and at the same time absence of empty resting cysts in the water column at station A (see figure 5.6 B). These results therefore suggest that, at least at station A, sexual reproduction occurred during

the 2004 sampling campaign. The temporal and spatial onset of resting cysts at station A showed that sexual reproduction: 1) coincided with high abundances of motile cells of *L. polyedrum*; 2) was confined to the surface of the water column; and 3) occurred during a narrow temporal window. Together these findings suggest that sexual reproduction was induced in a temporally and spatially synchronized way during a late stage of pre-bloom development. In line with the suggestion in chapter 3 (Frommlet et al. 2008) that *L. polyedrum* may actively expose itself to high irradiances in surface waters in order to induce the sexual life cycle, at the time sexual reproduction occurred *in situ* the population was at the water surface and presumably exposed to high irradiance levels. Whether, as hypothesized in chapter 3, the surface accumulation of the population was due to a change in DVM is still an open question that can not be answered based on the sampling conducted. The observation that the population was found at a depth of several meters (atypical for station A) a week before sexual reproduction was detectable suggests a great complexity of processes involved in controlling sexuality in *L. polyedrum*. An important next step to answer some of the open questions would be a study of the daily dynamics of vertical distribution of *L. polyedrum* populations before, during and after events of sexual reproduction.

5.4.4 Lingulodinium polyedrum population genetics in La Jolla Bay

The data obtained from La Jolla Bay showed that the population genetic dynamics within a geographic location can exceed that between widely separated sites. The following section therefore focuses on an in depth assessment of *L. polyedrum* population genetics in La Jolla Bay.

As discussed in the previous sections, sampling in La Jolla included two bloom events, in 2003 and 2005, and covered a period of environmental change from well stratified waters to mixing conditions in 2004. Changes in the abundance of *L. polyedrum* in 2004 were very dynamic and there was strong evidence that sexual reproduction occurred in the population. Both the corrected success rates of single cell microsatellite genotyping and the genetic diversity (Nei 1972) in 2004 were generally very high and stable. However, at the beginning of the sampling campaign both parameters showed reduced values. The lower success rate suggests the presence of null alleles, which means that the genetic makeup of a part of the population was inaccessible to genetic analysis and the coinciding reduced genetic diversity may therefore be due to this inaccessibility of

assessing some of the population genetic diversity.

A somewhat elevated level of genetic diversity coincided with the highest population density and sexual reproduction. The fact that genetic diversity was high independent of the stage of population development (non-bloom, pre-bloom or bloom), suggests that bloom populations are not comprised of only one or a few clonal lineages. The sudden increase of cells with two LP65 PCR products a week before resting cysts were found in the water column at station A support the previous suggestion that these products may represented true alleles of LP65 amplified from diploid planozygotes (Chapter 2; Frommlet and Iglesias-Rodríguez 2008).

The UPGMA dendrogram shown in figure 5.9 revealed four main clusters. Cluster 1 contained both the bloom samples (2003 and 2005) and one non-bloom sample from 2004. The fact that both bloom samples clustered together may have solely been due to stochastic interannual population genetic differences but alternatively may indicate that certain genotypes are more common during blooms then during non-bloom conditions. For example, Mayali (2007) showed that numbers of algicidal bacteria in 2003 and 2005 in La Jolla Bay increase dramatically during *L. polyedrum* blooms and that susceptibility of *L. polyedrum* to bacterial infection varied intraspecifically. If such intraspecific variation was based on genetic differences between lineages in *L. polyedrum*, the selective pressure of algicidal bacteria on *L. polyedrum* may explain shifts in population genetic structure during blooms. At this stage there is however not enough data available to support or disprove that this or any other intraspecific variability in *L. polyedrum* may cause population genetic composition to change between non-bloom and bloom stages.

Interestingly, the 2004 sample that clustered with the bloom samples from 2003 and 2005 coincided with evidence of cyst resuspension from the sediment further onshore. The fact that there were no cysts found at station A at this point in time does not disprove that excysted cells from further onshore may have entered the population of motile cells at station A. If this was the case, then the clustering of the 2003 and the 2004 sample may have been due to the introduction of genotypes from the cyst bed of the previous bloom year into the 2004 sample. Similarly, the 2005 bloom may have been seeded by cysts from the 2003 bloom. Detailed investigations of the genetic composition of cyst beds locally in La Jolla Bay and also on a larger geographic scale in Southern California may help to improve the understanding of these processes. The

applicability of the developed single cell genotyping technique to resting cysts of *L. polyedrum* (chapter 2; Frommlet and Iglesias-Rodríguez 2008) may be of great significance for such future studies.

Another cluster that could be identified in the UPGMA dendrogram was cluster 2. This group of samples contained the Avila Beach sample as well as the two earliest and one of the last samples that were genotyped from La Jolla Bay in 2004. This clustering gives further support to the idea that genetic material from Avila Beach may be introduced into La Jolla Bay by the California Current System (CCS). The fact that the two earliest as well as one of the latest samples from La Jolla Bay were genetically closest to the Avila Beach sample may be due to the seasonal variability of the velocity and the course of the CCS (Strub and James 2000). Further investigations into the dynamics of the CCS at the time of sampling in 2004 would be needed to study this relationship in more detail.

Cluster 3 was composed entirely of samples that were collected prior to the storm between the 20/10/2004 and the 23/10/2004, whereas cluster 4.1 exclusively contained samples that were isolated after the storm. This pattern suggests that local changes of environmental factors very quickly (probably in a matter of days) affect population genetic composition of *L. polyedrum* in La Jolla Bay. This is only possible if the different genotypes in a population vary in phenotypic traits that are differentially selected by the changing environmental conditions. Since the storm in 2004 may have affected a multitude of factors, such as nutrient availability, shear stress, light conditions, stratification, and many more, the current results do not allow the identification of a particular environmental variable and its effect on the population genetics of *L. polyedrum* but clearly shows that the storm had a selective effect on the *L. polyedrum* population.

The clustering of samples in the UPGMA dendrogram showed further that at some stages the population genetic composition was homogenous with depth, whereas at other times there was a change of population genetic composition with depth. Since these groupings did however not show a clear pattern, the vertical genetic structure of the population is considered in more detail in the following section that describes the temporal and vertical dynamics of individual genotypes.

5.4.5 Dynamics of individual L. polyedrum genotypes in La Jolla Bay

Out of the 37 different genotypes that could be identified during sampling In La Jolla Bay during 2004, genotype N was by far the most abundant. The dominance of this genotype at the beginning and at the end of the sampling campaign under very different environmental conditions may be due to certain phenotypic traits that enable this clonal lineage to thrive under a wide range of environmental conditions. Based on the environmental data there is no clear indication as to what these traits might be, but a certain flexibility regarding irradiance seems to characterize this clonal lineage because it was abundant from high irradiance surface waters to depths below the thermocline and from late summer to early winter. Interesting in this context is that genotype N represents the clonal lineage of M22, a strain that in chapter 4 has been shown to have the ability to acclimate to a wide range of photon flux densities. A genotype, defined by microsatellite fingerprinting, does not necessarily represent a certain phenotype since microsatellites are mainly neutral markers that are positioned in non-coding regions (Estoup and Angers 1998, Bennett 2000). The dominance of genotype N under a wide range of environmental conditions and the wide range of light intensities the strain M22 was able to acclimate to, may however be more than a coincidence. The other *L. polyedrum* strain that was studied in chapter 4 was LP2810. This strain did not have the same ability as strain M22 to acclimate to a broad range of photon flux densities and seemed to be best adapted to medium light conditions. Could the observation that genotype c, the clonal lineage of LP2810, was less abundant in surface waters be connected to the relatively narrow range of photon flux density LP2810 could acclimate to? At this stage any claim of such links between phenotypic traits of the two studied clonal strains M22 and LP2810 with their corresponding clonal lineages in natural populations is premature. Only the study of more clonal strains of *L. polyedrum* will show how genotypic and phenotypic diversity may affect the population ecology of the species as a whole. The low abundance genotypes that were shown in figure 5.12 are a good example of just how dynamic the population genetic structure of *L. polyedrum* can be over time and how structured its populations can be vertically in the water column. Interesting strains for future physiological studies may be identified based on an in depth study of the dynamics of these clonal lineages in the natural environment.

Chapter 6

General discussion

The aim of this dissertation was to improve the understanding of the population ecology of *Lingulodinium polyedrum* (F. Stein) J.D. Dodge, a typical bloom-forming dinoflagellate species. This chapter provides a summary of the main findings in this project, highlights the limitations of the work, reports on ongoing and potential future work and concludes with some final remarks on the project.

6.1 Single cell microsatellite genotyping

The population genetic aspects of this study were initially planned to be conducted on clonal cultures, as is common practice in phytoplanktonic organisms (see introduction to chapter 2 for references). For this purpose, a total of more than 15,000 single cells were isolated during the main sampling campaign in 2004. Unfortunately, only a relatively small number of approximately 400 clonal cultures could be established, which represents a success rate of less than 3%. A population genetic study based on these 400 surviving cultures would have been questionable, since a selection of more robust genotypes during culturing would likely give a different representation of diversity from that existing in the population. The development of a single cell genotyping technique was an obvious alternative to the use of clonal cultures, but also a challenging task. In chapter 2, the development of such an approach based on the combination of microsatellite genotyping with a novel single cell PCR technique is described. This method allowed the amplification of up to six microsatellite loci together with either the complete ITS1-5.8S-ITS2 region or a partial 18S region of the ribosomal gene of *L. polyedrum* from single motile cells and resting cysts, providing a novel tool for population genetic studies of *L. polyedrum*. The development of this method took more than three years and hundreds of PCRs to test and optimize the conditions for amplifying microsatellite markers in multiplex PCRs from single cells. Ultimately, this huge effort resulted in only one microsatellite marker that combined all the necessary attributes to be employed as a population genetic marker for *L. polyedrum*. Nonetheless,

as shown by the application of this microsatellite marker in chapter 5, even based on a single marker, single cell microsatellite genotyping has the potential to reveal phytoplankton population genetic dynamics in remarkable temporal and spatial detail. Therefore, although the application of the method may still be at an early stage of development, single cell microsatellite genotyping is a very powerful tool for the study of phytoplankton population genetics and is one of the major achievements of this project. The main shortcoming of the method (the small number of informative loci that could be identified) was due to common problems in the development of microsatellite markers (Squirrell et al. 2003) such as: non-polymorphic loci, loci with high numbers of null alleles as well as loci that created stutter bands, making their sizing difficult. Since the identification of informative microsatellite loci is a matter of trial and error, the development of future protocols should be based on a larger number of microsatellites than the 17 loci that were initially identified during this study (chapter 2; Frommlet and Iglesias-Rodriguez 2008). This ought to result in the identification of larger numbers of informative microsatellite markers and improve the power of the method in discriminating different genotypes.

6.2 Life cycle control in *L. polyedrum*

In chapter 3, new information on the life cycle of *L. polyedrum* and the environmental control of life cycle changes in this species were presented. Specifically, it was shown that ecdysis can be induced by nutrient-replete exposure to low light, while nutrient stress-induced gametogenesis in *L. polyedrum* can be considerably enhanced by high light and prevented by low light. To the best of my knowledge, this study represents the only work that has shown a clear influence of an environmental factor (in this case light) other than nutrient limitation on gametogenesis in a dinoflagellate species. These findings are therefore of significance in the study of the environmental control of the sexual life cycle in dinoflagellates. Also for the first time, this study gave an indication of the involvement of the photosynthetic apparatus in triggering the sexual life cycle in dinoflagellates, and provides the first indication of an involvement of reactive oxygen species (ROS) in triggering gametogenesis. Currently, experiments that employ ROS scavengers such as the enzyme catalase are being conducted to study whether it is indeed the amount of ROS that induces gametogenesis in *L. polyedrum*.

Another important finding was that gametes of *L. polyedrum* are formed by a single mitotic cell division and that, by means of a second mitotic cell division, each gamete can divide once again to produce a total of four gametes per vegetative cell. Since each of the two cell divisions results in a doubling of cell numbers, this novel model of gametogenesis in dinoflagellates may play an important role in increasing encounter rates of gametes (Wyatt and Jenkinson 1997). The study also gave first indications for intraspecific variability in the ecdysis of *L. polyedrum* under low light, as well as intraspecific morphometric and physiological differences. Whether these differences were due to general intraspecific variability or whether they represent sex-specific differences could not be clarified, since only one reproductively compatible pair of clonal cultures was studied. To answer this open question, future investigations should be aimed at comparing larger numbers of *L. polyedrum* strains of both sexes.

6.3 Photophysiology and light acclimation in *L. polyedrum*

As discussed above, a total of 400 clonal cultures were successfully isolated from a natural *L. polyedrum* population in 2004. A large number of these cultures were contaminated with a parasitic protist, which adhered to cells of *L. polyedrum*. Parasitic infections of *L. polyedrum* have not been described previously, and although this parasite may affect population dynamics of *L. polyedrum* profoundly, it was beyond the scope of this project to characterise this parasite further. In order to develop a holistic approach to understanding *L. polyedrum* population ecology, future studies will however also have to consider biological interactions such as parasitism, competition and bacterial/viral infections. Sixty cultures, that were not infected with the parasite, were screened for differences in pigmentation in order to identify intraspecific differences within *L. polyedrum* (results were not shown).

Two of the cultures that showed pronounced differences in pigmentation were M22 and LP2810. These two strains were selected for a more detailed study of intraspecific variability of photophysiology and light acclimation. These experiments showed for the first time the existence of *L. polyedrum* strains with different light requirements, which have different light acclimation capabilities and employ fundamentally different light acclimation mechanisms. These differences were so distinct that under the lowest irradiance tested only one of the strains was able to grow. At the same time, the two

tested strains shared identical ITS sequences and were reproductively compatible, suggesting that these differences represented true intraspecific variability of photophysiology and light acclimation in *L. polyedrum*. Only one of the strains showed pigment changes during light acclimation that are typical of *L. polyedrum*. Interestingly, measurements of the effective absorption cross-section of PS II ($\sigma_{PS\ II}$) during light acclimation challenge the current theory that *L. polyedrum* acclimates to changing PFDs by regulating PSU size. Since *L. polyedrum* is an important model organism for dinoflagellate photophysiology (Prézelin 1987), these discrepancies between the current theory and the results obtained in this study suggest that a re-examination of photophysiology, not only in *L. polyedrum* but in dinoflagellates in general ought to be considered, and should take into account intraspecific diversity.

6.4 Population ecology of *L. polyedrum* in Southern California

In chapter 5, single cell microsatellite genotyping was employed in the first population genetic analysis of *L. polyedrum*. The aim was to assess the population genetics of *L. polyedrum* over a range of temporal and spatial scales in Southern California and to study how population genetic parameters change in relation to environmental change and population development.

At a geographic level, the study indicated that (1) population genetic exchange of *L. polyedrum* in Southern California is not prevented by a dominant hydrographic boundary at Point Conception; (2) population genetic distance of *L. polyedrum* in Southern California is not related to geographic distance; and (3) large scale circulation patterns in the Southern California Bight affect population genetic exchange of *L. polyedrum*. Low levels of genetic differentiation also suggested that population genetic exchange in Southern California is high and that *L. polyedrum* in the region forms one large population. The low population genetic differentiation of *L. polyedrum* in Southern California may be a result of the combined effects of the meroplanktonic life cycle and the water circulation patterns that may favour genetic exchange within the species *L. polyedrum* in Southern California. The only other microsatellite based study of biogeography in a free-living dinoflagellate species showed significant population genetic differentiation in *Alexandrium tamarense* along the Japanese coast (Nagai et al. 2007). The water currents along the Japanese coast do not cause circular water

movements such as the anticlockwise California Countercurrent in the Southern California Bight (Nagai et al. 2007, U.S. GLOBEC 1994, Strub and James 2000), which may explain why the genetic differentiation of dinoflagellate populations in these two geographic regions shows these differences.

At a local level, in La Jolla Bay, remarkable temporal and spatial patterns in the genetic structure of populations were revealed and certain changes in population genetic distances and frequencies of individual genotypes gave a first indication that vertical gradients of environmental factors in the water column and temporal changes in environmental conditions strongly affect the population genetic composition of *L. polyedrum*. The laboratory experiments on the life cycle and its environmental control (chapter 3) and on the photophysiology and light acclimation of *L. polyedrum* (chapter 4) allowed interesting comparisons with *in situ* population dynamics. However, at this stage any claim of causal links between phenotypic traits of the tested strains in the laboratory and their clonal lineages in natural populations would be premature.

6.5 Final remarks

This thesis represents an attempt to approach *L. polyedrum* population ecology from a holistic point of view and was designed to integrate aspects of life cycle control and genotypic/phenotypic diversity into the study of *L. polyedrum* population dynamics. In the process of building a basis for such an integrated approach, this project has contributed to the development of novel methods for the study of phytoplankton population genetics, has given unique insights into the photophysiology and light acclimation of *L. polyedrum* and has significantly advanced the knowledge on the life cycle of this species and the environmental control of its life cycle transitions. The integration of these lines of research has given a first glimpse into the population ecology of this important bloom-forming species on a range of spatial and temporal scales in Southern California. The complexity of interactions that shape the population ecology of *L. polyedrum* require still extensive work and the work presented here is without doubt not the last chapter in the exciting story about *L. polyedrum* population ecology. The study of phytoplankton ecology in general is entering an exciting era that sees more and more the integration of different disciplines and many of today's puzzling questions will soon be uncovered.

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